◂**Fig. 6** Rac1 activity and cell migration are altered in CMT2B fibroblasts carrying the RAB7^{V162M} mutation. **a** Lysates of two controls and three CMT2B fibroblasts were analyzed by immunoblotting using anti-AKT, anti-pAKT, anti-ERK and anti-pERK. Bands were quantified using NIH ImageJ and normalized against total protein. All data represent the mean ± SEM of at least three experiments. Statistical analysis was performed using Student's *t* test with control fibroblasts as referring sample. *$p < 0.05$, *$p < 0.01$, ***$p < 0.001$. **b** Fibroblasts from control and CMT2B patients were imaged during wound healing assay. Images of initial time point (T0), 15 h and 24 h after the scratch are shown. Accumulated distance, the total distance that the cell traveled in a certain amount of time, is shown. Data represent the mean ± SEM of four experiments. Statistical analysis was performed using Student's *t* test with control fibroblasts as referring sample. *$p < 0.05$; ***$p < 0.001$. **c** Lysates of control and CMT2B fibroblasts (patient 1) were subjected to RAC1 activation assay and then subjected to western blot analysis using anti-RAC1 and anti-tubulin antibody. Quantification of active RAC1 in control and CMT2B cells is shown. Data represent the mean ± SEM of at least three experiments. **$p < 0.01$. **d** Gelatin zymography was performed using conditioned medium of fibroblasts derived from a healthy individual and three CMT2B patients. Representative results are shown. **e** Lysates of two controls and three CMT2B skin fibroblasts were analyzed by immunoblotting using anti-ARHGEF6 and anti-RACGAP1 antibodies. Bands were quantified using NIH ImageJ and normalized against tubulin. All data represent the mean ± SEM of at least three experiments. Statistical analysis was performed using Student's *t* test with control fibroblasts as referring sample. *$p < 0.05$

agreement with lower abundance of ESCRT proteins. Furthermore, it has been demonstrated that one of the RAB7 mutant causing CMT2B, the RAB7^{K157N}, interacts weakly with a subunit of the retromer complex that controls endosome-to-Golgi retrieval of CI-MPR receptor, thus reducing the efficiency of endosomal protein sorting [75]. Therefore, increased expression of CI-MPR in CMT2B cells could be due to a compensatory mechanism to counteract the presence of the RAB7^{V162M} mutant protein.

We observed increased degradative activity in CMT2B cells. Our data are in agreement with previous findings demonstrating that CMT2B-causing RAB7 mutants show increased interaction with ORP1L (cholesterol sensor oxysterol-binding protein-related protein 1L), and RILP (Rab-interacting lysosomal protein), two RAB7 effector proteins controlling transport of endosomes from the periphery of the cell toward the MTOC (MicroTubule Organizing Center) during endosome maturation [34]. Accordingly, it was shown that the expression of CMT2B-causing mutants restores EGFR degradation that was inhibited after RAB7 silencing [35, 36]. Moreover, higher lysosomal activity and increased degradation of EGFR in CMT2B cells suggest increased degradation also of other signaling receptors and consequent inhibition of signaling. Notably, expression of RAB7 mutant proteins affects the axonal transport by modifying trafficking and signaling of NGF (nerve growth factor) and its receptor, TrkA [76]. Importantly, it was also demonstrated that CMT2B-causing RAB7 mutants cause a reduction of TrkA surface level, which was hypothesized to be the consequence of premature degradation of TrkA induced by lysosomal activity [76]. In fact, hyperactivation of degradation within axons could induce a premature termination of TrkA signaling, with consequent inhibition of retrograde signaling, possibly contributing to axonal degeneration [76]. Finally, it was reported that increased endocytic flux in neurons induces neurodegeneration in Alzheimer's disease, as a consequence of reduced neurotrophin receptors expression and signaling, [67, 69]. Thus, we hypothesize that the increased lysosomal activity could affect signaling because of premature degradation of signaling receptors, thus contributing to the axonal degeneration occurring in CMT2B disease.

