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# Reduced cathepsins B and D cause impaired autophagic degradation that can be almost completely restored by overexpression of these two proteases in Sap C-deficient fibroblasts

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**Saposin (Sap) C deficiency, a rare variant form of Gaucher disease, is due to mutations in the Sap C coding region of the prosaposin (*PSAP*) gene. Sap C is required as an activator of the lysosomal enzyme glucosylceramidase (GCase), which catalyzes glucosylceramide (GC) degradation. Deficit of either GCase or Sap C leads to the accumulation of undegraded GC and other lipids in lysosomes of monocyte/macrophage lineage. Recently, we reported that Sap C mutations affecting a cysteine residue result in increased autophagy. Here, we characterized the basis for the autophagic dysfunction. We analyzed Sap C-deficient and GCase-deficient fibroblasts and observed that autophagic disturbance was only associated with lack of Sap C. By a combined fluorescence microscopy and biochemical studies, we demonstrated that the accumulation of autophagosomes in Sap C-deficient fibroblasts is not due to enhanced autophagosome formation but to delayed degradation of autolysosomes caused, in part, to decreased amount and reduced enzymatic activity of cathepsins B and D. On the contrary, in GCase-deficient fibroblasts, the protein level and enzymatic activity of cathepsin D were comparable with control fibroblasts, whereas those of cathepsin B were almost doubled. Moreover, the enhanced expression of both these lysosomal proteases in Sap C-deficient fibroblasts resulted in close to functional autophagic degradation. Our data provide a novel example of altered autophagy as secondary event resulting from insufficient lysosomal function.**

## INTRODUCTION

Saposin (Sap) C deficiency is a rare variant form of Gaucher disease (GD), the most common lysosomal storage disorder (LSD), generally caused by a deficit of the lysosomal enzyme glucosylceramidase (GCase). Sap C deficiency is due to mutations in the Sap C coding sequence of the prosaposin (*PSAP*) gene, which also encodes for other saposins (Sap A, B and D) that originate from sequential lysosomal proteolysis of PSAP. Sap C is an 80 amino acid glycoprotein containing six cysteine residues involved in three intramolecular disulfide bridges that render the structure of the protein remarkably stable to heat, acid environment and proteolytic degradation (1,2). Sap C promotes a rearrangement of the organization of lipids in lysosomal membrane and provides GCase greater accessibility to the glucosylceramide (GC) substrate (3–5). Deficit of Sap C or GCase leads to the

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accumulation of undegraded GC and other lipids in the lysosomes of the monocyte/macrophage lineage. GD main clinical manifestations include hepatosplenomegaly, anemia, thrombocytopenia, bone crisis, pulmonary problems and neurological involvement in some cases. Clinically, GD phenotypes are differentiated in non-neuronopathic variant (type 1), acute neuronopathic variant (type 2) and chronic neuronopathic variant (type 3) (6).

To date, only a few cases of Sap C deficiency have been reported worldwide. Mutations of human Sap C present as a variant of GD. A neuronopathic (type 3) variant phenotype was associated with a mutation involving a cysteine residue (p.C382F, p.C382G and p.C315S), whereas an apparent non-neuronopathic (type 1) variant occurred in the presence of an L349P substitution (7–14). In the first patient (p.C382F), one mutant allele remained unidentified; in the second patient (p.C382G), a new mutation (p.Q430X) in the Sap D domain was reported in the other allele (7–12). The third patient (p.C315S) exhibited compound heterozygous genotype: he carried one allele with a mutation in the Sap C domain, resulting in a single deficit of Sap C, concomitant with a *PSAP* null mutation in the other allele. The phenotype of a new identified patient, homozygous for the deletion of seven amino acid residues (p.del.FDKMCSK 342–348), involving a cysteine residue, could not yet be assessed, due to her young age (15).

Recently, we reported that the fibroblasts from two patients, P4 ($PSAP^{p.del.FDKMCSK342-348/del.FDKMCSK342-348}$) and P3 ($PSAP^{p.M1V/C315S}$) with Sap C deficiency, carrying mutations involving different cysteine residues, had an increased rate of autophagy (16,17). In the present work, these patients were named P1 and P2, respectively. Activation of autophagy may represent a responsive mechanism by which cytoplasmic materials including organelles reach lysosomes for degradation. The initial step of this recycling process is the formation of double-membrane structures, which engulf portions of cytoplasm. The resulting autophagosomes fuse with lysosomes to form single-membrane bound vesicles called autolysosomes, where contents are degraded to regenerate nutrients. Finally, lysosomes are then re-derived from autolysosomes by a process named autophagic lysosome reformation (ALR).

In order to explore the mechanisms of autophagic activation in Sap C deficiency, we investigated responses of fibroblasts from two GD patients to robust induction of autophagy by drug treatments or starvation and subsequent nutrient replenishment. For comparative analysis, we also studied GCase-deficient fibroblasts, homozygous for the most common mutations (p.N370S and p.L444P) and we observed that autophagic disturbance was not present in these cells. Our results suggest that in Sap C-deficient fibroblasts, the increased autophagy is not due to the storage of GC and other lipids, which are also present in GCase-deficient cells, but to a secondary phenomenon resulting from the complete lack of Sap C. The impairment of autolysosome degradation seems partially due to reduced amount and enzymatic activity of cathepsins (Cath) B and D. As a consequence, altered lysosomal proteolysis might lead to delayed ALR that restores lysosome homeostasis. Transient overexpression of Cath B and Cath D in Sap C-deficient fibroblasts results in almost complete recovery of autolysosome degradation.

## RESULTS

### Levels of Beclin 1, Atg5 and Atg7 do not differ between control and Sap C-deficient fibroblasts

We previously demonstrated the activation of autophagy in fibroblasts from two patients with Sap C deficiency. In particular, we observed that the level of LC3-II (microtubule-associated protein 1 light chain 3) was high in P1 cells after starvation (induced autophagy), whereas this protein was already elevated in P2 cells grown in normal medium (basal autophagy) (16,17). It has been reported that in some LSDs, such as neuronal ceroid lipofuscinosis (NCL), Niemann–Pick type C and $GM_1$ gangliosidosis, enhanced autophagy is due to the increased level of Beclin 1 (18–20). In order to investigate whether the activation of autophagy was due to increased autophagosome formation, some proteins involved in upstream steps of this pathway were examined (Fig. 1). Western blotting analysis did not reveal up-regulation of Beclin 1, Atg5 and Atg7 in Sap C-deficient fibroblasts. These results indicate that autophagic vesicle accumulation does not result from the increased level of proteins involved in autophagosome formation.

### Autophagic activity is enhanced by rapamycin treatment in Sap C-deficient fibroblasts

Autophagy is strongly induced by suppressing mammalian target of rapamycin (mTOR) kinase activity with rapamycin or nutrient deprivation (21). Exposure of control and mutant fibroblasts to rapamycin (1 μM) for 24 h promotes a decrease in p-mTOR in these cells, whereas total mTOR expression is not influenced (Fig. 2A). As expected, rapamycin elevated LC3-II levels in control and pathological fibroblasts, particularly in P1 and P2 cells. Of note, a significantly higher level of LC3-II was already present in fibroblasts from P2 patients in basal conditions (Fig. 2B), indicating a constitutive activation of autophagy in these cells, as reported previously (16,17). The same results were obtained by fluorescence microscopy: in rich-nutrient medium, green fluorescent protein-LC3 (GFP-LC3) was visualized as a diffuse cytoplasmic staining in control and P1 fibroblasts or in a punctate pattern in P2 cells. These data confirmed that basal autophagy was increased in P2 fibroblasts. After rapamycin exposure, an increase in the GFP-LC3 puncta number in P1 and even more dramatic in P2 cells was observed compared with control fibroblasts (Fig. 2C, left panels). Quantitative analysis indicated that the average number of autophagosomes in control cells was markedly reduced (mean = 10.5, SD = ± 1.8, $n$ = 30) compared with mutant cells, 32.4 (± 2.3, $n$ = 30) in P1 and 51.4 (± 3.5, $n$ = 30) in P2 fibroblasts (Fig. 2C, right panel).

