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RESEARCH ARTICLE

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Loss of ATP2C1 function promotes trafficking and degradation of NOTCH1: Implications for Hailey-Hailey disease

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Abstract

Hailey-Hailey disease (HHD) is a rare autosomal dominantly inherited disorder caused by mutations in the ATP2C1 gene that encodes an adenosine triphosphate (ATP)-powered calcium channel pump. HHD is characterized by impaired epidermal cell-to-cell adhesion and defective keratinocyte growth/differentiation. The mechanism by which mutant ATP2C1 causes HHD is unknown and current treatments for affected individuals do not address the underlying defects and are ineffective. Notch signalling is a direct determinant of keratinocyte growth and differentiation. We found that loss of ATP2C1 leads to impaired Notch1 signalling, thus deregulation of the Notch signalling response is therefore likely to contribute to HHD manifestation. NOTCH1 is a transmembrane receptor and upon ligand binding, the intracellular domain (NICD) translocates to the nucleus activating its target genes. In the context of HHD, we found that loss of ATP2C1 function promotes upregulation of the active NOTCH1 protein (NICD-Val1744). Here, deeply exploring this aspect, we observed that NOTCH1 activation is not associated with the transcriptional enhancement of its targets. Moreover, in agreement with these results, we found a cytoplasmic localization of NICD-Val1744. We have also observed that ATP2C1-loss is associated with the degradation of NICD-Val1744 through the lysosomal/proteasome pathway. These results show that ATP2C1-loss could promote a mechanism by which NOTCH1 is endocytosed and degraded by the cell membrane. The deregulation of this phenomenon, finely regulated in physiological conditions, could in HHD lead to the deregulation of NOTCH1 with alteration of skin homeostasis and disease manifestation.

KEYWORDS
ATP2C1, Hailey-Hailey disease, keratinocytes, NOTCH

1 | INTRODUCTION

Hailey-Hailey disease (HHD) is an autosomal dominant skin disorder characterized by painful erosions and fissures that occur most often at sites of skin folds such as the neck, axillae, groin, perineum, and under the breast. The disease is caused by loss of ATP2C1 function[1]; nevertheless, the downstream signalling pathways elicited by ATP2C1-loss remain unclear. The *ATP2C1* gene encodes a 4.5-kb transcript that is alternatively spliced into four variants, named ATP2C1a–d.[1–5] The encoded proteins belong to the SPCA (secretory pathway Ca^{2+}/Mn^{2+} ATPase) subfamily of P-type ion motive ATPases. SPCA pumps possess only one of the two high-affinity Ca^{2+} binding sites characteristics of SERCA (sarco/endoplasmic reticulum Ca^{2+}-transport ATPase). However, these can transport not only a single Ca^{2+} ion but also a Mn^{2+}. Such characteristics separate SPCA from the P-type SERCA and plasma membrane Ca^{2+} pumps.[6] To date, around 180 different *ATP2C1* mutations have been described in patients with HHD (www.hgmd.cf.ac.uk/ac/index.php). Among these, 20% are nonsense mutations, 19% are splice site mutations, 30% are frameshift mutations leading to premature termination codons, 28% are missense mutations, and approximately 3% are in-frame deletions or insertions.[7–10] *ATP2C1* is ubiquitously expressed in several mammalian cells, but its expression is particularly high in epidermal keratinocytes.[1] While Mn^{2+} levels have not been evaluated in HHD skin, the role of Ca^{2+} in disease development is better documented.[10] Because calcium regulates the proliferation and differentiation of keratinocytes both in vivo and in vitro,[11] it is not surprising that *ATP2C1* mutations selectively affect the skin. The reason for this is still not well understood and is probably complex, and although intracellular Ca^{2+} regulation is likely disturbed in HHD keratinocytes, the downstream mechanism remains to be defined. Furthermore, the clinical manifestations of HHD patients are highly heterogeneous, and there is no correlation between the type of specific mutation causing disease and the clinical characteristics of the associated phenotype. This holds true for various clinical facets, e.g., age of onset, distribution pattern of cutaneous lesions, disease severity, progression, or relapse and, most importantly, the effects of different therapeutic measures.[11] Although HHD manifestation is primarily caused by *ATP2C1* mutations, it is likely that the disturbance of additional molecular pathways as well as environmental factors might influence the phenotypic outcome of the clinical manifestation of the disease. In line with this notion, it has been indicated that HHD-ATP2C1 mutations do not exert a dominant-negative effect on wild-type hSPCA1.[12] Furthermore, HHD patients are heterozygous for the *ATP2C1* gene, indicating that loss of one functional *ATP2C1* allele alone is not sufficient to cause clinically overt HHD because (i) affected individuals do not usually manifest skin symptoms before the third or fourth decade of life and (ii) skin lesions in patients with HHD can be elicited in response to external stimuli such as trauma or friction. Therefore, the additional disturbance of as yet unknown molecular pathways due to dominant *ATP2C1* mutations in conjunction with additional genetic or nongenetic pathogenetic factors, e.g., 'second hits', may be required for the clinical manifestation of HHD. In line with this notion, it has been reported that loss of the healthy wild-type allele (loss of heterozygosity) due to somatic recombination leads, in a segmental pattern following the lines of Blaschko, to onset of a more severe phenotype of HHD earlier in life.[13] Interestingly, we reported that skin lesion keratinocytes had lower levels of ATP2C1 protein compared with keratinocytes derived from normal-appearing skin.[14] As both cell types carry one functional and one nonfunctional allele, an additional mechanism is needed to explain the downregulated expression of the functional one. Our hypothesis is that the presence of molecular or genetic modifiers either upstream or additional to *ATP2C1* mutation down-regulates expression of the functional allele of *ATP2C1*. Thus, compensatory mechanisms cannot adequately maintain cellular homeostasis, thereby depriving keratinocytes of key mediators of progression through the differentiation program. However, there could be additional extrinsic signals that give rise to HHD development. We reported that reactive oxygen species (ROS) contribute to the HHD manifestation by altering signalling pathways involved in skin homeostasis. An extrinsic signal, e.g., inflammatory mediators, might induce the formation of ROS, thus promoting DNA damage and the acquisition of heritable defects in a cellular context of reduced ability to counteract oxidative-stress, resulting from *ATP2C1* haploinsufficiency.

We previously demonstrated the complexity of HHD in which multi-hit combinations of altered signal pathways may explain the wide spectrum of defects. In our studies, we found that p63 and NOTCH1, two important factors that regulate proliferation, differentiation, cell adhesion, and other functions in keratinocytes, are negatively regulated in keratinocytes derived from lesional skin of HHD-patients.[14] Similarly, Li et al.[15] reported that the expression of levels of both NOTCH1 and p63 was lower in the lesional skin tissues of HHD patients compared to the healthy control skin. Having identified some of the relevant pathways in the HHD pathogenesis, a more careful dissection of specific components and underlying molecular mechanisms may now be addressed. This is particularly important from a therapeutic perspective since current treatment approaches for affected individuals do not address the underlying defects. Novel therapy strategies targeting the underlying mechanisms are therefore crucial for HHD treatment.