We also observed an increased migration of CMT2B fibroblasts, though EGFR degradation was higher in these cells. These results may appear contradictory, as it was shown that the downstream signaling pathways of numerous receptor tyrosine kinases, including EGFR, are involved in the regulation of cell motility [77, 78]. Indeed, extracellular-regulated kinase (ERK), Jun kinase and tumor protein (p)38 affect various cell functions, including migration [79]. Also phosphatidylinositol-3 kinase (PI3K) controls cell motility through the activation of protein kinase B (Akt) and other targets [80, 81]. However, cell migration does not exclusively depend on EGFR signaling. In fact, it was demonstrated that inhibition of EGFR activation in tumor cells leads to activation of a β1 integrin pathway that promotes migration, undermining EGFR blockade [82]. Furthermore, there are many mechanisms that promote migration independently of Akt, and one of the most important is the remodeling of the actin cytoskeleton mediated by RAC1 [83, 84]. In fact, it was found that β1 integrins locally activate RAC1 [85]. In CMT2B cells, we found an increased RAC1 activation, while we did not detect any difference in RAC1 total protein amount. Therefore, our data are in agreement with previous findings showing the existence of alternative pathways that regulate cell motility. Furthermore, it was previously reported that RAC1 promotes MMP-2 activation [63] and we observed higher activation of this gelatinase in CMT2B patients. Thus, a decrease in EGFR signaling due to its higher degradation could trigger β1 integrin pathway that leads to RAC1 and MMP-2 activation, causing increased migration of CMT2B cells in an EGFR-independent manner.

We previously found that the expression of CMT2B-causing RAB7 mutants negatively affects the role of RAB7 in autophagy [43]; in particular, in CMT2B fibroblasts we observed a reduced localization of RAB7 on autophagosomes after autophagy induction, a decreased number of LC3B-positive vesicles and a reduced autophagic flux, demonstrated by bafilomycin A_1 treatment [43]. We also established that the expression of the RAB7^{V162M} does not affect autophagosomal biogenesis, but it only alters later phases of autophagosome maturation [43]. Considering

