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Sortilin turnover is mediated by ubiquitination

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ABSTRACT

Sortilin is a transmembrane domain protein that has been implicated in the sorting of prosaposin and other soluble cargo from the Golgi to the lysosomal compartment. While the majority of the receptor is recycled back to the Golgi from endosomes, it is known that upon successive rounds of transport, a proportion of sortilin is degraded in lysosomes. Recently, it was shown that sortilin is palmitoylated and that this post-translational modification prevents its degradation and enables sortilin to efficiently traffic back to the Golgi. Thus palmitoylation can be used to modulate the amount of receptor and hence cargo reaching the lysosome. In this work, we demonstrate that non-palmitoylated sortilin is ubiquitinated and internalized into the lysosomal compartment via the ESCRT pathway for degradation. Furthermore, we identified Nedd4 as an E3 ubiquitin ligase that mediates this post-translational modification. We propose a model where palmitoylation and ubiquitination play opposite roles in the stability and turnover of sortilin and serve as a control mechanism that balances the amount of lysosomal sorting and trafficking in cells.

1. Introduction

Sortilin is a member of the Vps10p family of sorting receptors [1] and cycles mainly between the Golgi apparatus and endosomes where it functions as a lysosomal sorting receptor [2]. It has been shown to traffic soluble lysosomal cargo such as prosaposin, GM2-activator protein and acid sphingomyelinase [2,3]. A small proportion of sortilin also localizes to the plasma membrane where it interacts with and internalizes lipoprotein lipase for lysosomal degradation [4]. In order to exit the Golgi, sortilin is packaged into clathrin coated vesicles by cytosolic factors such as adaptor protein-1 (AP1) [5] and the Golgi-localized, gamma-ear-containing Arf-binding proteins (GGAs) [6]. When these trafficking vesicles reach the endosomes, cargo dissociates from the receptor due to the more acidic pH in this organelle [7], which is then able to reach the lysosomes while, the majority of the receptor is recycled back to the Golgi for another round of cargo binding and trafficking [8]. The efficient endosome-to-Golgi sorting and trafficking of sortilin is mediated by retromer [5], a heteropentameric complex first discovered in yeast where it was shown to have a role in the endosome-to-Golgi trafficking of Vacuolar Sorting Protein 10 (Vps10p), the yeast homologue of mammalian sortilin [9]. In mammalian cells, retromer is divided in two subcomplexes: the Vps26, Vps29 and Vps35 trimer implicated in cargo recognition [9] along with a combination of two sorting nexins (SNX1, SNX2, SNX5 and SNX6) responsible for membrane binding [8]. Both subcomplexes are required for the efficient endosome-to-Golgi trafficking of sortilin [10–12].

We have previously shown that sortilin is palmitoylated on its cytosolic tail at cysteine 783, and that this post-translational modification is required for its efficient endosome-to-Golgi trafficking [13]. Palmitoylation is a reversible post-translational modification which consists in the linkage of a 16-carbon chain of saturated palmitic acid on a cysteine residue [14]. Additionally, we identified DHHC15, a member of the recently discovered palmitoyltransferases that share a common catalytic domain composed of an aspartic acid, histidine, histidine and cysteine (DHHC) motif [15], as the palmitoyltransferase required to palmitoylate sortilin [13]. Functionally, we demonstrated that non-palmitoylated sortilin, either by mutating the site of palmitoylation or by depleting cells of DHHC15, was not recycled back to the Golgi and was rapidly degraded in lysosomes [13]. The palmitoylation of sortilin appears to provide an opportunity to control the amount of receptor available for sorting and trafficking and the amount of cargo that reaches the lysosomes.

In this work, we found that sortilin, when not palmitoylated, is monoubiquitinated and degraded in the lysosomal compartment. We also demonstrate that the ESCRT complex is required for the degradation of non-palmitoylated, ubiquitinated sortilin and that Nedd4 plays an important role in this process. We propose a model whereby palmitoylation and ubiquitination control the amount of intracellular sortilin are provide cells the opportunity to control lysosomal sorting and trafficking.