Notch signalling plays a complex role in skin homeostasis by controlling both keratinocyte proliferation and differentiation.[16,17] Similarly, the maintenance of genome stability is essential for keeping cellular homeostasis. Among the regulators of cellular response to DNA-damage, ATM is a multifunctional protein kinase with a key role in preserving genomic stability. We found that the deregulation of calcium homeostasis, resulting from the loss of ATP2C1 function produces ROS-induced DNA damage.[18] ATP2C1 loss would then trigger a mechanism that results in NOTCH1 activation and subsequent ATM down-regulation.[18] Increased ROS levels and ATM loss would produce DNA damage up to a threshold that keratinocytes cannot repair, which would then promote HHD-manifestation.[18]

However, there are still many unanswered questions; we showed that NOTCH1 expression is negatively regulated by ATP2C1

deficiency-mediated ROS induction in keratinocytes, both ex vivo and in vitro.[14,18–20] In line with this observation, we found that the expression levels of NOTCH1 are markedly reduced in HHD-derived keratinocytes.[14] In sharp contrast, we found that siRNA-mediated *ATP2C1* inactivation was associated with NOTCH1 activation.[18] The discrepancy in NOTCH1 expression between the lesion-derived and siRNA-ATP2C1-treated keratinocytes in our in vitro model may result from the pathways that were altered prior to lesion formation, that are likely causative, and other alterations in the lesioned area that are likely secondary and associated with tissue damage. However, it might also be possible that these alterations underlie the signalling pathways that are responsible for the initiation and progression of the lesions.

In order to better understand the discrepancy and the difference observed on NOTCH1 expression between the lesion-derived and siRNA-ATP2C1-treated keratinocytes, the mechanism of NOTCH1 activation in siRNA-ATP2C1 treated keratinocytes was further investigated. In this work, we report that loss of ATP2C1 through siRNA promotes an increased expression of the activated form of NOTCH1-Val1744. Interestingly, we found that neither ADAM10 nor ADAM17 is required for the increased expression of NOTCH1-Val1744 indicating a ligand-independent NOTCH1 activation mechanism. In ligand-independent receptor activation, the E3 ubiquitin ligase DELTEX is implicated in the regulation of NOTCH endocytic trafficking.[21–23] DELTEX stabilizes the NOTCH receptor in the endocytic compartment allowing upon its cleavage to form NICD-Val1744 that it is free to translocate to the nucleus and promote activation of its target genes. We uncovered that loss of ATP2C1 increases the expression of DELTEX-1 and treatment with endocytosis inhibitor led to reduced NICD-Val1744 expression in ATP2C1-defective cells. Collectively, our data support the hypothesis that in ATP2C1-defective keratinocytes, the increased DELTEX expression promotes NOTCH endocytosis and its ligand-independent activation. Furthermore, we found that in ATP2C1-defective keratinocytes the activated form of NOTCH1-Val1744 has a cytosolic localization and it is degraded through proteasome/lysosomal pathways. When ATP2C1-defective keratinocytes were analysed by nuclear and cytoplasmic fraction experiments, NOTCH1-Val1744 appears to be strongly enriched in the nucleus after chloroquine treatment. Furthermore, the chloroquine treatment increased the expression levels of Hes1, Hes4; overall, our findings support the hypothesis that, in ATP2C1-defective keratinocytes, NOTCH1-Val1744 might be regulated post-translationally by a proteasome/chloroquine-sensitive pathway that leads to its degradation rather than being shuttled to the nucleus.

2 | METHODS

2.1 | Cell culture and transfection

HaCaT keratinocytes (70%–80% confluent) were maintained in a modified low-calcium medium (EpiLife, Thermo Fisher) and transfected using the Lipofectamine-RNAiMAX transfection Reagent according to manufacturer's instructions (Thermo Fisher Scientific). Keratinocytes were transfected with 100 nmol L^{-1} small interfering RNAs (siRNAs) for validated human ATP2C1, ADAM 10, and ADAM 17 (L-006119-00, L-004503-00, and L-003453-00-Horizon Discovery Ltd, Dharmacon), and the corresponding control scrambled siRNAs cells were analysed by Western blot at the indicated times after transfection.

2.2 | Cell treatment

siATP2C1 or siCTR transfected cells were treated with 10 μM γ-secretase inhibitor IX (DAPT; Calbiochem Merck KGaA), 25–50 μM of Dynasore, 100 μM Chloroquine diphosphate salt (Sigma Aldrich), and 500 nM Carfilzomib (Cell Signaling Technology) as indicated in figure legends. Vehicle treated cells served as control.

2.3 | Protein extraction and western blot analysis

Whole cell extracts were prepared according to the manufacturer's instructions for detection of phospho-ERK (Cell Signaling Technology).

Nuclear and cytoplasmic lysates were prepared using three different buffers. Briefly, buffer A containing 10 mM Hepes, 50 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 500 μM Sucrose, 250 μM PMFS, and protease inhibitor cocktail was used to release nuclei and to recover the supernatant retained as cytoplasmic fraction; buffer B containing 10 mM Hepes, 50 mM NaCl, 0.1 mM EDTA, and 25% glycerol was used to rinse nuclei; buffer C containing 10 mM Hepes, 350 mM NaCl, 0.1 mM EDTA, 25% glycerol, 250 μM PMFS, and protease inhibitor cocktail was used to re-suspend clean nuclear pellets and sonicate for 10 s at full power.

For western blot analysis, lysates were denatured at 95°C and resolved by SDS-PAGE. After transfer to a polyvinylidene difluoride (PVDF) membrane, proteins were immunoblotted using standard procedures.

Blots were incubated with the following primary antibodies: mouse monoclonal ATP2C1 (Santa Cruz Biotechnology), rabbit polyclonal Lamin B, rabbit polyclonal ADAM17 (AbCam), rabbit polyclonal NOTCH1Val1744, rabbit polyclonal NOTCH1, rabbit polyclonal ADAM 10 (Cell Signaling Technology), and mouse monoclonal Tubulin (Sigma Aldrich).

2.4 | RNA analysis and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from cells in guanidine isothiocyanate (Trizol reagent; Thermo Fisher Scientific) and further processed by reverse transcriptase polymerase chain reaction (RT-PCR) as described.[18] Each sample was analysed in triplicate by qRT-PCR and in at least two independent experiments. qRT-PCR was performed at the

opportune annealing temperature with the primers indicated in Table 1, with SensiFAST SyBr Hi-ROX kit (Bioline) or with specific TaqMan MGB primers/probe using Taqman gene expression assay (Thermo Fisher Scientific).

2.5 | Flow cytometer analysis

Single-cell suspensions of siCTR or siATP2C1 HaCaT were washed twice and resuspended in PBS; then, cells were stained with mouse monoclonal anti-NOTCH1-PE (BD Bioscience) for 30 min on ice and analysed by FACS Calibur flow cytometer (BD Biosciences). Isotype control was used at the same concentration as control.