### Recovery from starvation is delayed in Sap C-deficient fibroblasts

Another well-known inducer of autophagy by inhibiting mTOR kinase activity is nutrient starvation. In order to assess whether the autophagic process is altered in Sap C deficiency, control and P1/P2 cells were starved for 2 h with Earle's balanced salt solution (EBSS). This treatment reduced level of p-mTOR compared with the level of mTOR in control and pathological fibroblasts (Fig. 3A). Nutrient

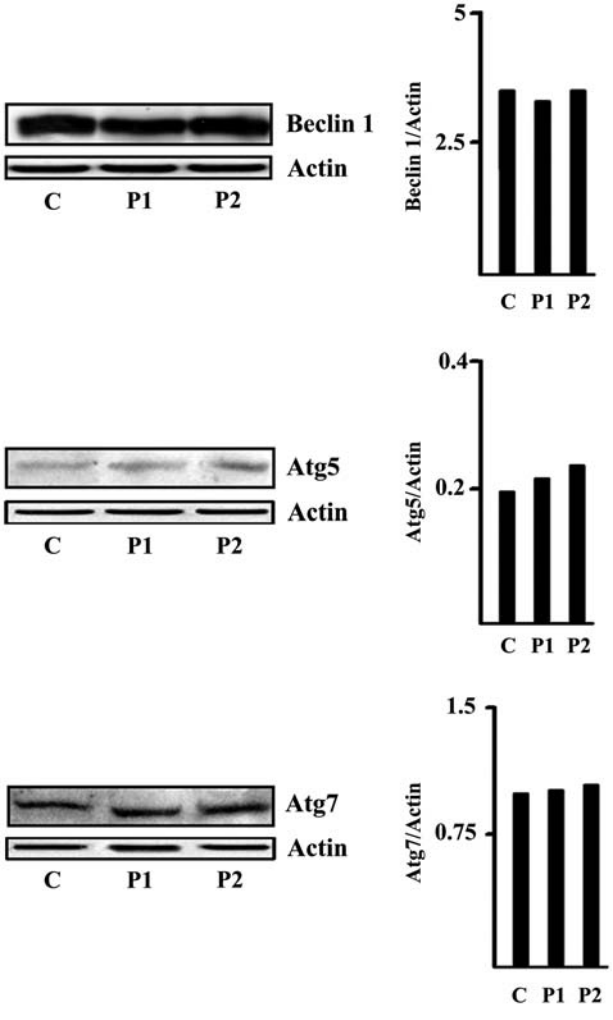


**Figure 1.** Beclin 1, Atg5 and Atg7 levels in Sap C-deficient fibroblasts. A representative western blot of Beclin 1, Atg5 and Atg7 in control and Sap C-deficient fibroblasts from three independent experiments is shown. Relative protein levels were evaluated by densitometric analysis of the bands and normalized to the actin.

deprivation caused a dramatic increase in the LC3-II level in Sap C-deficient fibroblasts compared with control cells. Of note, while the restoration of complete cell culture medium promoted the total disappearance of the LC3-II form in control fibroblasts after a 40 min recovery, such recovery appeared partial in P1 and P2 cells (Fig. 3B). Moreover, GFP-LC3 puncta were considerably higher after 2 h of starvation in Sap C-deficient cells compared with control fibroblasts (Fig. 3C, left panels). Quantification analysis revealed that the average number of autophagosomes in control cells was significantly reduced ($41.3 \pm 6.0$, $n = 30$) compared with mutant cells, which showed the average number of autophagosomes increased to 60.3 ($\pm 2.5$, $n = 30$) in P1 and to 73.2 ($\pm 5.2$, $n = 30$) in P2 fibroblasts. After a 20 min recovery, the puncta were decreased in both mutant fibroblasts. Following 40 min recovery, the number of punctate dots was significantly greater in P1 ($31.8 \pm 2.6$, $n = 30$) and P2 ($42.1 \pm 3.0$, $n = 30$) cells versus zero in control cells (Fig. 3C, right panel). These results indicated that the clearance of autophagosomes is delayed in Sap C-deficient fibroblasts. Impaired clearance of autophagosomes was also evaluated by treatment with lysosomal protease inhibitors, such as leupeptin. Cells were treated for 24 h with leupeptin (10 μg/ml), followed by short recovery time of 1 h in leupeptin-free cell culture medium. As expected, the levels of p-mTOR and mTOR were comparable in untreated and treated fibroblasts (Fig. 4A). Leupeptin treatment increased the LC3-II level more than 3–4-fold in P1 and 5–6-fold in P2 fibroblasts compared with control cells. After 1 h recovery, the LC3-II form disappeared in control fibroblasts but not in Sap C-deficient fibroblasts (Fig. 4B). The persistence of LC3-II after recovery in leupeptin-free medium strongly supported the idea that the autophagosome clearance was delayed. These results were confirmed by GFP-LC3 analysis. Leupeptin treatment led to an increased number of LC3 puncta in P1 and P2 cells compared with control fibroblasts. After 1 h recovery, the LC3 puncta decreased almost completely in control cells, but not in mutant cells (Fig. 4C, left panels). Quantitative analysis revealed that the average number of autophagosomes in control cells after leupeptin exposure was 51.3 ($\pm 3.1$, $n = 30$), whereas in mutant cells the average number of autophagosomes increased to 112.5 ($\pm 4.0$, $n = 30$) in P1 and to 141.0 ($\pm 6.2$, $n = 30$) in P2 fibroblasts (Fig. 4C, right panel). These findings are all consistent with a defect in the degradative function of lysosomes.

### Level and enzymatic activity of Cath B and Cath D are reduced in Sap C-deficient fibroblasts, but not in GCase-deficient fibroblasts

Degradation of autophagic substrates takes place inside the lysosomal compartment by means of acidic proteases, such as Caths. To determine whether the decrease in lysosomal degradative capacity might be accounted to a reduction in the acid protease content, we analyzed three widely expressed lysosomal proteases, Cath B, Cath D and Cath L (22). The level and the enzymatic activity of Cath L were comparable in control and pathological cells (data not shown). On the contrary, the levels of Cath B and Cath D resulted decreased in affected cells (Fig. 5A). Consistently, enzyme assays revealed that Cath B and Cath D activities were considerably reduced in Sap C-deficient fibroblasts (50 and 45%, respectively; Fig. 5B). Decreased Cath B and Cath D enzymatic activities may impair protein degradation. Consistent with these findings, immunofluorescence analysis documented a reduced Cath B and Cath D punctate pattern in pathological cells compared with control fibroblasts (Fig. 5C). The reduced lysosomal activity of both enzymes was confirmed by the proteolytic assay measuring red BODIPY dye conjugated to bovine serum albumin (DQ-BSA) (23). Control and pathological cells incubated with DQ-BSA in complete medium showed normal degradation capacity (Fig. 5D, left panels). Cells displayed a different behavior upon 12 h of starvation in EBSS medium: while control cells remained still positive for DQ-BSA, showing an intense punctate pattern which filled the whole cytoplasm, pathological cells lost their positivity for DQ-BSA, showing a much lower intensity of staining with a poor number of dots inside the cytoplasm (Fig. 5D, right panels). These findings indicated that lysosomal

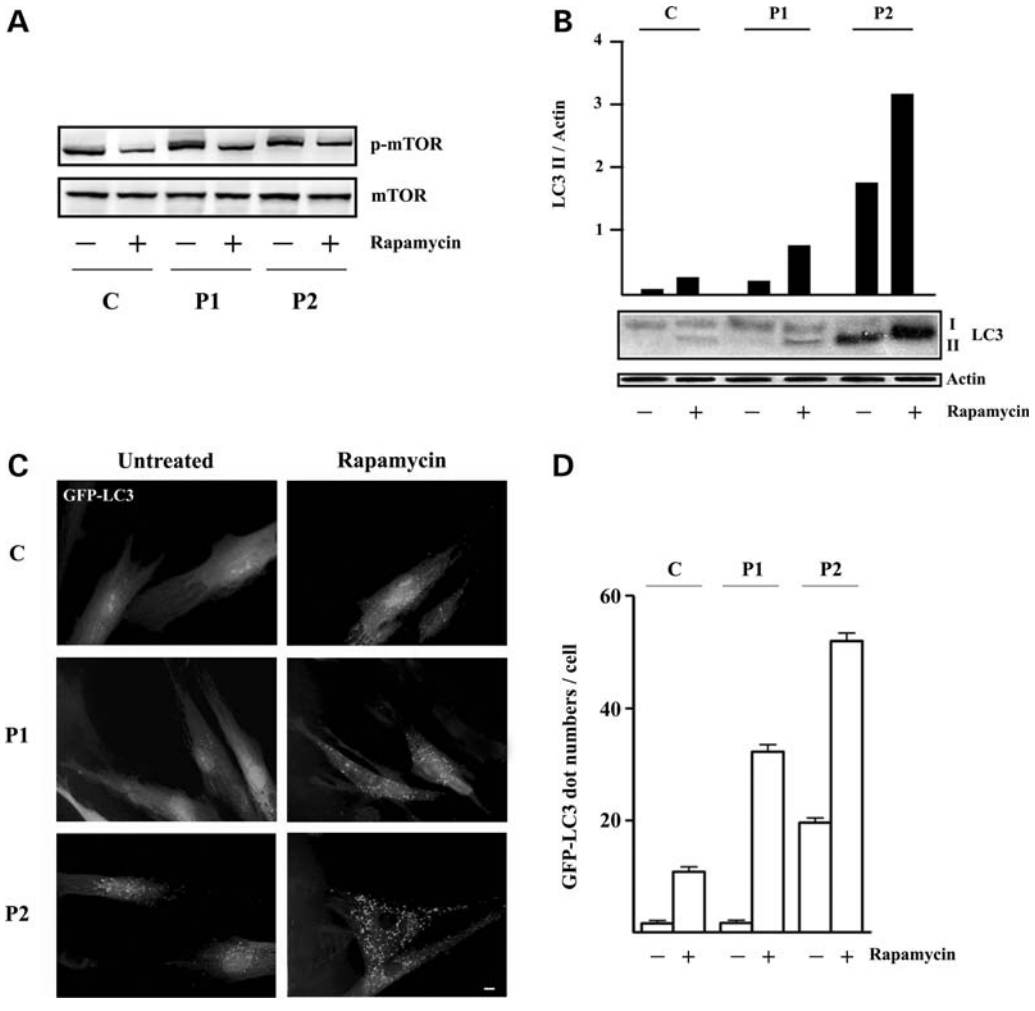


**Figure 2.** Autophagy induction by rapamycin in Sap C-deficient fibroblasts. (**A**) Western blot of p-mTOR relative to total mTOR in control and Sap C-deficient fibroblasts from untreated cells or following 24 h rapamycin (1 μM) treatment. (**B**) Western blot of LC3 in control and Sap C-deficient fibroblasts from untreated or treated cells. The blot was reprobed for actin as a loading control. Representative blots from three independent experiments are shown. Relative levels of LC3-II were evaluated by densitometric analysis of the bands and normalized as the LC3-II/actin ratio. (**C**) The fluorescence microscopy of GFP-LC3 in control and Sap C-deficient fibroblasts from untreated cells or treated with rapamycin. Relative quantification of GFP-LC3 dots per cell (mean ± SD) with or without rapamycin. Dot counts were performed on three independent experiments and 30 cells were analyzed in each experiment. Scale bar, 10 μm.