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2. Materials and methods

2.1. Antibodies, cDNA constructs and siRNA

Unless otherwise noted, all reagents were purchased from Sigma–Aldrich (Oakville, ON). The following mouse monoclonal antibodies were used: anti-myc (MMS-150P) and anti-HA (MMS-101P) (Covance, Emeryville, CA), anti-TSG101 (ab83, Abcam, Cambridge, MA), anti-ubiquitin (550944), anti-Actin Ab-5 (612657) and anti-Nedd4 (611481) (BD Biosciences, Mississauga, ON). The HA-Nedd4 (Plasmid 11426) was purchased from Addgene (Cambridge, MA). HA-c-Cbl was a generous gift from Dr. Hamid Band (Brigham and Women's Hospital, Harvard Medical School, Boston, MA). The sortilin-myc and sortilinC783S-myc were previously described [13]. To generate sortilinC783S,K818R-myc, site directed mutagenesis was performed as previously described [13]. RNAi against TSG101 (TSG101HSS111013, TSG101HSS111014, TSG101HSS111015) and against Nedd4 (NEDD4HSS107062, NEDD4HSS107063, NEDD4HSS107064) were purchased from Life Technologies (Burlington, ON).

2.2. Cell culture

All cell culture reagents were purchased from Life Technologies (Burlington, ON). Cells were grown in DMEM (11995) supplemented with 10% FBS (12483) and penicillin–streptomycin (15,140). Transfections were performed using Lipofectamine Transfection Reagent (18324) and Plus Reagent (11514) in 10 cm^2 plates according to manufacBD Biosciences, Mississauga, ON). The HA-Nedd4 turer's instructions. RNA interference was performed using Oligofectamine Transfection Reagent (12252011) using 100 nM of RNAi on 2 consecutive days. Assays were subsequently performed 48 h after the second RNAi treatment.

2.3. Whole cell lysate preparation

Cells were lysed in cell lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 0.5% Triton X-100 and protease inhibitor cocktail) for 30 min on ice and centrifuged at 3300 rcf for 15 min at 4 °C to remove debris.

2.4. Cycloheximide chase

Cells were incubated with or without 50 μg/ml of cycloheximide (C7698) for 6 h prior to cell lysis (described above).

2.5. Co-immunoprecipitation

Cell lysates were incubated overnight at 4 °C with 3 μg of mono-HA or 2.1 μg of mono-myc antibody followed by a one-hour incubation at 4 °C with protein G-Sepharose beads (17-0618-01, GE Healthcare, Baie-d'Urfé, QC). The samples were centrifuged to remove the supernatant and the beads were washed 3 times for 5 min with ice-cold lysis buffer. Laemmli sample buffer was added to the beads and samples were analysed by immunoblotting as described above.