2.6 | Immunofluorescence

HaCaT cells were seeded on 12-mm diameter glass coverslips and used at a confluence of 70%–90%. siCTR and siATP2C1 treated cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Incubation with rabbit polyclonal anti-NOTCH1 (Cell Signaling Technology) and mouse monoclonal anti-LAMP1 (Sigma-Aldrich) was carried out at room temperature for 1 h. After washing with PBS, the cover slips were incubated with FITC or Texas red-conjugated goat anti-rabbit/mouse secondary antibody (Jackson ImmunoResearch Laboratories) for 30 min in the dark. After final washing, nuclei were counterstained with DAPI (Sigma-Aldrich). The slides were then mounted in Moviol or Vecta Shield and examined by immunofluorescence microscopy.

All experiments were carried out at least three times.

2.7 | Statistical analysis

Each experiment was repeated at least two times independently. All results were expressed as means ± SD, and $p < 0.05$ was used for significance. One-Way ANOVA analysis for a comparison of two groups was used to determine statistical significance.

TABLE 1 Primer sequences and Taqman gene expression assays used for qPCR.

h-p21 Fw	5′-GGAAGACCATGTGGACCTGT-3′
h-p21 Rev	5′-GGCGTTTGGAGTGGTAGAAA-3′
h-Involucrin Fw	5′-GCCAGGTCCAAGACATTC-3′
h-Involucrin Rev	5′-GGGTGGTTATTTATGTTTGGGTGG-3′
h-Deltex Fw	5′-GGTGTGGGAGTGGCTGAATG-3′
h-Deltex Rev	5′-CCTGGCGAAACTGGTGCAT-3′
h-Hey1 Fw	5′-AGCAGGTAATGGAGCAAGGA-3′
h-Hey1 Rev	5′-CGAAATCCCAAACTCCGATA-3′
h-GAPDH Fw	5′-TGCACCACCAACTGCTTAG-3′
h-GAPDH Rev	5′-GAGGCAGGGATGATGTTC-3′
hGAPDH	Hs02758991_g1
hHes1	Hs00172878_m1
hHes4	Hs00368353_g1

3 | RESULTS

3.1 | Canonical ligand-dependent pathway involving proteolytic release and nuclear translocation of the NOTCH1-IC is impaired by ATP2C1 inhibition

We previously reported that keratinocytes, derived from lesional skin of HHD-patients, retain their characteristics after culture.[14,18] Interestingly, the expression of ATP2C1 was strongly downregulated in cells derived from lesional skin areas but not in cultured keratinocytes originating from unaffected skin areas of the same patients.[14] We and others also observed that keratinocytes as well as the skin tissue derived from lesional skin of HHD-patients have a reduced expression of NOTCH1 protein.[14,15,18,20] Conversely, reducing the expression of *ATP2C1* gene by siRNA-mediated silencing, we found increased NOTCH1 expression levels.[18] Thus, it could be that the discrepancy in NOTCH1 expression between siRNA-ATP2C1-treated and the lesion-derived keratinocytes underlies a differential effect on NOTCH1 activity by short- and long-term inactivation of ATP2C1 and could represent a pathogenetic mechanism of the disease manifestation. We hypothesized that the early activation of NOTCH1 in siRNA-ATP2C1 keratinocytes might represent a mechanism required to compensate the ATP2C1 loss that could be progressively counteracted by a pathogenic fixation mechanism that can contribute to this disease manifestation. Therefore, to analyse the underlying mechanism that might be involved in the differential NOTCH1 activation in these two different contexts, we first confirmed that siRNA-mediated *ATP2C1* inactivation increased NOTCH1 activation (Figure 1A). As previously observed,[18] inhibition of *ATP2C1* expression in keratinocytes resulted in increased levels of activated NOTCH1 (Figure 1A). At the plasma membrane, the interaction between NOTCH and its ligands exposes a cleavage site (S2) on the extracellular side of the NOTCH1 receptor for metalloprotease 10 (ADAM10) or 17 (ADAM17).[24,25] ADAM cleavage produces a short extracellular truncation fragment, which serves as a substrate for the latter cleavage (S3) mediated by the γ-secretase activity.[23–25] S3-cleavage produces NICD-Val1744 that translocates to the nucleus where it binds to its downstream transcription factor RBPJK (CSL) and drives canonical NOTCH-mediated gene transcription.[26–28] Thus, to analyse whether inhibition of *ATP2C1* expression in keratinocytes resulted in increased levels of activated NOTCH1 through influencing canonical ligand-mediated receptor activation, siATP2C1-treated keratinocytes were analysed after γ-secretase inhibition. Strikingly, when we inhibited γ-secretase by adding a specific inhibitor (γ-secretase-inhibitor, GSI), the NICD-Val1744 levels were no longer increased in the siATP2C1-treated cells (Figure 1A). Canonical ligand-mediated NOTCH activation relies on conformational changes in the receptor that are induced by the ligand to unmask the protected S2/S3 sites for cleavage by