degradation capacity was altered. We reasoned that the reduction in these two proteases in mutant cells might be due to either loss of Sap C and/or to the lysosomal storage of lipids. Since GD fibroblasts with enzyme or activator deficit display almost similar lipid storage, we utilized fibroblasts from patients homozygous for the most common mutations in *GBA* gene (N370S associated with phenotype 1, and L444P associated with phenotypes 2 and 3) to monitor whether autophagic dysfunction was present in these cells. Western blot analysis did not reveal an increase in the LC3-II level in basal and starved conditions in comparison with control fibroblasts (Fig. 6A). Similar results have been reported under basal conditions by Pacheco *et al*. (19). Next, we examined Cath B and Cath D levels and activities: the Cath B level was almost doubled in GCase-deficient cells compared with control fibroblasts, as observed in other GD models; whereas the Cath D level was comparable among all of the cell lines (Fig. 6B). The enzymatic activities of both Cath B and Cath D well fitted with results obtained by western blot analysis (Fig. 6C). These data were also supported by immunofluorescent staining of the two enzymes (Fig. 6D), indicating that the reduction in the two lysosomal proteases might represent a secondary event due to the complete deficit of Sap C and not to the accumulation of GC or other lipids in lysosomes.

Defective Cath B and Cath D in Sap C-deficient fibroblasts and consequent retarded autophagic degradation might be responsible for the delay in the reformation of lysosomes.

### Reformation of lysosomes is delayed in Sap C-deficient fibroblasts

As recently reported by Yu *et al*. (24), genetic or chemical inhibition of autolysosomal protein degradation abrogates lysosomal reformation, leading to the accumulation of enlarged and long-lived autolysosomes. To better investigate how the

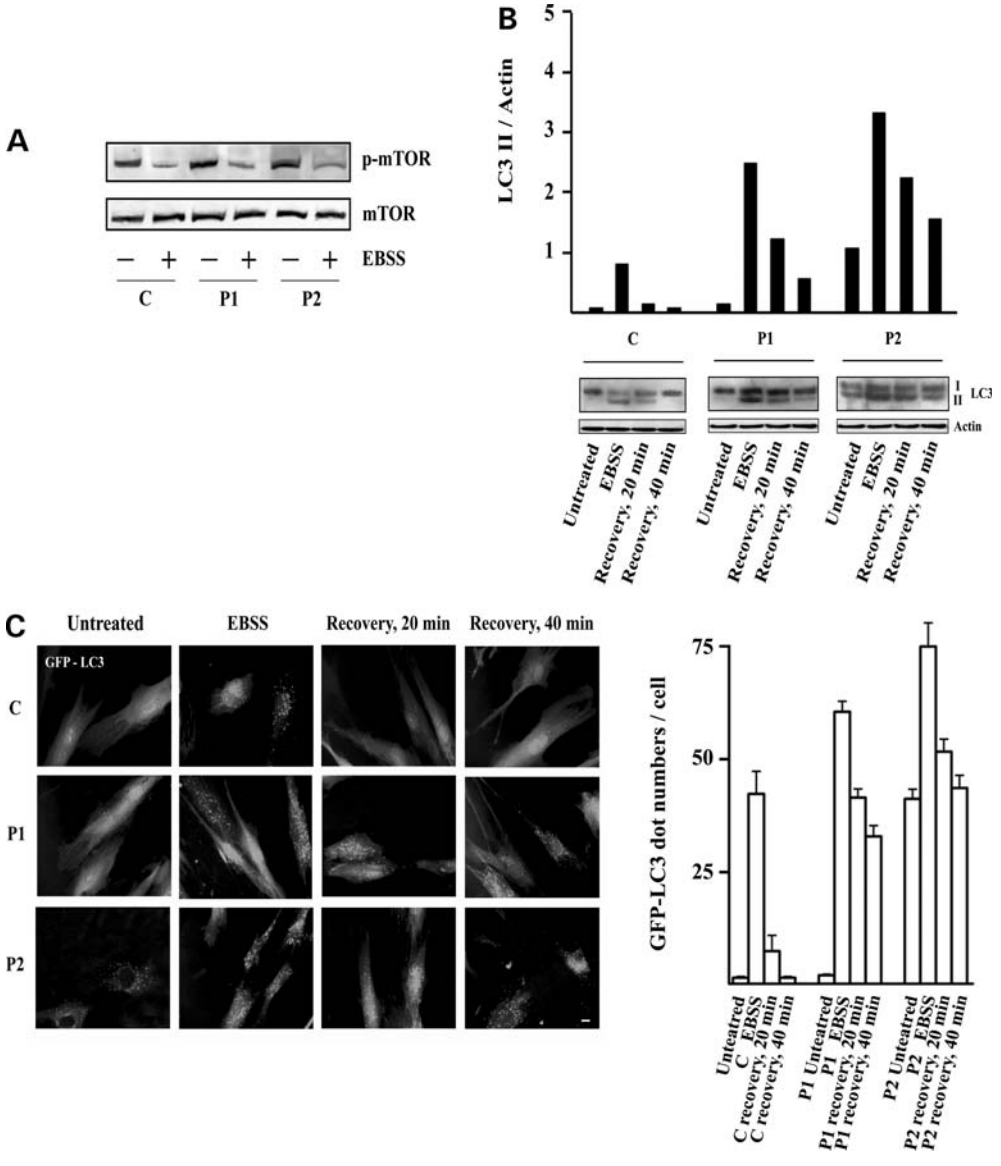


**Figure 3.** Autophagy induction by starvation in Sap C-deficient fibroblasts. (**A**) Western blot of p-mTOR relative to total mTOR in control and Sap C-deficient fibroblasts from untreated cells or following 2 h EBSS starvation. (**B**) Western blot of LC3 in control and Sap C-deficient fibroblasts from untreated cells, cells starved for 2 h in EBSS or cells starved for 2 h followed by 20 or 40 min of recovery. The blots were reprobed for actin as a loading control. Representative blots from three independent experiments are shown. Relative levels of LC3-II were evaluated by densitometric analysis of the bands and normalized as the LC3-II/actin ratio. (**C**) The fluorescence microscopy of GFP-LC3 in control and Sap C-deficient fibroblasts from untreated, EBSS starved for 2 h or starved and then recovered cells for 20 or 40 min. The number of GFP-LC3 dots per cell (mean ± SD) in each condition was quantified. Dot counts were performed on three independent experiments and 30 cells were analyzed in each experiment. Scale bar, 10 μm.

reduced autophagosome degradation observed in our Sap C-deficient fibroblasts might alter recycling of proto-lysosomal membranes into new functionally active lysosomes, cells were starved in EBSS for different times. In control fibroblasts, the level of LC3-II increased, reaching the maximum after 4 h of starvation and then lowered significantly. Differently, the level of LC3-II did not decrease as much as that of untreated cells in pathological cells (Fig. 7A). The same results were obtained examining GFP-LC3 puncta: after 4 h of starvation, there was an increase in punctate dots in all three cell lines that disappeared after 8 h in control fibroblasts but not in pathological cells (Fig. 7B, left panels). Furthermore, the number of GFP-LC3 positive cells was similar in all samples after 2 h of starvation (data not shown); in the following hours (4 h), the increase was different, being more dramatic in P2 (∼80%) than in P1(∼60–70%) compared with control fibroblasts (∼40%). GFP-LC3 was negative after 24 h in control cells, whereas the amount of positive cells was ∼30 and ∼10% in P2 and P1, respectively (Fig. 7B, right panel). After all, the pathological cells had the same behavior, since basal autophagy was present in P2. These results support the altered recovery obtained after a short period of starvation and after leupeptin treatment. We also observed that long starvation (24 h) did not affect cell viability, since only 0.5–1% dead fibroblasts