3. Results and discussion

3.1. Non-palmitoylated sortilin is ubiquitinated on lysine 818

As we have previously demonstrated that sortilin is degraded in lysosomes when it is not palmitoylated [13], we tested if this degradation is mediated by ubiquitination, as transmembrane domain proteins are known to be monoubiquitinated prior to lysosomal degradation, whereas polyubiquitination usually results in proteasomal degradation [16]. Ubiquitination, is a reversible enzyme-mediated post-translational modification that adds a ubiquitin (Ub) polypeptide to lysine residues on target proteins [17] which serves as a degradation signal for transmembrane domain proteins. To test if non-palmitoylated sortilin is ubiquitinated, we transfected HeLa cells with wild-type sortilin-myc which was then purified from whole cell lysates by immunoprecipitation. Immunoprecipitated proteins were migrated on a 12% gel and the ubiquitination status of wild-type sortilin was assessed by staining with an anti-ubiquitin antibody (Fig. 1A). We were able to detect a weak signal for ubiquitin (Fig. 1A, lane 2) which suggested that a very small proportion of sortilin is ubiquitinated and this population probably corresponds to the fraction destined for lysosomal degradation which occurs during successive rounds of trafficking. To ensure that the anti-ubiquitin antibody could efficiently recognize ubiquitin, we ran a total cell lysate and performed a Western blot. We found the characteristic ubiquitin "smear" suggesting that this antibody did in fact recognize ubiquitin (Fig. 1A, lane 1). Next, we tested the hypothesis that the fraction of ubiquitinated wild-type receptor was not palmitoylated. To test this, we transfected HeLa cells with sortilinC783S-myc, a mutant form of sortilin that cannot be palmitoylated [13], and repeated the immunoprecipitation assay (Fig. 1A). In this experiment, the amount of ubiquitinated sortilin was significantly increased (Fig. 1A, lane 3) compared to wild-type sortilin-myc. Our results suggest that when sortilin is not palmitoylated, it is modified by ubiquitin and subsequently degraded in lysosomes. The interplay between palmitoylation and ubiquitination provides cells the ability to control the amount of sortilin and therefore the amount of lysosomal cargo that reaches the lysosomes. Since the site of palmitoylation is in the cytosolic tail of sortilin (Fig. 1B, Box 1), we rationalized that the site of ubiquitination could also be in this region. Using bioinformatics, we identified a lysine at position 818 (Fig. 1B, Box 2) in the cytosolic tail of sortilin that we mutated to arginine to determine if sortilin could still be ubiquitinated. To determine if lysine 818 is the site of ubiquitination of sortilin, we immunoprecipitated sortilinC783S-myc or sortilinC783S,K818R-myc from whole cell lysates and Western blotted for ubiquitin. We found that non-palmitoylated receptor (sortilinC783S) is ubiquitinated (Fig. 1C) while non-palmitoylated receptor with the lysine mutation (sortilinC783S,K818R) is no longer modified by ubiquitin (Fig. 1C). This confirmed our hypothesis that sortilin, when not palmitoylated, is ubiquitinated and that the site of ubiquitination is in its cytosolic tail at lysine 818. The fact that we observe one band, as opposed to a smear in these Western blots, suggests that sortilin is monoubiquitinated, which is consistent with the mechanism of degradation of transmembrane domain proteins in lysosomes [18]. Our results so far support our hypothesis that palmitoylation prevents ubiquitination of sortilin and enables its recycling back to the Golgi apparatus. When cells need to degrade sortilin, they could block the palmitoylation of the receptor by inhibiting the function of DHHC15, previously identified as the sortilin palmitoyltransferase [13], which would then be ubiquitinated on lysine 818 and degraded in lysosomes.

3.2. Non-palmitoylated sortilin is degraded through the ESCRT pathway

Transmembrane domain monoubiquitinated proteins interact with the endosomal sorting complexes required for transport (ESCRT) machinery, which is involved in sequestering cargo destined for lysosomal degradation [18]. There are five distinct ESCRT complexes (ESCRT-0, -I, -II, -III and Vps4) that act sequentially to internalize cargo into multivesicular bodies for eventual degradation in lysosomes [19]. To determine if the ESCRT machinery is involved in

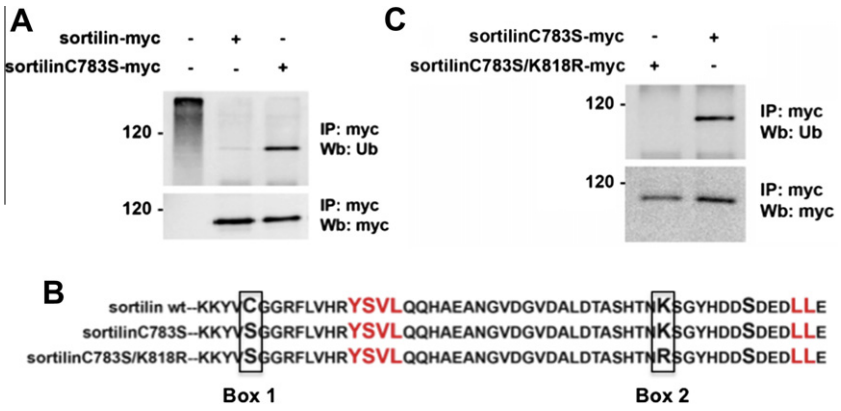

Fig. 1. Non-palmitoylated sortilin is ubiquitinated at lysine 818. (A) HeLa cells were transfected with either sortilin-myc or sortilinC783S-myc as indicated. Following an immunoprecipitation (IP) using anti-myc antibody, the samples were run on a 12% polyacrylamide gel and Western blotted (Wb) with either anti-ubiquitin or anti-myc antibodies. The first lane is total cell lysate used as a positive control for the anti-ubiquitin antibody. (B) The amino acids sequence of the cytosolic tail of wild-type sortilin, sortilinC783S and sortilinC783S,K818R highlighting the site of palmitoylation (cysteine 783, Box 1) and the predicted site of ubiquitination (lysine 818, Box 2). (C) HeLa cells were transfected with either sortilin-myc or sortilinC783S,K818R. Whole cell lysate was immunoprecipitated (IP) with anti-myc antibody and Western blotted (Wb) with anti-ubiquitin and anti-myc antibodies.