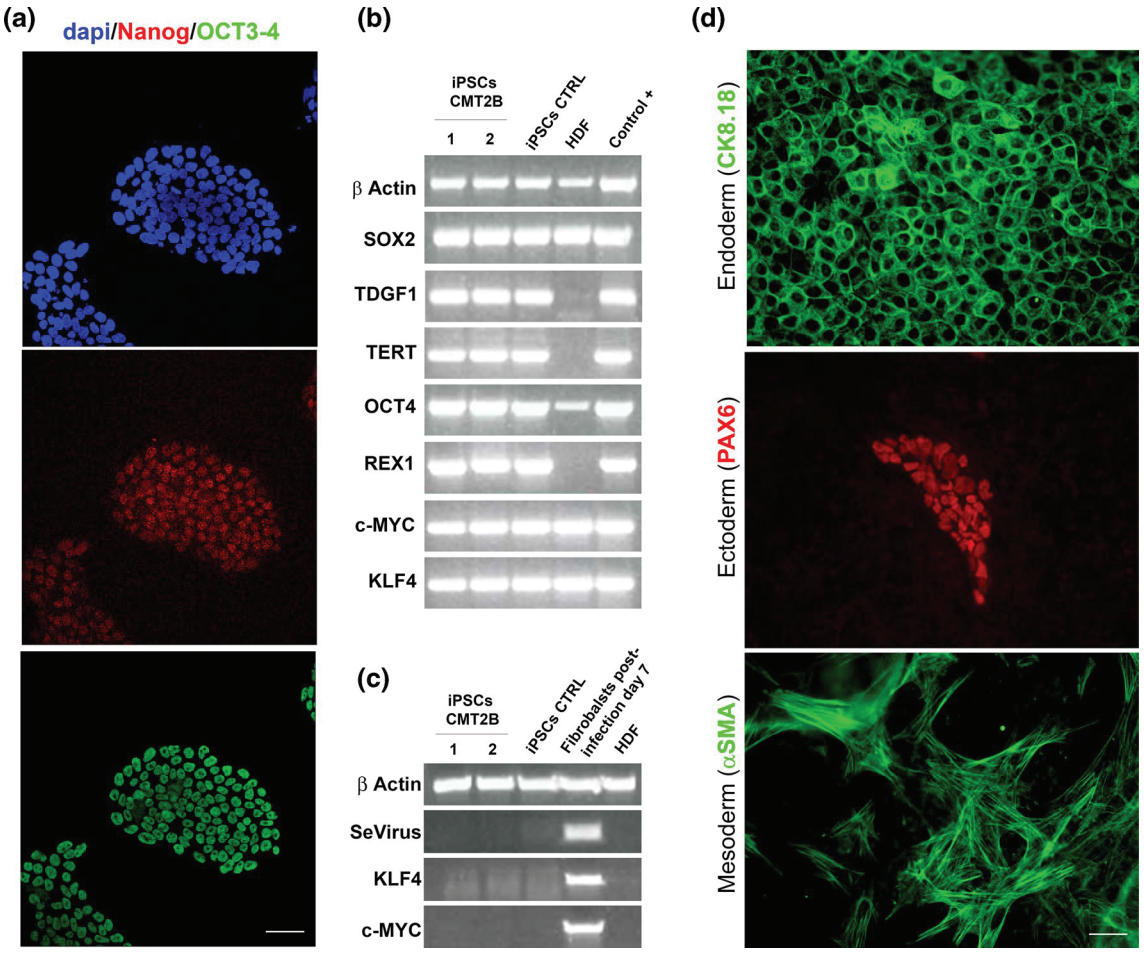

Fig. 7 Expression of human embryonic stem cell markers and pluripotency of control and CMT2B hiPS cells. **a** Immunohistochemistry for embryonic stem (ES) cell markers of CMT2B iPS cells. Bars 50 μm. **b** RT-PCR analysis of ES cell-marker genes in human iPS cells, human dermal fibroblast (HDF) and previously derived iPS cell as positive control. Primers used for Oct3/4, Sox2, Klf4, and c-Myc specifically detect the transcripts from the endogenous genes, but not from the retroviral transgenes. **c** RT-PCR for expression of retroviral transgenes in human iPS cells, HDF, and HDF 7 days after the transduction with the four retroviruses as a positive control. **d** Immunohistochemistry of iPS cells spontaneously differentiated show markers for the three germ layers. Bars 50 μm

that RAB7 controls fusion of autophagic vacuoles with late endosomes and lysosomes, the present data, showing increased expression of lysosomal membrane proteins and enzymes and increased lysosomal activity, seem to be in contrast. However, it was reported that thapsigargin distinguishes membrane fusion in the late stages of the two pathways, endocytosis and autophagy, indicating that the two pathways, although sharing many components of the membrane traffic machinery, can be independently regulated [86, 87]. In addition, CMT2B mutations do not generate classical gain or loss of function RAB7 mutants. It was reported that RAB7 mutants would cause the disease due to the misregulation of native RAB7 activity [34]. Indeed, these mutant proteins display a higher nucleotide K_{off} and thus they tend to release nucleotides (both GTP and GDP) earlier as compared to the wt protein [34–36]. The k_{off} is higher for GDP than for GTP and, considering that GTP concentration in the cell is approximately one order of magnitude higher than GDP, these mutants are mainly in the GTP-bound form, as each time that they release GDP prematurely they have a higher probability to bind GTP. However, due to the increased K_{off} for GTP compared to the wt protein, they also tend to release GTP prematurely and this affects negatively the GTPase activity per binding event [34–36]. Thus, these mutant proteins are dysfunctional, as they have unregulated nucleotide exchange and activation [34]. They can stimulate a process as they are GTP bound but, at the same time, if the reaction controlled by RAB7 requires being stimulated by RAB7-GTP for a certain amount of time, premature release of GTP could block the controlled process. Hence, depending on the kinetic requirements of the regulated process, these mutant proteins could behave as active or inhibitory mutants. These considerations may explain the apparently conflicting data present in the literature. Indeed,