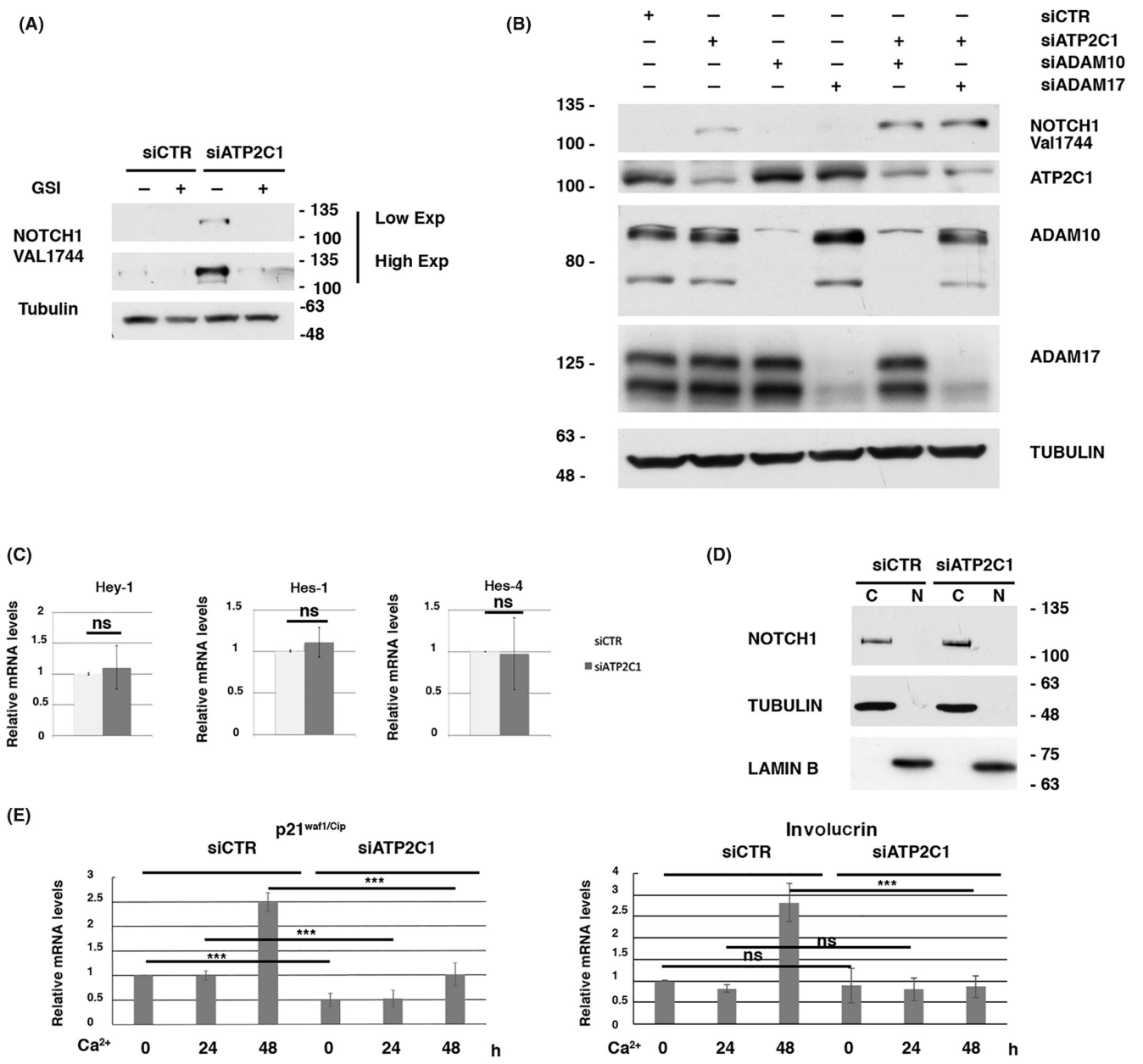

FIGURE 1 Effect of ATP2C1 silencing on NOTCH1 signalling in HaCaT cells. (A) Inhibition of ATP2C1 increases γ-secretase processing of NOTCH1 resulting in increased expression of endogenous cleaved NOTCH1 (NICD-Val1744). Immunoblotting for NOTCH1 (NICD-Val1744) in HaCaT keratinocytes treated with either siCTR or siATP2C1 untreated and treated with GSI (10 μM). Tubulin was used as loading control. (B) Inhibition of ATP2C1 increases γ-secretase processing of NOTCH1 in an ADAM10-17 independent manner. Immunoblotting for NOTCH1, ATP2C1, and ADAM10-17 in HaCaT cells after treatment with the indicated specific siRNAs. (C) Loss of ATP2C1 promotes an increased expression of the activated form of NOTCH1-Val1744 but not an increased expression of NOTCH1 transcriptional targets. Real-Time qPCR analysis of indicated NOTCH1 targets in HaCaT keratinocytes treated with either siCTR or siATP2C1. (D) Increased cleaved NOTCH1 (NICD-Val1744) displays a cytoplasmic localization in response to ATP2C1 inhibition. Immunoblotting for NOTCH1 in cytoplasmic (C) and nuclear (N) fractions of HaCaT keratinocytes treated with either siCTR or siATP2C1. Tubulin and Lamin B were used as loading control of cytoplasmic and nuclear compartments, respectively. (E) Inhibition of ATP2C1 and defective NOTCH1 signalling are associated with impaired calcium-induced keratinocyte differentiation. Real-Time qPCR analysis of p21 and Involucrin expression in siCTR or siATP2C1 keratinocytes treated with 1 mM Ca^{2+} for the indicated time. The significance of the differences was calculated using one-way ANOVA. ***$p < 0.005$ is the significance of indicated time-points; ns, not significant.

ADAMs and then γ-secretase. To test the requirement of ADAMs-mediated cleavage for the activation of NOTCH1 observed in siATP2C1-treated keratinocytes, we used siRNA to target both ADAM10 and ADAM17.

siRNA-ATP2C1 keratinocytes were treated with either ADAM10- or ADAM17-specific siRNA or siCTR. Depletion of neither ADAM10 nor ADAM17 protein alters NICD-Val1744 levels compared to that detected for either siATP2C1- or siCTR-treated keratinocytes

(Figure 1B), indicating that in ATP2C1-defective keratinocytes, NOTCH1 activation might occur via a ligand-independent mechanism. After S3-cleavage, NICD-Val1744 translocates to the nucleus where it binds to its downstream transcription factor RBPJK (CSL) and drives canonical NOTCH-mediated gene transcription.[26–28] Surprisingly, no significant change in the expression of the NOTCH1 target genes was detected in siATP2C1 treated cells (Figure 1C). The nuclear and cytoplasmic distribution of the NOTCH1 protein was therefore analysed. Strikingly, in siATP2C1-treated cells, we found a predominant cytoplasmic localization of NICD-Val1744 expression (Figure 1D).

These data indicate that the canonical ligand-dependent pathway involving proteolytic release and nuclear translocation of the NICD-Val1744 is impaired by ATP2C1 inhibition.

During keratinocyte differentiation, NOTCH1 signalling is essential to direct exit of keratinocytes from cell cycle and entry into differentiation by promoting the induction of $p21^{WAF1/Cip1}$ expression and expression of 'early' differentiation markers.[17,29] To ascertain whether ATP2C1 depletion could impair NOTCH1 signalling and in turn keratinocytes differentiation, HaCaT cells which were maintained with serum-free, low-calcium medium, were subjected to Ca^{2+} treatment to promote differentiation. As expected, we found that, in siCTR cells Ca^{2+} treatment elicited expression of both p21 and Involucrin. In contrast to the siRNA-control, ATP2C1 silencing abrogated the expression of both p21 and Involucrin (Figure 1E). These results suggest that ATP2C1 depletion impairs both NOTCH1 signalling and keratinocytes differentiation.

3.2 | Loss of ATP2C1 promotes proteasome/lysosomal-dependent degradation of the NOTCH1 intracellular domain

The reduction in the pool of nuclear NICD-Val1744 despite its increased expression in ATP2C1 inhibited cells could reflect changes in either the rate of internalization, degradation, or recycling of the NOTCH receptor. To determine whether the post-endocytic destiny of NOTCH1 is influenced by loss of ATP2C1 function, we first examined NOTCH1-IC expression levels in the presence of both lysosomal and proteasome inhibitors. HaCaT cells were treated with either siCTR or siATP2C1 and then incubated in the presence of Chloroquine and Carfilzomib to inhibit lysosomal- and proteasomal-mediated protein degradation respectively. A steady accumulation of intracellular NICD-Val1744 was observed with both treatments (Figure 2A,B). Interestingly, after both Chloroquine and Carfilzomib treatment, an increase in NICD-Val1744 nuclear localization was observed (Figure 2C). We next examined the effect of NICD-Val1744 relocalization on transcriptional activation of NOTCH1 target genes. Following treatment with Chloroquine and Carfilzomib, real-time PCR was used to measure the expression of NOTCH1 target genes. In cells treated with Chloroquine, an increase in the expression of HES-1 and HES-4 was observed in both siCTR and siATP2C1 treated cells (Figure 2D). Conversely, although Carfilzomib leads to increased levels of NICD-Val1744, the expression of both HES-1 and HES-4 was reduced (Figure 2D). Although it is not clear whether ubiquitination affects binding of NICD-Val1744 to the DNA-binding protein CSL or whether it masks a transactivation domain in NICD-Val1744, our results are consistent with a previous finding that proteasome inhibitors increased the levels of NICD-Val1744, but, at the same time, resulted in the transcriptional inhibition of NOTCH targets.[30]

Interestingly, in HaCaT cells treated with siATP2C1, endogenous NOTCH1 colocalized with lysosomal associated glycoprotein LAMP1,[31] indicating a distribution of NOTCH1 through the endolysosomal organelles (Figure 3). These results imply that inhibition of ATP2C1 reduces the nuclear pool of activated NOTCH1 by promoting NOTCH1-IC degradation.