the degradation of non-palmitoylated ubiquitinated sortilin, we depleted HeLa cells of TSG101, one of the subunits of the ESCRT-I complex [18], using siRNA and performed a cycloheximide chase experiment, to determine the degradation of sortilinC783S-myc by inhibiting *de novo* protein synthesis. Since previous reports had shown significant degradation of non-palmitoylated sortilin after 6 h when palmitoylation was inhibited [13], the kinetics of which is similar to the rate of degradation in retromer-depleted cells [10,11], we compared the levels of sortilinC783S-myc at 0 h and after a 6 h incubation with cycloheximide. We were able to efficiently deplete cells of TSG101 as shown by Western blotting (Fig. 2A, middle panel). As expected, we found significant degradation of sortilinC783S-myc in mock-depleted cells (Fig. 2A, top panel) with only 45% of the receptor remaining after 6 h (Fig. 2B, white bar). However, in TSG101-depleted cells, the degradation was not as significant as more than 70% of the receptor was remaining after a 6 h incubation with cycloheximide (Fig. 2B, white bar). Our results show that non-palmitoylated sortilin is stabilised in TSG101-depleted HeLa cells suggesting that that degradation of ubiquitinated sortilin normally occurs through the ESCRT pathway. Although the ubiquitin and ESCRT machinery are well characterized in the degradation of transmembrane proteins originating from the plasma membrane, the relatively short time frame and taking into consideration that 90% of sortilin cycles between the Golgi and endosomes, our interpretation of this data is that the majority of the non-palmitoylated sortilin being degraded originated from the Golgi and not the plasma membrane. To our knowledge, this is the first demonstration of a transmembrane protein originating from the Golgi using this degradation machinery in mammalian cells. Interestingly, it was very recently reported that in plant cells, a ubiquitinated reporter protein exiting the Golgi also used ubiquitination and the ESCRT degradation machinery [20]. Based on that study and our own work, we propose a novel mechanism of degradation using the ubiquitin/ESCRT pathway for proteins originating from the Golgi as well as the plasma membrane.

3.3. The E3 ligase Nedd4 participates in the degradation of non-palmitoylated sortilin

Since we found that non-palmitoylated sortilin was ubiquitinated and degraded in lysosomes, we next wanted to identify the E3 ligase responsible for the ubiquitination of non-palmitoylated sortilin. Ubiquitination is a 3 step process where an Ub-E1 activating enzyme activates Ub which is then transferred to the active site of a Ub-E2 conjugating enzyme and finally, a Ub-E3 ligase, which determines the specificity of the reaction, transfers the Ub moiety onto the target protein. At least 3 different Ub E3 ligases (AIP4, Nedd4 and c-Cbl) localize to the endosomes and act on transmembrane proteins destined for lysosomal degradation. In mammalian

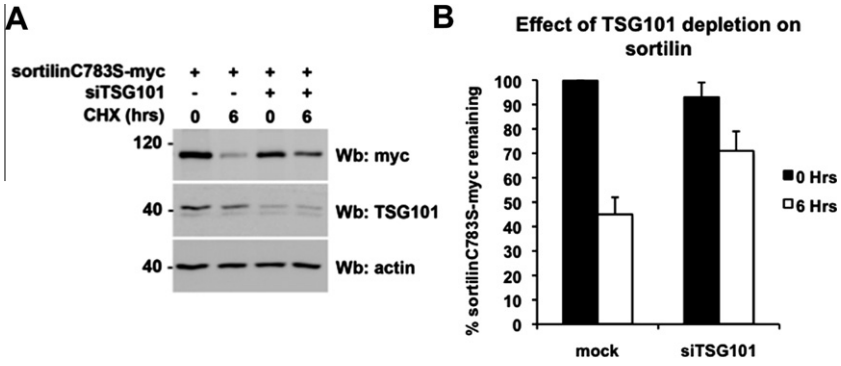

Fig. 2. Depletion of TSG101 prevents the degradation of sortilin. (A) HeLa cells were depleted or not depleted of TSG101 by siRNA and subsequently transfected with sortilinC783S-myc. The cells were then incubated with 50 μg/ml of cycloheximide for the time indicated. Equal amounts of protein were loaded on a 12% acrylamide gel and transferred to nitrocellulose membranes and subsequently Western blotted with anti-myc, anti-TSG101 and anti-actin (to serve as a loading control) antibodies. (B) Quantification of the level of sortilinC783S-myc from 3 separate experiments as described in (A). Error bars represent +/− standard deviation.

cells, Nedd4 has been implicated in the lysosomal trafficking of SIMPLE [21] and LAPTM5 [22] while c-Cbl has been implicated in the lysosomal degradation of the Epidermal Growth Factor receptor [23] and gp130 [19].