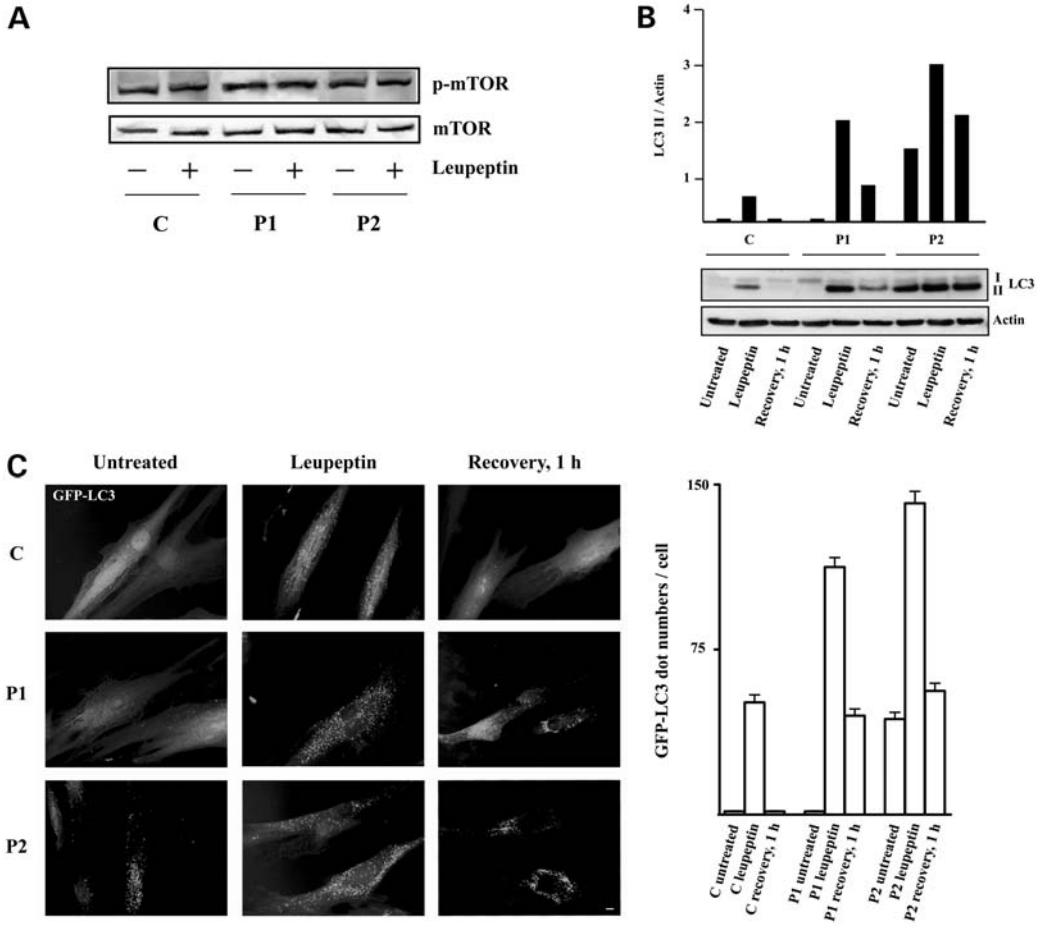


**Figure 4.** Autophagy induction by leupeptin in Sap C-deficient fibroblasts. (**A**) Western blot of p-mTOR relative to total mTOR in control and Sap C-deficient fibroblasts from untreated cells and following 24 h leupeptin treatment. (**B**) Western blot of LC3 in control and Sap C-deficient fibroblasts from untreated cells, cells treated with leupeptin for 24 h or cells treated for 24 h followed by 1 h in leupeptin-free medium. The blots were reprobed for actin as a loading control. Representative blots from three independent experiments are shown. Relative levels of LC3-II were evaluated by densitometric analysis of the bands and normalized as the LC3-II/actin ratio. (**C**) The fluorescence microscopy of GFP-LC3 in control and Sap C-deficient fibroblasts from untreated, treated and recovered cells. The number of GFP-LC3 dots per cell (mean ± SD) in each condition was quantified. Dot counts were performed on three independent experiments and 30 cells were analyzed in each experiment. Scale bar, 10 μm.

were present in control and pathological fibroblasts (data not shown); however, all three fibroblast cultures modified their morphology upon starvation, acquiring a more roundish shape and developing long cytoplasmic processes (Fig. 7C).

To further demonstrate the delayed ALR, we carried out the analysis of intracellular lysosomal localization in response to starvation. Korolchuk *et al*. (25) reported that peripheral lysosomes are correlated with basal nutrient conditions, whereas starvation causes perinuclear clustering of lysosomes. Therefore, untreated and starved fibroblasts were immunostained with Lamp1 (lysosomal-associated membrane protein 1), a marker of late endosomal–lysosomal organelles. Lamp1 positive lysosomes were dispersed widely throughout the cytosol in untreated cells (Fig. 7D, left panels), whereas lysosomes aggregated predominantly in the perinuclear region and became larger size in starved cells (Fig. 7D, middle panels). The perinuclear position of lysosomes lasted until 24 h of starvation in Sap C-deficient fibroblasts, whereas the restoration of peripheral localization began after 8 h and was complete after 24 h of treatment in control cells (Fig. 7D, right panels). Furthermore, Yu *et al*. (24) observed that p-mTOR is inhibited during the initiation of autophagy, but it is reactivated by prolonged starvation after degradation of autolysosomal products. Significantly, the pathological cells showed impaired mTOR reactivation compared with control fibroblasts, after long starvation (Fig. 7E).

## Overexpression of Cath B and Cath D almost completely restores autolysosome degradation in Sap C-deficient fibroblasts

In order to confirm that delayed autolysosomes degradation is due to decreased amount and enzymatic activity of Cath B and Cath D, we overexpressed these proteases by transient infection in Sap C-deficient fibroblasts. Immunofluorescence (Fig. 8A) and western blot analysis (Fig. 8B) showed the efficient enhancement of these two enzymes, whereas endogenous levels of Cath B and Cath D in comparison were quite low. In these transient infection experiments, we usually obtained between 40 and 50% infection efficiency as judged by GFP

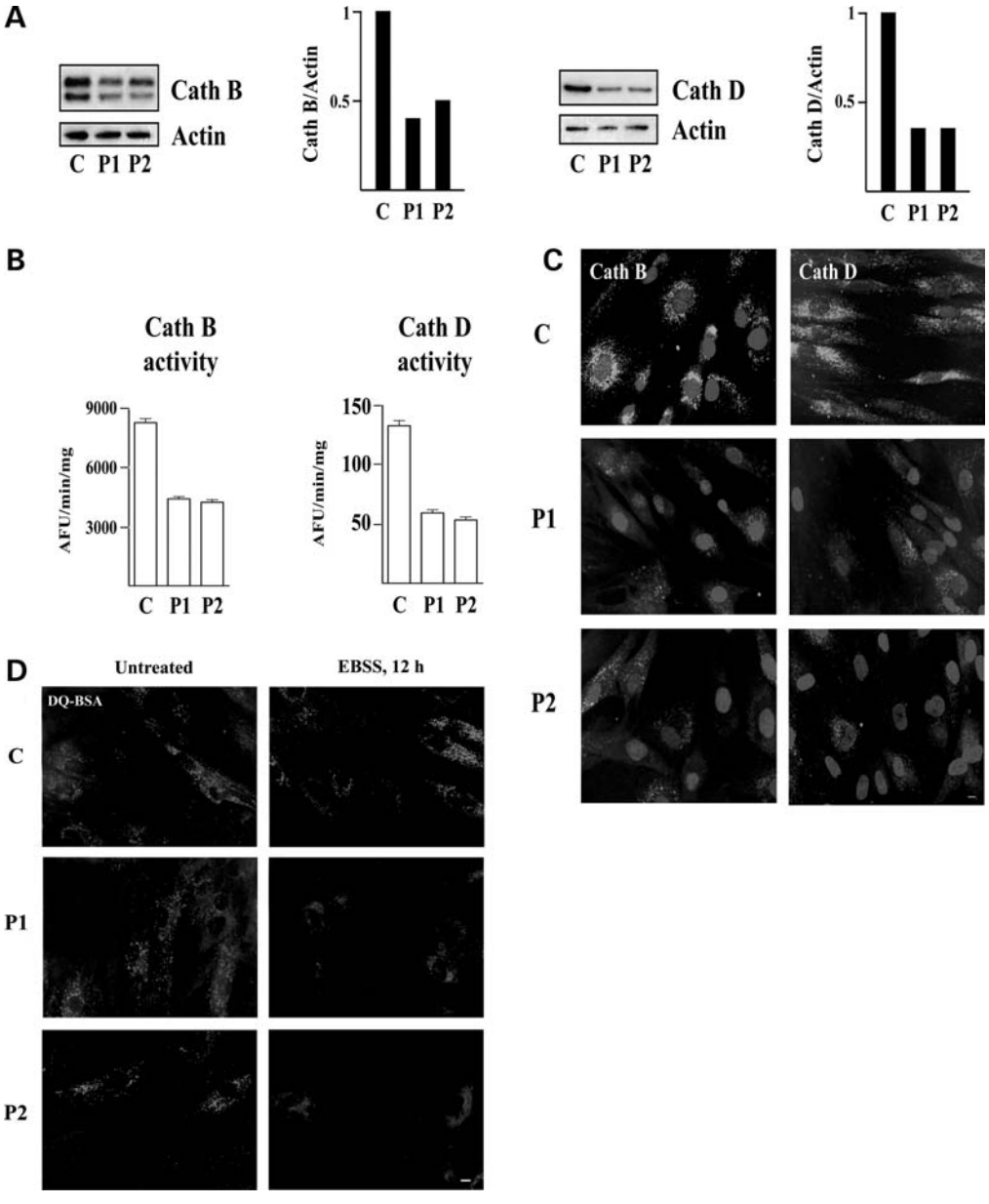


**Figure 5.** Level and enzymatic activity of Cath B and Cath D in Sap C-deficient fibroblasts. (**A**) Western blot and relative densitometric analysis of Cath B and Cath D in control and Sap C-deficient fibroblasts. Normalization of protein loading was performed using anti-actin antibody. Representative blots from three independent experiments are shown. (**B**) Enzymatic activity of Cath B and Cath D in control and Sap C-deficient cells was evaluated in three independent experiments. (**C**) Immunofluorescence of Cath B and Cath D in control and pathological cells. Nuclei were counterstained with Hoechst dye. (**D**) Control and Sap C-deficient fibroblasts were incubated with DQ-BSA in complete medium, starved for 0 or 12 h, and observed by a confocal microscopy. Scale bar, 10 μm.