3.3 | ATP2C1 inhibition alters post-endocytic trafficking of NOTCH1-IC

NOTCH receptors localized at the cell membrane are continuously internalized into early endosomes and then either recycled to the plasma membrane via recycling endosomes or degraded in the lysosomes.[32] These trafficking steps play an important role in the regulation of NOTCH signalling promoting either ligand-induced NOTCH activation or preventing inappropriate signalling from the pool of NOTCH that has been internalized but has not been activated by ligand. Additionally, the alterations of NOTCH trafficking might also have complex effects on its signalling as the delivery of receptor to the limiting membrane of the late endosome/lysosome might also promote NOTCH activation in a γ-secretase-dependent but ligand-independent manner.[32,33] Thus, we hypothesized that the loss of *ATP2C1*, by stimulating the routing of NOTCH1 through the endocytic compartments, could lead to the γ-secretase-dependent activation of the receptor followed by its lysosomal/proteasomal degradation. To address this model, we first examined the level of NOTCH1 expression at the cell surface using an antibody against its extracellular portion. Both HaCaT cells treated with either siCTR or siATP2C1 were analysed by FACS staining. The siATP2C1-treated cells showed higher cell-surface expression of NOTCH1 compared with siCTR-treated cells (53.38% vs. 41.44%) (Figure 4A); this increase was not due to an increase in *NOTCH1* transcription because the siATP2C1-treated cells displayed the same *NOTCH1* mRNA levels as siCTR treated cells (Figure 4B). Notch signalling is regulated by three successive proteolytic cleavages (S1–S3). The first occurs in the Golgi apparatus, at the S1-site by a furin-like protease creating a non-covalently associated NOTCH1 heterodimer which is then transported to the cell membrane. In mammals, dysregulation of the intracellular trafficking influences furin-cleavage of NOTCH1, resulting in an altered surface expression of the NOTCH1 receptor. Dynasore, a membrane-permeable inhibitor of dynamin, rapidly blocks the GTPase activity of dynamin and inhibits dynamin-dependent endocytosis and forward trafficking/recycling.[34,35] To clarify the effect of the

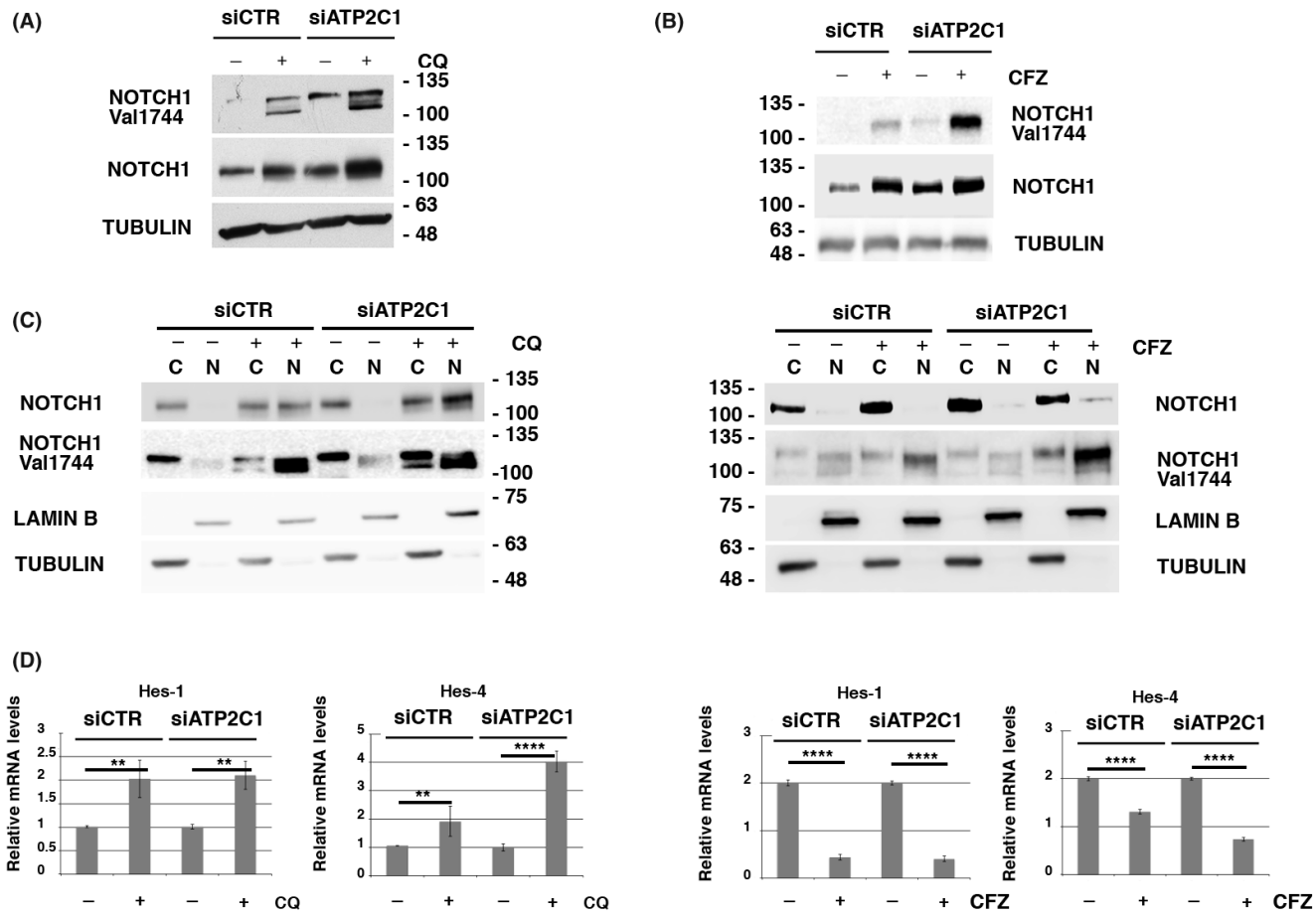

FIGURE 2 Lysosomal and proteasome inhibitors lead to increased levels and nuclear localization of NICD-Val1744 in siRNA-ATP2C1-treated cells but differentially affect the expression of NOTCH1 target genes. (A, B) In siCTR and siATP2C1 treated cells, lysosomal and proteasomal inhibition increase the expression levels of NICD-Val1744. Immunoblotting analysis of NICD-Val1744 and total NOTCH1-IC in whole cell extracts of siCTR or siATP2C1 HaCaT cells treated with 100 μM Chloroquine (24 h) or 500 nM Carfilzomib (5 h). NOTCH1-Val1744 indicates detection of endogenous levels of the NOTCH1 intracellular domain (NICD) only when released by cleavage of γ-secretase. NOTCH1 indicates detection of γ-secretase-cleaved and uncleaved NOTCH1 intracellular domain. (C) Lysosomal and proteasomal inhibition increase the nuclear localization of NICD-Val1744. Immunoblotting analysis of γ-secretase-cleaved (NICD-Val1744) and uncleaved (NOTCH1) NOTCH1 intracellular domain. NOTCH1 expression was analysed in fractionated extracts of either siCTR or siATP2C1 HaCaT cells treated with either Chloroquine or Carfilzomib. (D) Lysosomal and proteasome inhibitors differentially affect the expression of NOTCH1 target genes. HaCat cells were treated as described in panels A and B. Total mRNA was extracted after drug treatment, and the mRNA levels of the indicated *NOTCH1* target genes were analysed by Real-Time quantitative-PCR. The significance of the differences was calculated using one-way ANOVA. $^{**}p < 0.05$; $^{****}p < 0.0005$.