We tested for an interaction between sortilin and sortilinC783S with two different E3 ligases known to localize to endosomes, Nedd4 and c-Cbl using co-immunoprecipitation. We found no significant interaction between wild-type sortilin-myc and either HA-Nedd4 or HA-c-Cbl, but our results show an interaction between HA-Nedd4 (Fig. 3A) and sortilinC783S-myc. We did not observe an interaction between HA-c-Cbl and sortilinC783S-myc (Fig. 3A). This suggested that Nedd4 is a potential E3 ligase responsible for the ubiquitination of non-palmitoylated sortilin. It was not surprising that we did not observe an interaction between an E3 ligase and wild-type sortilin. Although a small proportion of sortilin is ubiquitinated (our data, Fig. 1) and degraded in each successive round of trafficking, the amount is very small and could be under the detection limit of our co-immunoprecipitation protocol.

3.4. Nedd4 overexpression increases sortilin degradation

Since the ubiquitination of sortilin leads to its degradation and we found an interaction between sortilinC783S-myc and HA-Nedd4, we tested whether or not the overexpression of HA-Nedd4 in HeLa cells would increase the degradation of sortilinC783S-myc. Compared to cells transfected with only sortilinC783S-myc (Fig. 3B), the degradation of non-palmitoylated sortilin is more significant after a 6 h cycloheximide chase in HeLa cells also overexpressing HA-Nedd4 (Fig. 3B) as only 20% of sortilinC783S-myc remained in cells overexpressing HA-Nedd4 compared to 40% in cells not transfected with HA-Nedd4 (Fig. 3C). Although we observed a significant difference in the amount of sortilinC783S-myc remaining after a 6 h cycloheximide chase in the presence of HA-Nedd4, our assay is probably underestimating the degradation of non-palmitoylated receptor as HA-Nedd4 is also being degraded (Fig. 3B, middle panel), which is consistent with its half life of 6 h [24]. To verify the results we found by overexpressing Nedd4, we performed the cycloheximide chase experiment in cells depleted of Nedd4 by siRNA. We were able to efficiently deplete more than 80% of Nedd4 from HeLa cells (Fig. 4A). Compared to mock-depleted cells, the degradation of sortilinC783S-myc in Nedd4-depleted cells was not as great (Fig. 4A) as we found more than 65% of sortilinC783S-myc remaining compared to 40% in mock-depleted cells (Fig. 4B). Based on our overexpression and siRNA experiments, our data supports a model whereby the E3 ubiquitin ligase Nedd4 would ubiquitinate non-palmitoylated sortilin resulting in its internalization into multivesicular bodies for lysosomal degradation. We did attempt to verify if overexpression of HA-Nedd4 or siRNA of Nedd4 would either increase or decrease the ubiquitination of sortilinC783S-myc. However, we could not detect a significant change in the ubiquitin signal probably due to the fact that the amount of sortilinC783S-myc we found ubiquitinated is already near saturation in the overexpression experiments and due to the remaining Nedd4 not depleted in by siRNA, a phenotype typical when trying to ascertain the function of an enzyme by siRNA. An *in vitro* system could be used to test definitively if Nedd4 can ubiquitinate non-palmitoylated sortilin, but we would need to use *Escherichia coli* produced proteins which would be difficult as Nedd4 is a large protein (1319 amino acids) and sortilin is a transmembrane domain protein. We could use only the cytosolic tail of sortilin, but it is unclear whether or not Nedd4 would recognize this short peptide even if we could produce enough bacterially expressed Nedd4 to attempt the *in vitro* experiment.