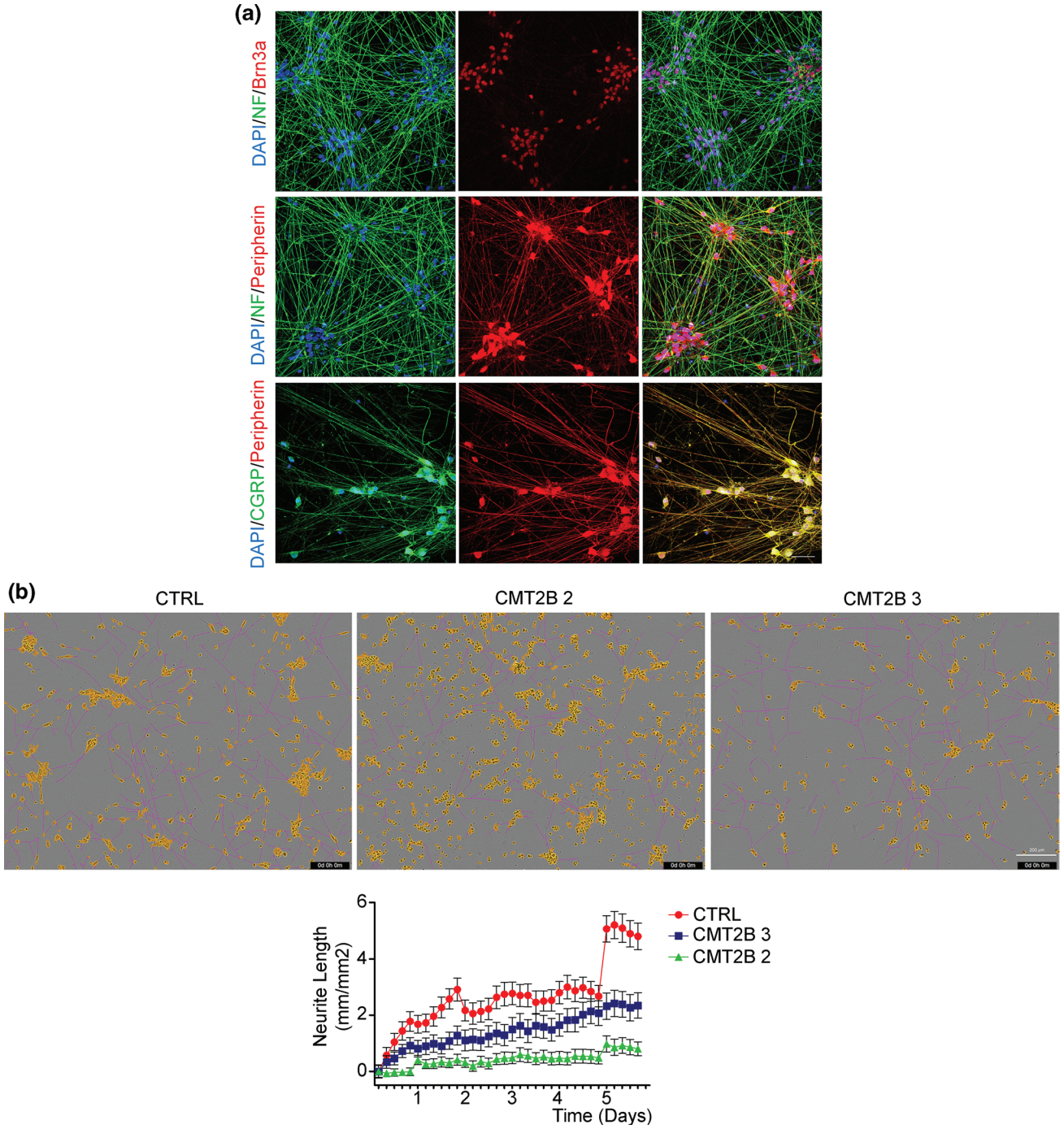

Fig. 8 CMT2B peripheral sensory neurons show inhibited neurite outgrowth. **a** Control iPS cells were differentiated in peripheral sensory neurons and analyzed by immunohistochemistry at 17 days of differentiation for Brn3a, Neurofilaments, Peripherin and CGRP. Bars 50 µm. **b** Phase contrast images of iPSCs-derived sensory neurons from control (CTRL) and CMT2B patients carrying the RAB7^{V162M} mutation (#2 and #3) 6 days after plating. The IncuCyte automated acquisition and analysis show the extension of neurites growth (pink). Neuron cell body clusters are marked in yellow. Bar 200 µm. Automated quantification of neurite length for a time window of 6 days after plating. Neurite length data were collected in units of mm of total neurites detected/mm^2 and all data are expressed relative to the respective day 0 of plating. Each group is represented by as mean ± SEM ($n = 32$ image)

for instance, in the Drosophila model of the disease, it was established that neurodegeneration was due to a partial loss of function of RAB7, while studies on zebrafish showed that CMT2B-causing RAB7 mutations induce defects in axon growth, similarly to the constitutively active form of RAB7, thus suggesting that defects observed in the neuropathy are