cotransfection, and an increase in cathepsin amount of at least 2- and 4-fold in P1 and P2 cells, respectively, for Cath B and 4- and 6-fold in P2 and P1 fibroblasts, respectively, in the case of Cath D. The enzymatic activities of Cath B and Cath D well fitted with the levels of these proteins presented in Figure 8B (data not shown). The restored lysosomal activity of both proteases was confirmed by proteolytic degradation of DQ-BSA. We reported above that, following 12 h of starvation, control fibroblasts were still positive for DQ-BSA staining, whereas the majority of Sap C-deficient cells were almost negative (Fig. 5D). When pathological fibroblasts were infected by lentiviral particles containing Cath B and Cath D, their response to starvation totally changed: cells incubated with DQ-BSA remained positive upon 12 h of EBSS medium, with an intense punctate staining filling the whole cytoplasm (Fig. 8C). Finally, we examined the effect of the high level of Cath B and Cath D on the recovery from starvation in pathological cells. We observed that the restoration of complete cell culture medium promoted the total disappearance of LC3-II in P1 fibroblasts and a substantial decrease in P2 fibroblasts (Fig. 8D). These results were also supported observing that p-mTOR was reactivated after prolonged starvation in infected cells (Fig. 8E). These data indicate that less amount and activity of Cath B and Cath D impair autophagic

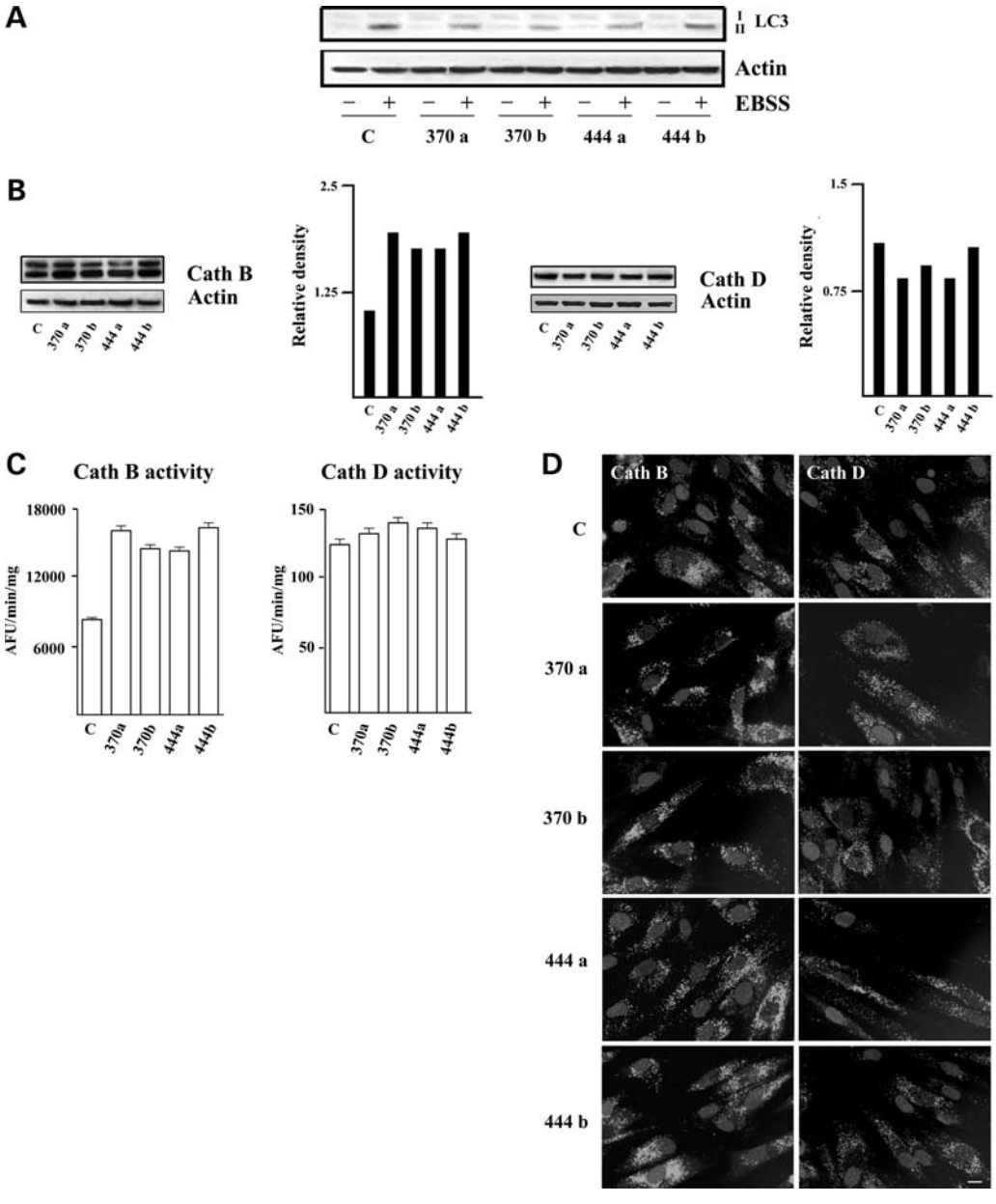


**Figure 6.** LC3, Cath B and Cath D levels and enzymatic activity of these two proteases in GCase-deficient fibroblasts. (**A**) Western blot of LC3 in control and GCase-deficient fibroblasts from untreated and starved cells for 2 h in EBSS. (**B**) Western blot and relative densitometric analysis of Cath B and Cath D in control and GCase-deficient fibroblasts. Normalization of protein loading was performed using anti-actin antibody. Representative blots from three independent experiments are shown. (**C**) Enzymatic activity of Cath B and Cath D in control and GCase-deficient cells was evaluated in three independent experiments. (**D**) Immunofluorescence of Cath B and Cath D in control and pathological cells. Nuclei were counterstained with Hoechst dye. Scale bar, 10 μm.

flux in Sap C-deficient fibroblasts that can be almost completely normalized enhancing the expression of these two lysosomal proteases.

## DISCUSSION

Our previous work documented enhanced autophagy in fibroblasts from two GD patients with Sap C deficiency, carrying mutations involving different cysteine residues in the Sap C-coding region of the *PSAP* gene. In contrast, normal autophagy was present in cells from two other patients with Sap C deficiency carrying a mutation that does not involve a cysteine residue. Based on our results, we hypothesized that the complete absence of Sap C leads to autophagy disturbance, whereas a small amount of this protein, present in patients $PSAP^{p.M1L/L349P}$, is probably sufficient to guarantee a normal autophagic pathway. Apparently, mutations involving cysteine residues and thus affecting the disulfide structure of Sap C lead to a neuronopathic phenotype (16,17).

In this report, we provide experimental evidence for the activation of autophagy in Sap C-deficient fibroblasts. In principle, enhanced autophagy may be due to either increased