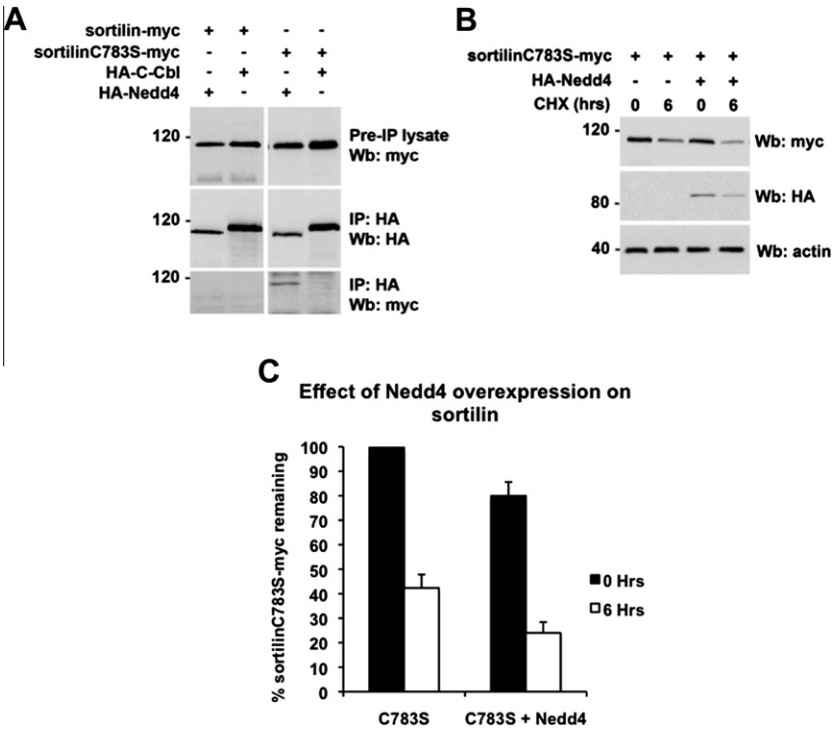

Fig. 3. Overexpression of Nedd4 results in increased degradation of sortilin. (A) Wild-type sortilin-myc or sortilinC783S-myc were transfected into HeLa cells with either HA-c-Cbl or HA-Nedd4. Whole cell lysate was immunoprecipitated (IP) with anti-HA antibody. Samples were loaded on a 12% acrylamide gel and membranes were Western blotted (Wb) with anti-HA and anti-myc antibodies. (B) HeLa cells transfected with sortilinC783S-myc were co-transfected or not with HA-Nedd4 and a 6 h cycloheximide chase was performed. Equal amount of proteins were loaded on a 12% acrylamide gel and membranes were blotted with anti-myc, anti-HA and anti-actin (to serve as a loading control) antibodies. (C) Quantification of the level of sortilinC783S-myc from 3 separate experiments as described in B. Error bars represent +/− standard deviation.