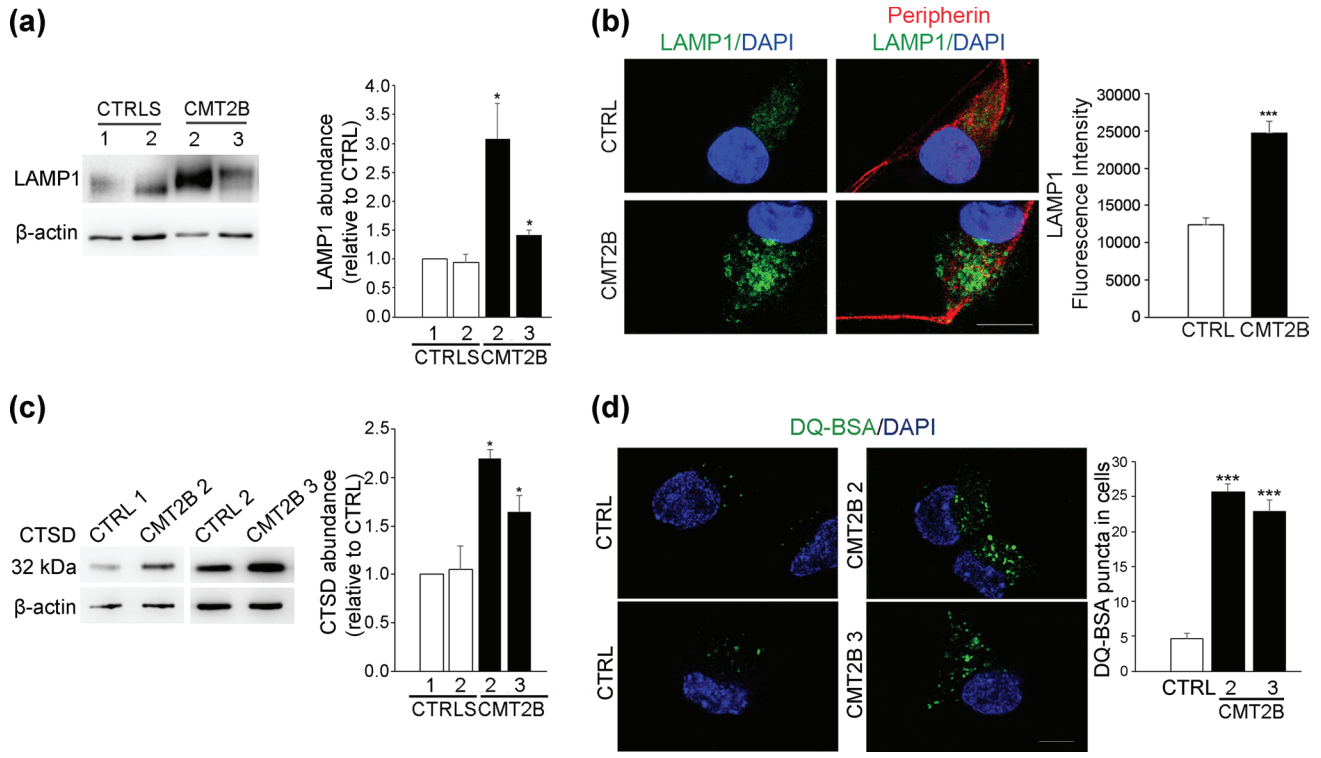

Fig. 9 Lysosomal activity in iPSC-derived sensory neurons. **a** Lysates of neurons derived from controls and CMT2B fibroblasts carrying the RAB7^{V162M} mutation (patient 2 and 3) were subjected to immunoblotting using anti-LAMP1 and anti-β-actin antibodies. Intensities of bands were measured by densitometry and normalized against β-actin. Data represent the mean ± SEM of three experiments. Statistical analysis was performed using Student's *t* test with control neurons as referring sample. *$p < 0.05$. **b** Control and CMT2B neurons from patient 2 were fixed and immunolabeled with anti-LAMP1 and anti-peripherin antibodies followed by Alexa488- and Alexa568-conjugated secondary antibody respectively while nuclei were stained with DAPI. Bars 10 µm. LAMP1 intensities were measured using ImageJ and Corrected Total Cell Fluorescence (CTCF) was calculated. Data represent the mean ± SEM of at least 50 cells analyzed. Student's *t* test was used for statistical analysis. ***$p < 0.001$. **c** Lysates of neurons derived from controls and CMT2B fibroblasts (patient 2 and 3) were subjected to immunoblotting using anti-cathepsin D and anti-β-actin antibodies. Intensities of bands were measured by densitometry and normalized against β-actin. Data represent the mean ± SEM of three experiments. Statistical analysis was performed using Student's *t* test with control neurons as referring sample. *$p < 0.05$. **d** Control and CMT2B neurons from patient 2 and 3 were treated with DQ-BSA for 24 h, then fixed, labeled with DAPI and observed on a confocal microscope. Bars 10 µm. DQ-BSA puncta in cells were measured using ImageJ. Data represent the mean ± SEM of at least 50 cells analyzed. For DQ-BSA quantification, Student's *t* test was used for statistical analysis. ***$p < 0.001$