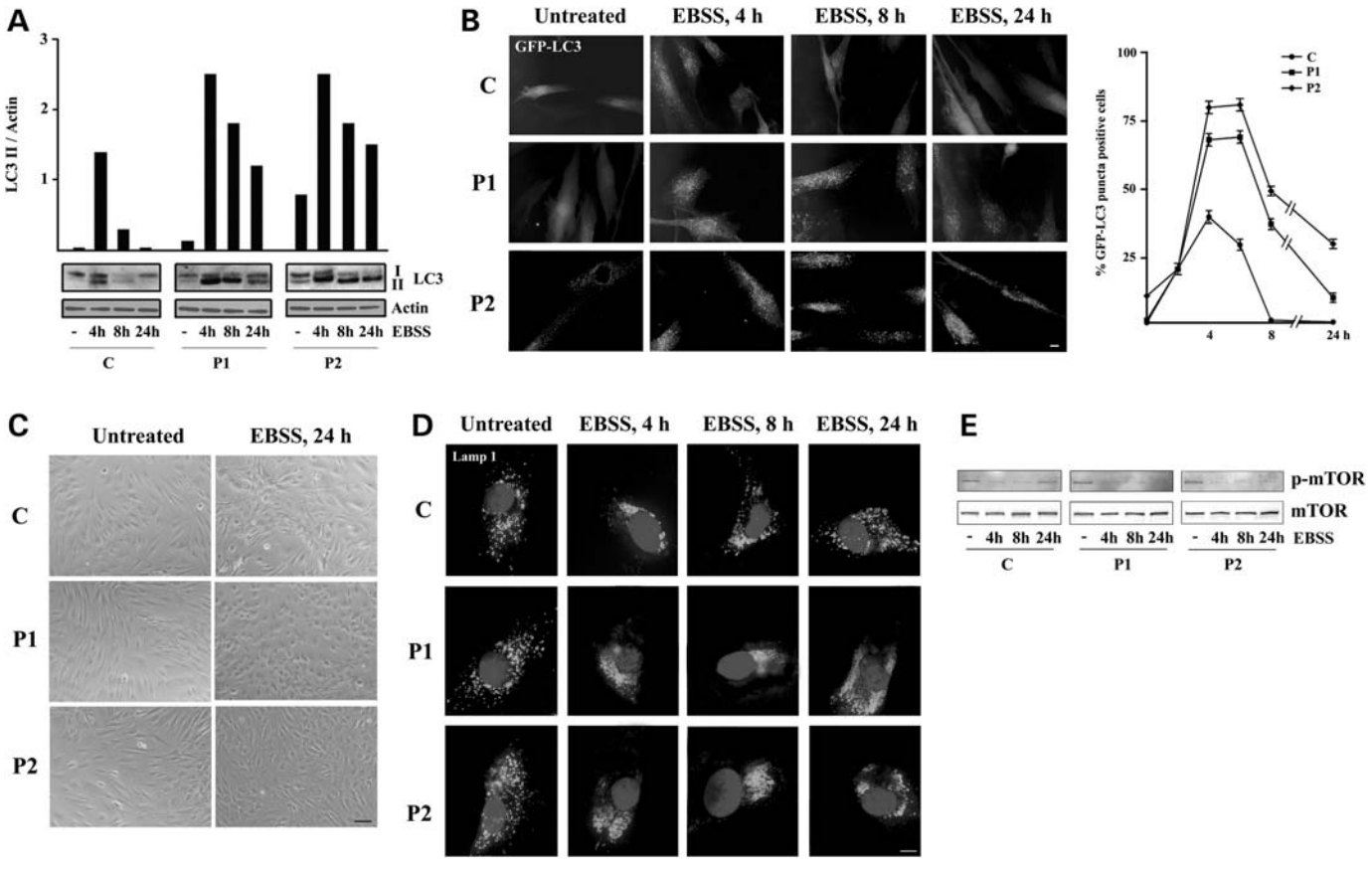


**Figure 7.** Delayed ALR and lysosomal positioning after long starvation in Sap C-deficient fibroblasts. (**A**) Western blot of LC3 in control and Sap C-deficient fibroblasts from untreated or EBSS starved cells for different times (4, 8 and 24 h). The blots were reprobed for actin as a loading control. Representative blots from three independent experiments are shown. Relative levels of LC3-II were evaluated by densitometric analysis of the bands and normalized as the LC3-II/actin ratio. (**B**) The fluorescence microscopy of GFP-LC3 in control and Sap C-deficient fibroblasts from untreated or EBSS starved cells. The percentage of cells with GFP-LC3 puncta was calculated at the indicate time points. (**C**) Phase-contrast images of control and Sap C-deficient fibroblasts before and after 24 h starvation. Scale bar, 100 μm. (**D**) Control and Sap C-deficient fibroblasts were either untreated or EBSS starved for different times (4, 8 and 24 h) and then immunostained with Lamp1 antibody and stained with Hoechst dye to visualize nuclei. Scale bar, 10 μm. (**E**) Western blot of p-mTOR relative to total mTOR in control and Sap C-deficient fibroblasts either untreated or EBSS starved for different times (4, 8 and 24 h).

autophagosome formation or impaired clearance of autophagosomes. Being the autophagosome intermediate structure in a dynamic process, the number of autophagosomes observed at any specific time point is a function of the balance between the rate of their generation and the rate of their conversion into autolysosomes.

We considered three mechanisms that might underlie altered autophagy in Sap C deficiency. One possibility could be the increased autophagosome formation. Beclin 1 is a critical component in the class III PI3 kinase complex involved in autophagosome formation (26). Up-regulation of Beclin 1 is often considered as a hallmark indicative of autophagic induction. Indeed, increased autophagosome formation and concomitant activation of Beclin 1 were observed in some LSDs, such as Niemann–Pick type C, $GM_1$ gangliosidosis, NCL (18–20). Based on the evaluation of Beclin 1, Atg5 and Atg7, however, no convincing evidence was found for enhanced autophagosome formation gene expression in Sap C-deficient fibroblasts. These findings are not consistent with hypothesis that autophagic dysfunction might be due to augmented autophagosome formation (16). Such hypothesis was based on a reduced number of experimental conditions (short starvation and lysosomal protease inhibitor treatment without recovery) showing an accumulation of autophagosomes. Another possible explanation of autophagic dysfunction could be the blockade of autophagosome fusion with lysosomes, which occurs in multiple sulfatase deficiency, mucopolysaccharidosis type IIIA, Pompe disease (27,28). This possibility was verified by treatment with leupeptin, an inhibitor of lysosomal proteases. Leupeptin up-regulated autophagy by strongly increasing LC3-II level and dot numbers of GFP-LC3 in pathological cells in comparison with control fibroblasts. This is consistent with the notion that the fusion of autophagosomes with lysosomes is not blocked in Sap C-deficient fibroblasts. The last mechanism could be a decreased activity of lysosomal proteases resulting in delayed lysosomal clearance. Deprivation of nutrients that induces a cellular stress similar to that elicited by rapamycin, an inhibitor of mTOR, determined an enhanced autophagy in P1 fibroblasts and more dramatic in P2 fibroblasts compared with control cells. We obtained the same results analyzing both the LC3-II level and the GFP-LC3 puncta number. We hypothesize that such