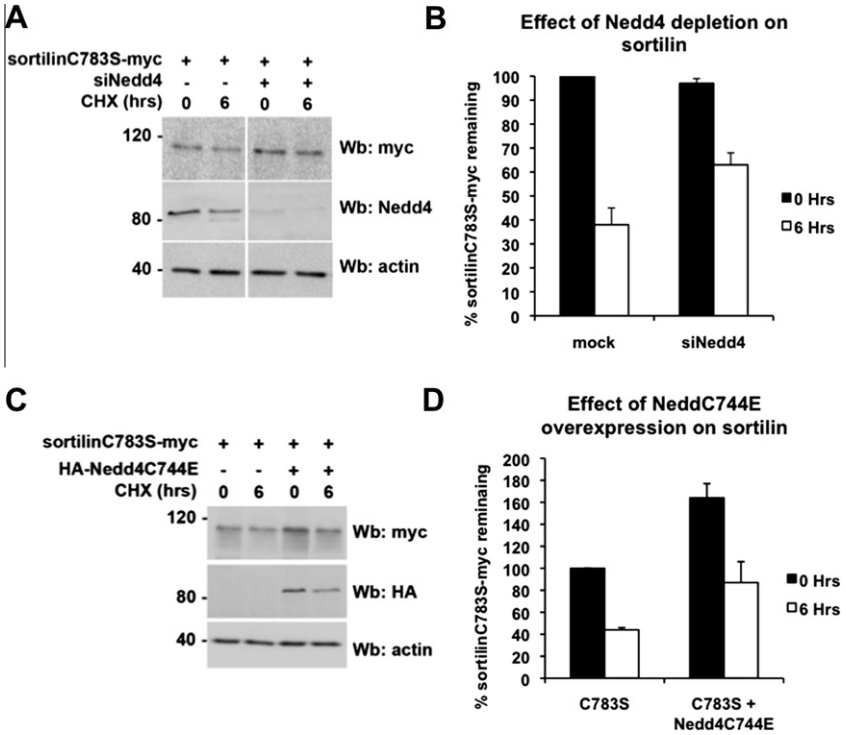

Fig. 4. The ubiquitin ligase activity of Nedd4 is required to degrade sortilin. (A) HeLa cells were depleted of Nedd4 or not as indicated and transfected with sortilinC783S-myc. A 6 h cycloheximide chase was performed and equal amounts of proteins were loaded on a 12% acrylamide gel. Membranes were blotted with anti-myc, anti-Nedd4 and anti-actin (to serve as a loading control) antibodies. (B) Quantification of the level of sortilinC783S-myc from 3 separate experiments as described in A. Error bars represent +/− standard deviation. (C) SortilinC783S-myc was co-transfected (or not) in HeLa cells with HA-Nedd4C744E and a 6-h cycloheximide chase was performed and equal amount of proteins were loaded on a 12% acrylamide gel. Membranes were blotted with anti-myc, anti-HA and anti-actin as a loading control. (D) Quantification of the level of sortilinC783S-myc from 3 separate experiments as described in (C). Error bars represent +/− standard deviation.

3.5. The ubiquitin ligase activity of Nedd4 is required to degrade non-palmitoylated sortilin

Although we were not successful in determining whether or not Nedd4 ubiquitinates non-palmitoylated sortilin, we tested the effect of overexpressing catalytically inactive Nedd4 which does not have the ability to ubiquitinate target proteins to further characterise the role of Nedd4 in the degradation of non-palmitoylated sortilin [25]. We performed a 6 h cycloheximide chase to test the stability of sortilinC783S in cells transfected or not with HA-Nedd4C744E. Our results show that in HeLa cells overexpressing HA-Nedd4C744E, the absolute amounts of sortilinC783S-myc is significantly increased compared to HeLa cells only expressing sortilinC783S-myc (Fig. 4C). In fact, we found that the level of expression of sortilinC783S-myc in cells overexpressing inactive Nedd4 is up to 160% at time 0 h (Fig. 4D). However, the level goes down to 80% after the cycloheximide incubation compared to cells not overexpressing Nedd4C744E, where we see 40% of remaining sortilinC783S after the cycloheximide incubation (Fig. 4D). Although the level of expression of sortilinC783S-myc is increased in cell also overexpressing inactive Nedd4, the degradation rate seems similar in both conditions. This could be explained by the fact that the cells overexpressing inactive HA tagged Nedd4 still express endogenous levels of wild-type Nedd4. Interestingly, like wild-type HA-Nedd4, HA-Nedd4C744E is also partially degraded in the cycloheximide chase experiment after 6 h (Fig. 4C, middle panel). It is possible that due to this decrease in expression, endogenous Nedd4 can ubiquitinate sortilinC783S-myc resulting in its lysosomal degradation. Regardless, our data suggests that the ubiquitin ligase activity is required for the degradation of non-palmitoylated sortilin in lysosomes.

Taken together, our results show that ubiquitination, along with palmitoylation, play key roles in regulating the amount of intracellular sortilin cycling between the Golgi and endosomes. Although cells could downregulate the synthesis of sortilin to control the intracellular amount, this would be long and time consuming and could be a mechanism for cells in the long term. However, for cells to respond more quickly for the need to downregulate intracellular levels of sortilin, we propose a model whereby cells could modulate and/or block the palmitoylation of sortilin by inhibiting the action of DHHC15, previously shown to palmitoylate sortilin [13]. This would lead to the degradation of sortilin (now non-palmitoylated) in the lysosomes due to ubiquitination by Nedd4 and an interaction with the ESCRT machinery.

4. Authors contribution

Karine Dumaresq-Doiron planned and performed the experiments. Karine Dumaresq-Doiron, Felix Jules, and Stephane Lefrancois conceptualized experiments, analyzed data and wrote the manuscript.

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