loss of ATP2C1 on NOTCH1 membrane localization, both pre-S1 cleaved and cell-surface expression of NOTCH1 were examined in dynasore-treated cells. Interestingly, in both siCTR and siATP2C1 treated cells, the surface level of NOTCH1 was reduced after treatment with 25 μM Dynasore (Figure 4A). Additionally, dynasore-treated cells showed an accumulation of the full-length non-furin processed NOTCH1 receptor, reflected in both decreased cell surface expression, and reduced expression of NOTCH1 ICD and as well as NICD-Val1744 (Figure 4C,D). This indicates an effect of loss of ATP2C1 function on the surface expression of NOTCH1 through an enhancement of the delivery of the furin- S1 cleaved NOTCH receptor trafficking to the cell membrane. The E3 ubiquitin ligase DELTEX is implicated in the regulation of NOTCH endocytic trafficking.[23] DELTEX stabilizes the NOTCH receptor in the endocytic compartment allowing upon its cleavage to form NICD-Val1744 that it is free to translocate to the nucleus and promote activation of its target genes.[23] Interestingly, we found that the siATP2C1-treated cells showed higher expression of DELTEX-1 compared with siCTR-treated cells (Figure 4E). Overall, our results indicate that loss of ATP2C1 increases the intracellular pool of activated NOTCH1 by increasing NOTCH1 delivery at the cell membrane and ligand-independent activation of NOTCH signalling followed by the lysosomal/proteasomal degradation of NICD-Val1744.

4 | DISCUSSION

Previous studies have shown that ATP2C1 plays an essential role in the maintenance of skin homeostasis, and the loss of ATP2C1 function has a causative role in HHD.[1,36] However, it is unclear how

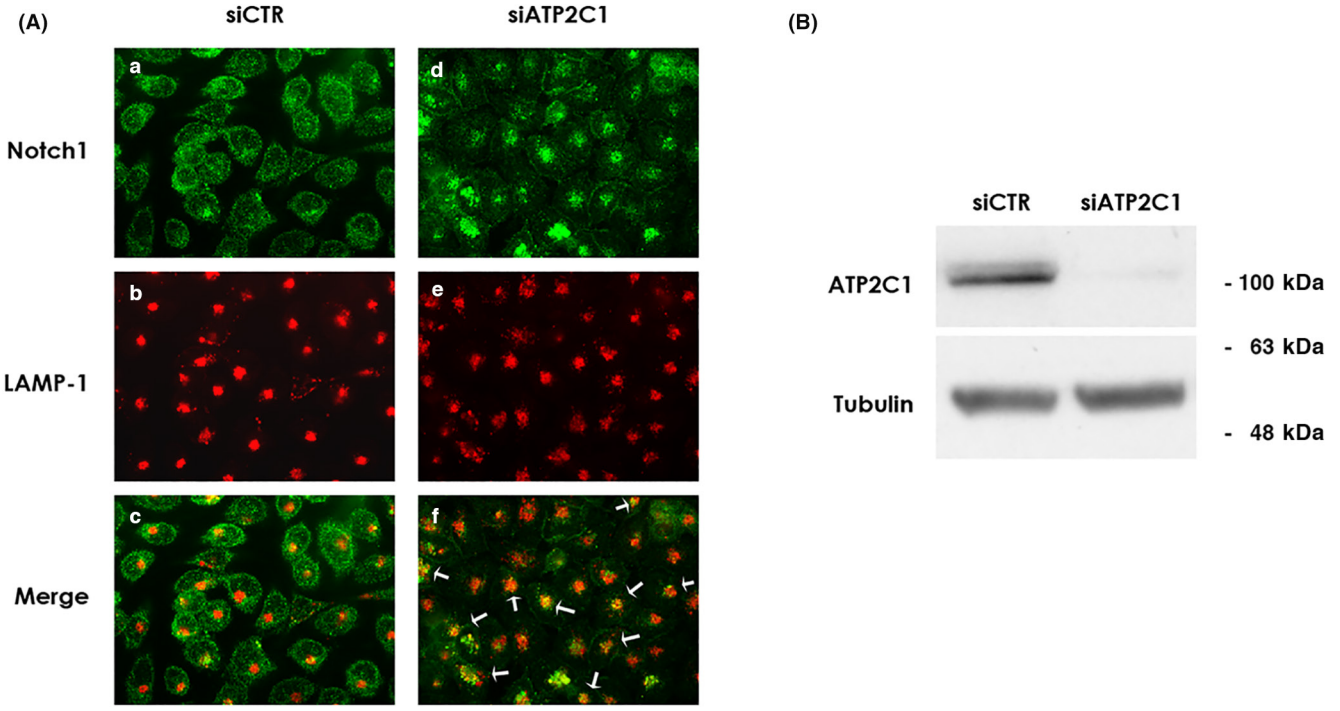

FIGURE 3 Endocytosed NOTCH1 co-localize with late endosomal/lysosomal marker LAMP1. HaCaT cells were treated with siCTR or siATP2C1, fixed, permeabilized and immunostained with anti-NOTCH1 antibody (green), followed by anti-LAMP1 antibody (red). Images were acquired under a confocal microscope. Arrows indicate NOTCH1 and LAMP1 colocalization. (A) Immunostaining for NOTCH1 and LAMP-1 in siCTR (a–c) or siATP2C1 (d–f) HaCaT cells. (B) Immunoblot for ATP2C1 as silencing control.