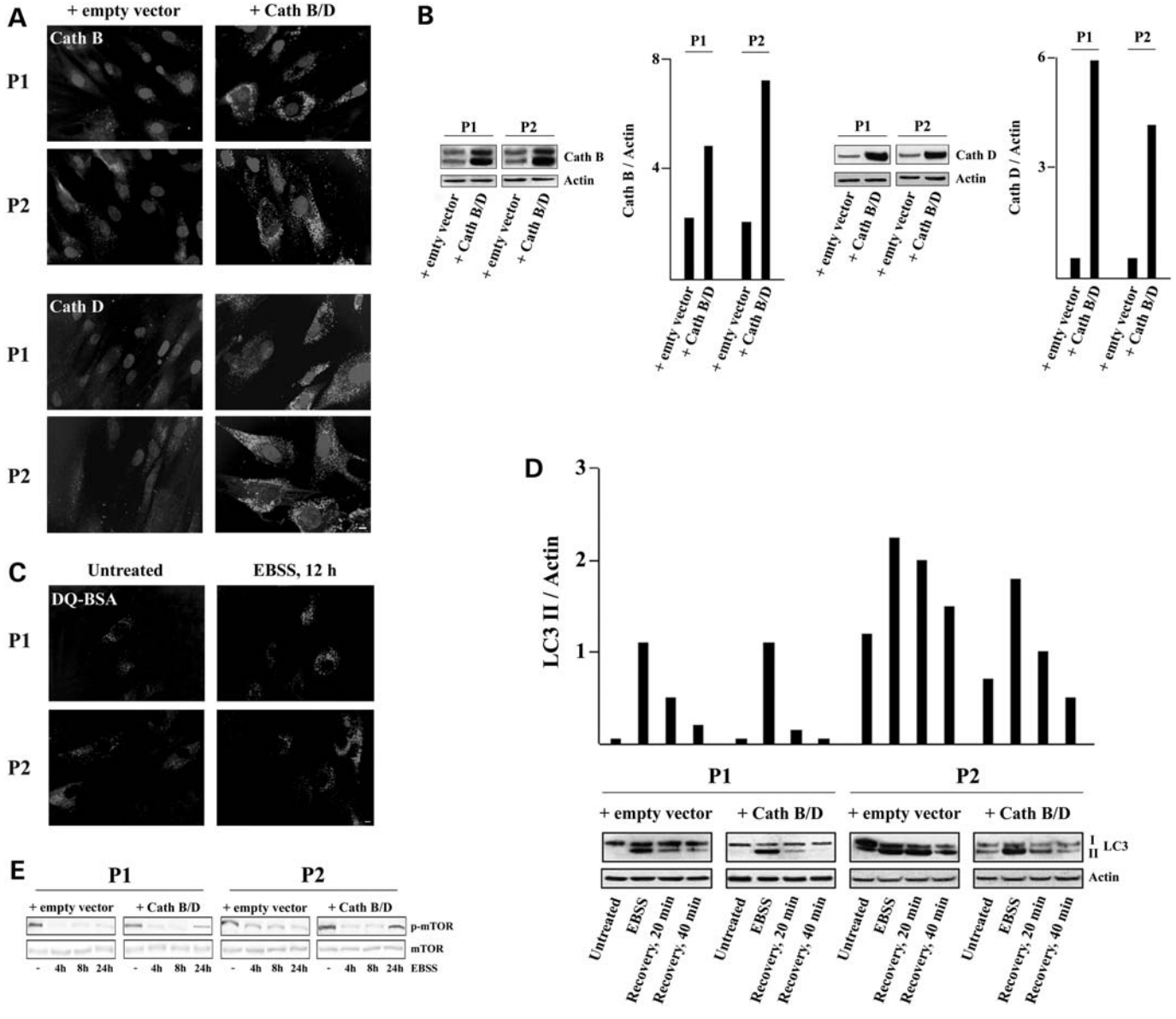


**Figure 8.** Effect of Cath B and Cath D overexpression in Sap C-deficient fibroblasts. Sap C-deficient fibroblasts were infected with expression plasmids for Cath B and Cath D or empty vector. Expression was checked by (**A**) immunofluorescence and (**B**) western blot. (A) Left panels, + empty vector cells; right panels, +Cath B/D cells. Nuclei were counterstained with Hoechst dye. Scale bar, 10 μm. (B) Western blot with relative densitometric analysis of Cath B and Cath D bands in +empty vector and +Cath B/D Sap C-deficient cells. Normalization of protein loading was performed using the anti-actin antibody. Representative blots from three independent experiments are shown. (**C**) +Cath B/D Sap C-deficient fibroblasts were incubated with DQ-BSA in complete medium, starved for 0 or 12 h and observed by a confocal microscopy. Scale bar, 10 μm. (**D**) Western blot of LC3 in +empty vector and +Cath B/D Sap C-deficient fibroblasts from untreated, EBSS starved for 2 h or starved and then recovered cells for 20 or 40 min. The blots were reprobed for actin as a loading control. Representative blots from three independent experiments are shown. Relative levels of LC3-II were evaluated by densitometric analysis of the bands and normalized as the LC3-II/actin ratio. (**E**) Western blot of p-mTOR relative to total mTOR in +empty vector and +Cath B/D Sap C-deficient fibroblasts from untreated or EBSS starved cells for different times (4, 8 and 24 h). Representative blots from three independent experiments are shown.

'phenotype' of P2 cells might be related to the more severe disease features of this patient compared with that of patient P1. Further biochemical and functional analyses are required to confirm this idea. Incomplete recovery was observed in starved pathological cells when cultured in nutrient-rich medium for short times, in comparison with control fibroblasts which instead had complete recovery. These findings indicated that the degradation of autophagosomes in Sap C-deficient fibroblasts was impaired. Moreover, no complete recovery was observed in pathological cells in contrast to control cells, when cultured in leupeptin-free medium. These data confirmed that the degradation of autophagosomal content was delayed. After the fusion of autophagosomes with lysosomes to form autolysosomes, intra-autophagosomal components are rapidly degraded by lysosomal hydrolases, including Cath B, Cath D and Cath L, the most abundant proteases (22). Our analysis revealed that the catalytic activities and the intracellular levels of mature forms of Cath B and Cath D were decreased

in pathological fibroblasts. Confocal microscopy analysis further confirmed the reduced amount of Cath B and Cath D in Sap C-deficient fibroblasts. These results were also corroborated by the poor degradation capacity to cleave DQ-BSA observed in mutant fibroblasts. On the contrary, we observed that GD fibroblasts with deficit of GCase do not show either autophagic dysfunction or a reduction in Cath B and Cath D, but even Cath B activity is almost doubled compared with control cells. These findings support our hypothesis that Sap C lack can be responsible for Cath B and Cath D down-regulation. Furthermore, earlier observations by Moran *et al*. (29), and lately by van Breemen *et al*. (30), revealed that several cysteine proteases, including Cath B, Cath K and Cath S, are overproduced by Gaucher storage cells. Recently, Vitner *et al*. (31) reported elevated protein levels and activities of different Caths in the brain of a mouse model of neuronopathic GD. Overexpression and not down-regulation of Cath B and/or Cath D were also found in other sphingolipidoses. For instance, Cath B and Cath D activities were increased in Niemann–Pick type C-deficient mice (32), and mRNA expression of Cath B, Cath C and Cath S was elevated in Tay–Sachs and Sandhoff diseases (33,34).

Based on this evidence, we suggest that the defect in Cath B and Cath D levels and activities may underlie the retarded autophagic progression. The mechanism(s) responsible for reduced amounts and activities of Cath B and Cath D remain to be established. Our results indicate that the complete lack of Sap C leads to autophagy impairment such as a manifestation of a more general phenomenon, due to lysosomal dysfunction. Altered lysosome function seems more serious in Sap C-deficient fibroblasts compared with that observed in examined GCase-deficient cells. It might be speculated that lysosomes are more lipid engorged in Sap C-deficient fibroblasts, since Sap C is not only required for GC hydrolysis and GCase stabilization but also for extraction and transport of lipids from lysosomes to other vesicles or compartments (16).

Recent studies regarding the autophagic pathway in LSDs established either the induction of autophagy or a defective autophagosome/lysosome fusion as cause of autophagosome accumulation. Our findings revealed for the first time enhanced autophagy associated with reduced expression and activity of two lysosomal proteases in one LSD. This represents a novel attempt to explain altered autophagy in neurodegenerative LSDs. Only the complete lack of Cath D enzyme, associated with a human neurodegenerative form of NCL disease with severe mental retardation, has been reported among LSDs (35–37). A similar neurological pattern is also observed in mice after the deletion of both Cath B and Cath L (38). Notably, Nogalska *et al*. (39) have reported results similar to ours in sporadic inclusion-body myositis (s-IBM). In cultured human s-IBM muscle fibers, the enhanced accumulation of autophagosomes is due to impaired activities of Cath B and Cath D (39). Instead, processing and activities of Cath B and Cath L are also reduced in rodent models of acute pancreatitis (40). Recent research confirmed Cath B enzymatic activity as an important regulator of autophagy flux (41).

The impaired catabolism of autolysosomes delays lysosome reformation and mTOR reactivation resulting in long-lasting autolysosomes. This is further confirmed by lysosomal positioning: during starvation, lysosomes are perinuclear, after recovery in nutrient-rich medium lysosomes localize peripherally, regulating mTOR signaling (25). Our results indicate that the perinuclear position of lysosomes lasted until 24 h of starvation in Sap C-deficient fibroblasts, whereas in control fibroblasts peripheral lysosomal localization is almost restored after 8 h of starvation. Moreover, mTOR reactivation was impaired in mutant cells. Based on our findings, we propose a model in which the defective amounts and enzymatic activities of Cath B and Cath D impair lysosomal function resulting in inefficient protein catabolism, reduced clearance of autolysosomes and delayed ALR (Fig. 9). Induction of autophagy and resultant enhanced protein degradation would protect the cell from toxic insults; instead, the accumulation of autophagosomes without their efficient clearance could contribute to cell death. Impairments of lysosomal function are mild to be tolerated in fibroblasts but not in tissues with a low regeneration rate such as the heart and muscle or in non-mitotic cells such as neurons that cannot remove intracellular aggregates by cell division. The accumulation of undegraded proteins in neurons could result in cell death and explains the prevalence of a neuronopathic phenotype in LSDs. Our data suggest that autophagic disturbance observed in Sap C deficiency most likely arises from impaired activity of Cath B and Cath D, decreased protein degradation and probably delayed reformation of lysosomes. These results are in line with Yu *et al*. (24) indicating that fibroblasts derived from patients with LSDs, Scheie syndrome, Fabry disease and aspartylglucosaminuria show impaired mTOR reactivation and defective lysosomal reformation.

In agreement with our observations, we found that transient overexpression of Cath B and Cath D improves the degradation of autolysosomes and almost totally restores autophagic flux in both Sap C-deficient fibroblasts.

Since no effective therapies are available for treatment for Sap C deficiency, our observations can be of significance in the design of therapeutic approaches. Actually, Tylki-Szymanska *et al*. (42) recently reported that the administration of Miglustat, a drug utilized for substrate reduction therapy, in one Sap C-deficient patient with a non-neuronopathic phenotype failed without any effect in the clinical conditions. Thus, based on our findings, the modulation of Cath B and Cath D expression might have therapeutic implication for Sap C deficiency and probably for other neurodegenerative disorders associated with up- or down-regulation of Caths.

## MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM), Nu-PAGE (polyacrylamide gel electrophoresis) gels and electrophoresis reagents were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Waltham, MA, USA). Complete$^{TM}$ (protease inhibitor mixture) was obtained from Roche Diagnostics (Mannheim, Germany). ECL western blotting reagents, Hyperfilm$^{TM}$ ECL were from GE Healthcare (Buckinghamshire, UK). EBSS, leupeptin and rapamycin were obtained from Sigma Aldrich (St Louis, MO, USA). ProLong anti-fade kit and DQ-BSA were obtained from Molecular Probes (Eugene, OR, USA). cDNA for human Cath B was obtained

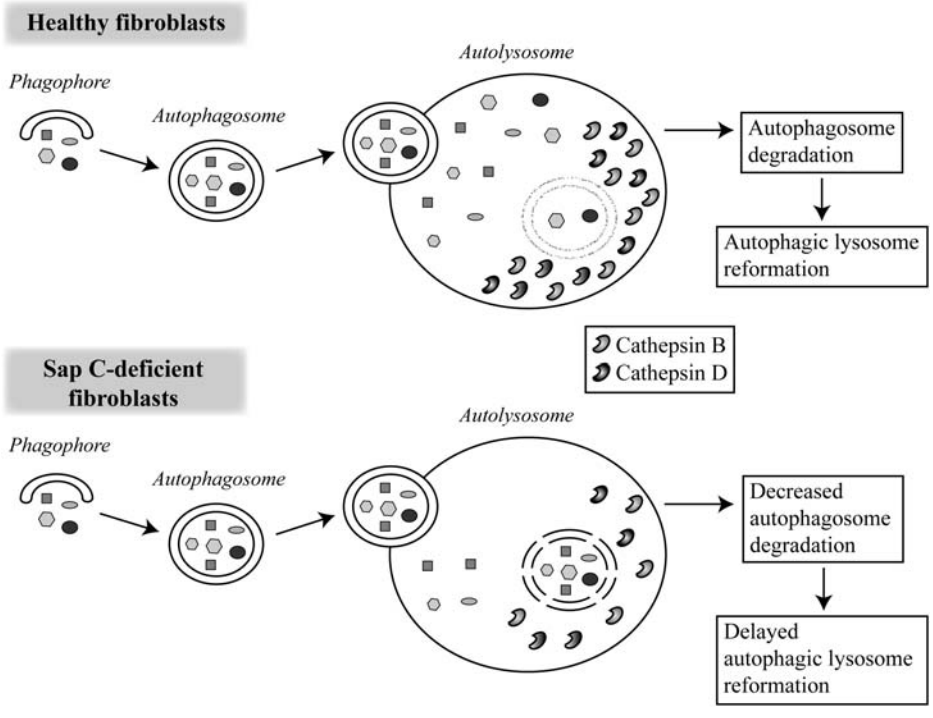


**Figure 9.** A proposed model of altered autophagy in Sap C-deficient patients. In healthy cells, the content of autolysosomes, derived by fusion of autophagosomes with lysosomes, is degraded by lysosomal proteases, leading to reformation of lysosomes. In mutant cells the complete absence of Sap C results in the accumulation of undegraded substrates in the lysosomes, altered autophagy and reduced amount and enzymatic activity of Cath B and Cath D. This alteration leads to delayed degradation of autolysosomes and to defects in lysosome reformation.

from Addgene-Lablife Plasmid Repository (Cambridge, MA, USA) and cDNA for Cath D was a kind gift of H. Rochefort (INSERM, Montpellier, France). Both cDNA for Cath B and Cath D were subcloned in the pBOB modified vector (Addgene-Lablife Plasmid Repository) by using *Xba*I and *Xho*I restriction enzymes (Promega, Fitchburg, WI, USA).

All other reagents were of the purest available grade.

### Cell cultures and infection

Six GD fibroblast lines were utilized: two from Sap C-deficient patients, P1 ($PSAP^{p.del.FDKMCSK342-348/del.FDKMCSK342-348}$) and P2 ($PSAP^{p.M1V/C315S}$), and four from GCase-deficient patients. Two enzyme-deficient cells were homozygous for the N370S mutation (370a and 370b) and two homozygous for L444P mutation (444a and 444b). Control fibroblasts were from normal subjects. Control and GD cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin. The fibroblasts were used between passages 4 and 12. Experiments were carried out when cell monolayers were 80% confluent. Fibroblasts were harvested, rinsed with phosphate-buffered saline (PBS) and lysed on ice in radioimmunoprecipitation assay buffer, pH 7.4, containing one tablet of protease inhibitor mixture/10 ml. Lysates were precleared by centrifugation at 10 000*g* for 15 min at 4°C and the supernatants used as fibroblast homogenates.

Subconfluent cells were infected by lentiviral particles containing GFP-LC3, Cath B and Cath D cDNAs or empty vector, as described by Di Bartolomeo *et al*. (43).

### Cell morphological examination

Cell morphology of control and Sap C-deficient fibroblasts was examined before and after 24 h of starvation using an inverted phase-contrast microscope.

### Drug treatment and starvation

To induce autophagy in an mTOR-dependent manner, fibroblasts were treated with rapamycin to a working concentration of 1 μM for 24 h or starved in EBSS by the removal of amino acids and serum for different times. To examine the recovery of cells from starvation, fibroblasts incubated in EBSS for 2 h were transferred to complete cell culture medium for the indicated times. To inhibit LC3-II degradation, cells were treated with leupeptin diluted in culture medium to a working concentration of 10 μg/ml for 24 h. For the autophagy recovery, cells were washed twice with PBS after inhibitor protease treatment and leupeptin-free medium was added.

### Antibodies

The primary antibodies used were: mouse monoclonal anti-actin, mouse monoclonal anti-cathepsin B, mouse monoclonal anti-cathepsin D (Sigma Aldrich), goat polyclonal anti-cathepsin D (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse monoclonal anti-Lamp1 (Developmental Studies Hybridoma Bank, maintained by the University of Iowa, USA), rabbit polyclonal anti-LC3 (MBL International, Woburn, MA, USA), rabbit polyclonal anti-Beclin 1, rabbit polyclonal anti-Atg5, goat polyclonal anti-Atg7 (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-p-mTOR and rabbit polyclonal anti-mTOR (Cell Signaling Technology, Inc., Danvers, MA, USA).

The secondary antibodies used were: goat anti-mouse, goat anti-rabbit and rabbit anti-goat conjugated to Alexa Fluor 488 or 594 (Molecular Probes) and horseradish peroxidase-conjugated anti-rabbit or anti-mouse (GE Healthcare).

### Western blot analysis

Fibroblast homogenates were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Italy). Membranes were blocked for 1 h with 5% non-fat milk powder in PBS containing 0.1% Tween-20 and incubated for 1 h with specific antibodies. Primary and secondary antibodies were diluted in blocking solution. Immunoreactive proteins were detected by an ECL Advance™ Western Blotting Detection kit, according to the manufacturer's instructions (GE Healthcare).

### Autophagy assays

Autophagy in Sap C-deficient fibroblasts was monitored analyzing LC3, the highly specific autophagosomal marker. Under basal conditions, LC3 shows a cytosolic distribution (LC3-I). Increased autophagic activity is reflected by enhanced conversion of LC3-I (cytosolic form) to LC3-II (lipidated form), whose amount correlate with the number of autophagosomes. LC3-II is the only known protein that specifically associates with autophagosomes and not with other vesicular structures (44,45). Autophagic activity was also monitored following changes in the distribution of GFP-tagged LC3 transiently expressed in control and Sap C-deficient fibroblasts. Normally, a low level of GFP-LC3 fluorescence is evenly distributed in the cytosol. Enhanced autophagy leads to the recruitment of GFP-LC3 to autophagosomes indicated by the increased number of GFP-LC3 puncta (46).

### Fluorescence and immunofluorescence microscopy

GFP-LC3 transfected cells were visualized by a fluorescence microscope. To determine the activation of autophagy, GFP-LC3-expressing cells were grown on Labteck chamber slides (Nunc, Naperville, IL, USA), incubated with rapamycin or leupeptin in culture medium or starved for the indicated times. Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) at 4°C for 20 min. Cells were analyzed at ×50 magnification. GFP-LC3 fluorescent staining was characterized by a typical punctate cytoplasmic pattern. The number of GFP-LC3 dots per cell were counted and reported in the graph. Thirty cells were analyzed in each experimental condition in at least three independent experiments. The obtained quantitative values were then statistically analyzed and compared.

For immunofluorescence, cells were grown and fixed as described above. Cells were then rinsed with PBS, permeabilized 7 min with 0.05% saponin and incubated 2 h with 3% BSA (w/v). Cells were incubated for 1 h with a specific primary antibody, rinsed twice with PBS and incubated 1 h with the secondary antibody (Alexa Fluor 594 or Alexa Fluor 488-conjugated goat anti-rabbit, goat anti-mouse or rabbit anti-goat). Finally, cells were rinsed twice with PBS and the nuclear staining was performed with Hoechst (Sigma Aldrich) 10 μg/ml in PBS for 5 min. After a final rinse with PBS, cells were mounted with ProLong antifade reagent and then analyzed by a fluorescence microscope (Olympus BX52) or a confocal microscope (Leica TCS SP2). Acquisition and processing were conducted by using the I.A.S.2000 and Adobe Photoshop software programs.

### Cath B and Cath D enzymatic activity

Cell pellets were lysed in PBS containing 0.05% (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate) (w/v) for 30 min on ice (47). After sonication for 20 s, lysates were centrifuged at 15 000*g* for 15 min at 4°C. The resultant supernatants were used for enzymatic assays. The activity of Cath D was measured utilizing the fluorogenic substrate MCA-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-$NH_2$ (Sigma Aldrich) as described by Yasuda *et al*. (47). The activity of Cath B was assayed using the fluorogenic substrate Z-Arg-Arg-AMC (Biomol International, Plymouth, PA, USA) as described by Barrett and Kirschke (48).

### Test of lysosomal function by degradation of a chromogenic BSA

A DQ-BSA was used for the detection of the degradation capacity of lysosomes and autolysosomes (23). This BSA derivative is so heavily labeled that the fluorophore is self-quenched. Proteolysis of this compound results in dequenching and release of brightly fluorescent fragments. Control and pathological fibroblasts were grown on Labteck chamber slides, incubated for 1 h at 37°C with DQ-BSA (10 μg/ml) in complete culture medium. Cells were washed twice with PBS to remove excess probe and then incubated in starvation medium (EBSS) for 12 h. After fixation in 4% paraformaldehyde, cells were immediately analyzed by a confocal microscopy.

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