caused by excess of RAB7 activity [37, 38, 88]. It is also worth noting that it was demonstrated that even though RAB7 mutants are inefficiently recruited to the endosomes, endosomal maturation is not impaired [38]. Moreover, it was shown that a dominant negative mutant of RAB7 induces endosomal accumulation of TrkA, enhancing TrkA signaling and neurite outgrowth, while CMT2B-causing RAB7 mutants inhibit this process, again behaving similarly to the constitutively active mutant [13, 40]. Thus, RAB7 mutants are dysfunctional and distinct regulatory processes are differently affected by their presence. Accordingly, CMT2B-causing RAB7 mutants impact differently on autophagy and endocytosis.

Several CMT genes affect the autophagy pathway at different stages, suggesting that autophagy may represent a common pathomechanism in inherited peripheral neuropathies, including CMT1A and CMT1E in which mutations in PMP22 (peripheral myelin protein 22) disrupt the initiation of autophagy [89]. However, despite these premises, autophagy modulation as a treatment for hereditary neuropathies remains elusive [89]. Our present data widen the current knowledge about pathomechanisms of CMT2B, demonstrating changes in the expression of different proteins along the endocytic degradative pathway that could become targets to revert the disease phenotype.

This study has been performed on skin fibroblasts from three CMT2B patients with the RAB7^{V162M} mutation and on iPS-derived sensory neurons of two patients. Although these data are consistent with previous and present data obtained on continuous cell lines transfected with plasmids for expression of CMT2B-causing RAB7 mutant proteins, the number of patients is very limited, due to the fact