ATP2C1 loss affects keratinocytes homeostasis. In our previous results, we showed that human keratinocytes respond to the loss of ATP2C1 function in a manner consistent with increased oxidative stress and NOTCH1 activation.[18] NOTCH signalling is an essential determinant of keratinocyte growth and differentiation.[17,37] We found that the DNA damage response (DDR) was consistently down-regulated in keratinocytes derived from the lesions of patients with HHD.[18] Although oxidative-stress activates the DDR, ATP2C1 inactivation down-regulates DDR gene expression.[18] We showed that the DDR response was a major target of oxidative stress-induced NOTCH1 activation.[18] These results indicate that an ATP2C1/NOTCH1 axis might be critical for keratinocyte function and cutaneous homeostasis, suggesting a plausible model for the pathological features of HHD. Given the key role for NOTCH1 signalling in maintaining tissue integrity and epidermal organization, here, we analysed the mechanism of increased NOTCH1 activation in the ATP2C1-defective keratinocytes to identify the potential involvement of specific factors/mechanisms responsible for its activation. An examination of the expression of NOTCH1 target genes revealed that there was no obvious difference between their expression levels in siCTR and siATP2C1 treated cells. Therefore, although substantial evidence indicates that *ATP2C1* loss increases NOTCH1-IC expression, cellular signalling directly or indirectly interferes with the transcriptional activity of NOTCH1-IC. The observed transcriptional inactivity of NOTCH1-IC prompted us to investigate whether the membrane-nucleus shuttling, which is essential for NOTCH1-IC to elicit its transcription/biological effects, is impaired in ATP2C1-defective cells. Remarkably, we found cytoplasmic accumulation of NOTCH1-IC protein instead of nuclear localization, indicating that the loss of ATP2C1 interferes with the nuclear shuttling of NOTCH1-IC. Endocytosis plays an important role in the regulation of NOTCH signalling as it is linked to downstream signalling outcomes influencing recycling, activation, and degradation of the receptor.[21–23] In basal condition, the NOTCH receptor is continuously internalized. This removal from the cell surface is balanced by endosomal recycling pathways that return the majority of the endocytosed NOTCH receptor to the plasma membrane. The balance between internalization and recycling controls the amount of NOTCH1 on the cell surface and likely contributes to the strength of NOTCH1 signalling. However, while the majority of internalized NOTCH receptor is directly transported back to the plasma membrane, the minority is further transported towards the endocytic compartments and shunted through to lysosomes for degradation.[32] Interestingly, while this last aspect may be physiologically important to prevent aberrant NOTCH activation and maintenance of NOTCH signalling homeostasis, it has been shown that disruption of endosomal and lysosomal trafficking promotes ligand-independent activation of NOTCH receptor in lysosomes (Ref. 32 and the references therein).

NOTCH1 and its ligands are expressed in the HaCaT keratinocytes and cultured in vitro at a low calcium concentration they retain a basal phenotype. Furthermore, under basal conditions, HaCaT cells are capable of expressing differentiation-specific markers, such as Involucrin and Keratin1,[38] reflecting the fact that HaCaT cells are per se prone to differentiate spontaneously in culture. Low level of

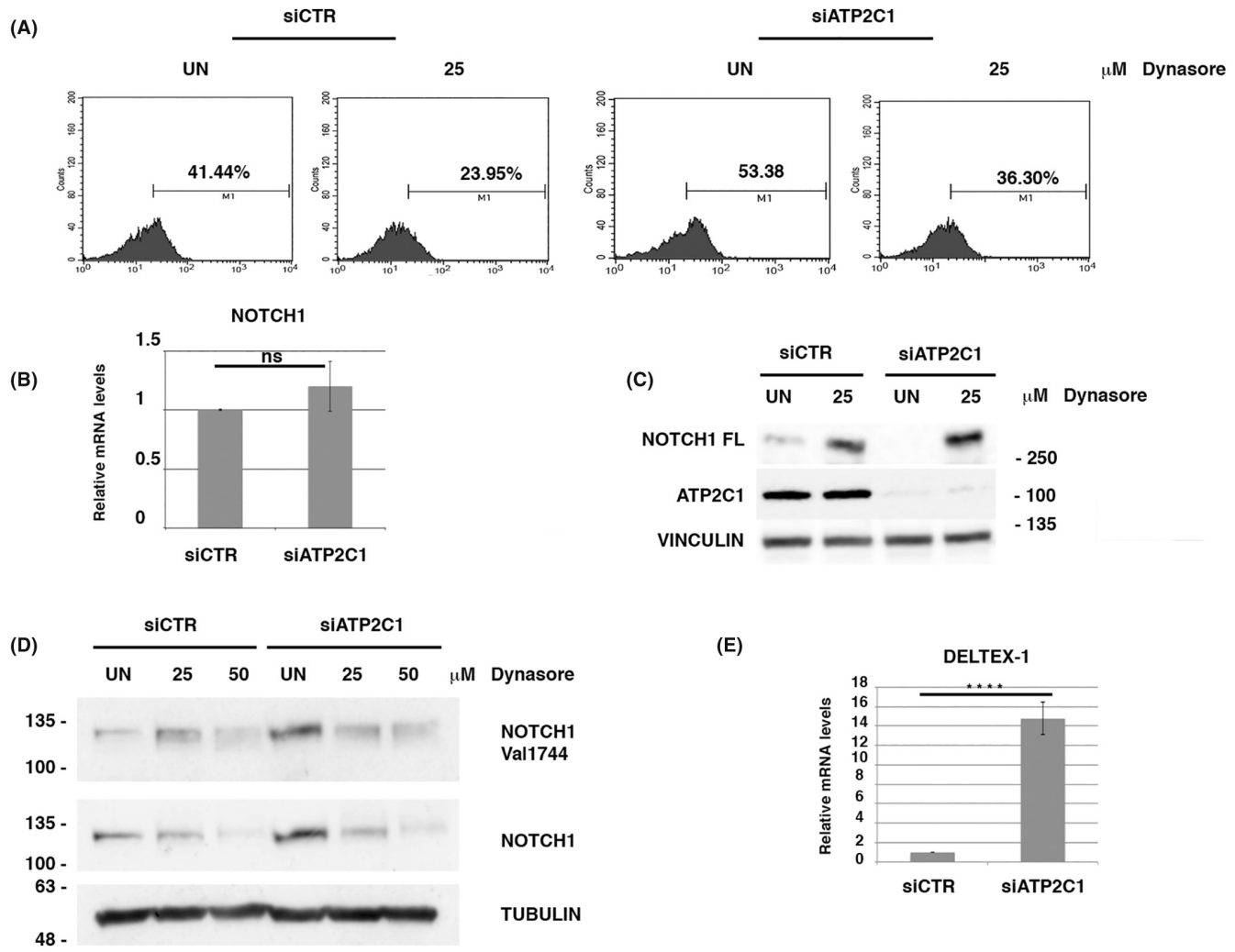

FIGURE 4 Effect of dynamin-dependent endocytosis inhibition on NOTCH1 levels in siATP2C1 treated HaCaT cells. Cells were stained with NOTCH1 extracellular domain specific antibodies and analysed by flow cytometry. The cell surface levels of NOTCH1 were compared between siCTR and siATP2C1 treated HaCaT cells. (A) Surface NOTCH1 expression on siCTR and siATP2C1 treated HaCaT cells after treatment with 25 μM Dynasore for 8 h. All data shown are representative FACS plots from three independent experiments. (B) HaCaT cells were treated with siCTR or siATP2C1, total mRNA was extracted and Notch1 mRNA levels were analysed by Real-Time quantitative-PCR. (C) Effect of Dynasore treatment on NOTCH1 processing in siCTR and siATP2C1 treated HaCaT cells. Immunoblot was performed in HaCaT cells treated as in A, the blot was incubated with an antibody against the C-terminus of NOTCH1 that recognizes the unprocessed NOTCH1 precursor (full-length pre-S1 cleaved NOTCH1). (D) Immunoblotting analysis of NICD-Val1744 in whole cell extracts of siCTR or siATP2C1 HaCaT cells after treatment with Dynasore (25–50 μM) for 8 h. (E) Analysis of the E3 ubiquitin ligase Deltex-1 expression in siCTR and siATP2C1 treated HaCaT cells. Deltex-1 mRNA expression in siCTR and siATP2C1 treated HaCaT cells was analysed by quantitative PCR. The significance of the differences was calculated using one-way ANOVA. ****$p < 0.0005$; ns, not significant.