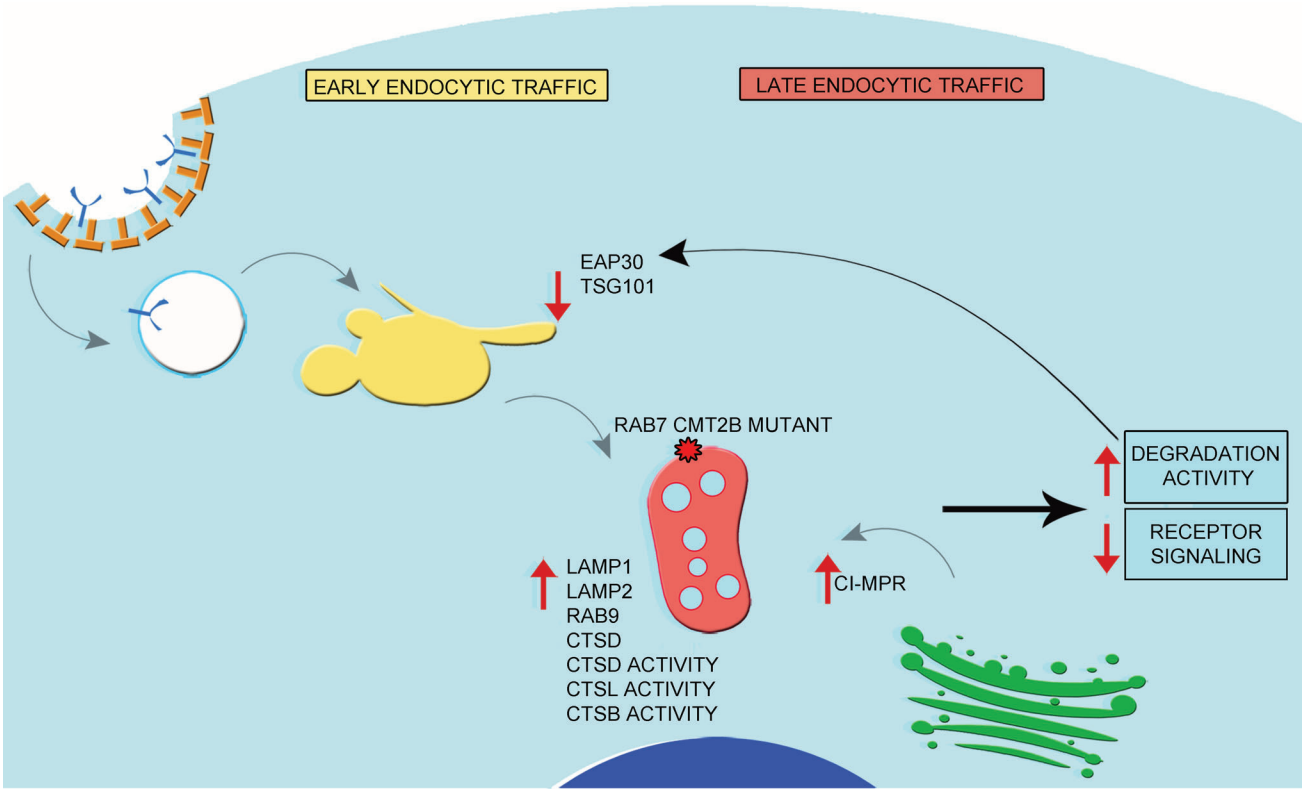

Fig. 10 Model of the Impact of the CMT2B-causing RAB7 mutant protein on the endocytic pathway. In CMT2B the presence of $RAB7^{V162M}$ causes an increase of late endosomal proteins and enzymes as well as of lysosomal activity. This, in turn, activates a feedback control mechanism demonstrated by the decrease of ESCRT proteins. Because of the presence of the RAB7 mutant, CMT2B cells fail to respond to this regulatory mechanism, showing higher lysosomal activity

that CMT2B is a rare form of a rare disease. Furthermore, the three patients were from a single family and there may be modifier variants in this family. Thus, it is fundamental to expand in the future the number of patient cell lines to strengthen our findings.

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Author contributions CB, LS and SCP conceived the study. CB and SCP supervised the study. CB, RR, MDL, SCP and EE designed the experiments. RR, CR, MDL, RT, RB and EE performed the experiments. CB, RR, CR, MDL, RT, FM, MN, SCP and EE analyzed the data. CB wrote the paper. RR, CR, MDL and EE prepared the figures. All authors reviewed, edited and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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