activated NICD-Val1744 in HaCaT keratinocytes under basal conditions can be detected, suggesting that activation of NOTCH1, which has been shown to be a critical determinant of keratinocytes differentiation,[17] might support their differentiation under basal conditions. Here, we reported that treatment of HaCaT keratinocytes with either lysosomal and proteasomal inhibitors is associated with an accumulation of intracellular NICD-Val1744. The increased level of NICD-Val1744 in cells treated with proteasome inhibitor is likely the result of the stabilization of the ligand-driven NOTCH1 activation that might occur in differentiating keratinocytes under basal conditions. Interestingly, accumulation of NICD-Val1744 in lysosomal inhibitor treated cells indicates that internalized receptor generates a basal noise of ligand-independent NOTCH1 activation that is either suppressed or buffered by a mechanism that shunts NICD-Val1744 through to lysosomes for degradation. Interestingly, it has been shown that overexpression of DELTEX bypasses the requirement for NOTCH-ligands and promotes NOTCH activation through an endocytic-dependent mechanism.[39–41] Here, we have also reported that ATP2C1-defective keratinocytes are characterized by increased DELTEX 1 expression. Our data demonstrate that ATP2C1 loss increases the delivery to the cell membrane of the NOTCH1 receptor. Thus, increased DELTEX expression might represent the mechanism that mediates and rises the rate of NOTCH1 internalization upon ATP2C1 loss. We also found that the increased

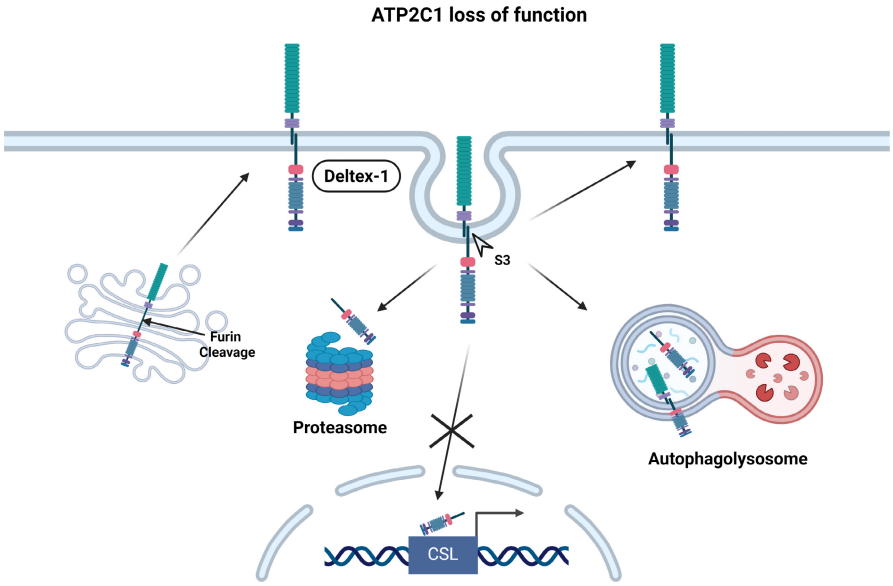

FIGURE 5 Speculative model of the effects of the loss of ATP2C1 function on NOTCH1 signalling. ATP2C1/SPCA1 is crucial for both the structure and function of the secretory pathway that ensures proper protein folding and modification as well as transport to compartments such as the extracellular space, plasma membrane and lysosome. For example, in yeast, loss of PMR1/ATP2C1 affects outer chain glycosylation, proteolytic processing and increases trafficking of proteins in the secretory pathway.[45] Additionally, endocytosis serves as an important regulatory mechanism of several processes initiated at the cell membrane. Endocytic and secretory pathways contribute to the plasma membrane recycling pathways.[46,47] Endocytosis is involved in the turnover and degradation of plasma membrane receptors and dynamin plays a significant role in this process. During maturation and transport of the receptor to the membrane, NOTCH1 is first cleaved by furin-like in the Golgi apparatus at Site-1 (S1), resulting in a heterodimeric biologically active transmembrane receptor. In our model we suggest that in normal cells, when canonical NOTCH1 signalling is switched off, the receptor is endocytosed and might be either returned to the cell surface or degraded. Our hypothesis is therefore that deficiency of ATP2C1 in keratinocytes causes secretory pathway defects, inducing the delivery of NOTCH1 to the membrane associated with an increased rate of its endocytosis. The overload of the endocytic/recycling machinery results in the re-routing of NOTCH1 from a constitutive recycling pathway in a lysosomal/proteasomal compartment leading to its abortive activation that precedes its degradation (Image Created with BioRender.com).

rate of NOTCH1endocytosis enhances its ligand-independent activation. However, ATP2C1 loss promotes an "abortive" activation of NOTCH1 since it is trafficked to the lysosomal/proteasome pathway (Figure 5). An interesting possibility is that ATP2C1 loss triggers an exacerbation of a mechanism required to either suppress or buffer basal noise of ligand-independent NOTCH1 activation. Thus, loss of ATP2C1 function impairs NOTCH1 signalling depriving keratinocytes of a key regulator of growth and differentiation.

5 | CONCLUSION

Hailey-Hailey disease represents a disease in which mutation in ATP2C1 on its own is not sufficient to cause the HHD symptoms. Both genetic modifiers and environmental factors likely influence the disease manifestation and the severity of the phenotype of HHD. Although not well understood, our results support a model in which loss of ATP2C1 increases the delivery of NOTCH1 to the cell membrane. Increased DELTEX expression might promote the internalization of NOTCH1 receptor that is then sorted into an endo/lysosomal compartment leading to its abortive activation that precedes its degradation. These results fit with previous observations indicating that loss of ATP2C1 altered both intracellular trafficking and endo/lysosomal compartment function.[9,42–44] Thus, it is likely that the disease manifestation might be influenced by both polymorphisms and environmental triggers that promote intracellular trafficking stress, influencing those pathways, as NOTCH1 signalling, for which intracellular trafficking is central in their mechanism of action.

AUTHOR CONTRIBUTIONS

Azzurra Zonfrilli, Federica Truglio, Alessandra Simeone, Maria Pelullo, Valeria De Turris, and Samantha Cialfi performed experiments. Saula Checquolo, Diana Bellavia, Rocco Palermo, and Isabella Screpanti analysed and interpreted the data. Claudio Talora, Samantha Cialfi and Azzurra Zonfrilli drafted and revised the manuscript. Dario Benelli and Daniela Uccelletti revised the work critically for important content. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors confirm that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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