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1 **Role of the cytosolic domain of occludin in trafficking and HCV infection**

2 **Running Title:** OCLN C-terminus and HCV entry

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10

11 **Synopsis**

12 The importance of the C-terminal part of OCLN for HCV infection was assessed by a
13 serial deletion approach. The characterization of OCLN mutants revealed that the
14 first 18 residues of the C-terminal cytosolic tail constitute the minimal region required
15 for HCV infection in non-polarized and polarized hepatocyte, despite a loss of TJ
16 localization. Among these 18 residues, I279 and W281 were crucial for cell surface
17 expression of OCLN mutant and modulated its ability to mediate HCV infection.

18

19 **Abstract**

20 The role of the tight-junction (TJ) protein occludin (OCLN) in hepatitis C virus (HCV)
21 entry remains elusive. Here, we investigated the OCLN C-terminal cytosolic domain
22 in HCV infection. We expressed a series of C-terminal deletion mutants in Huh-7
23 cells KO for OCLN and characterized their functionality in HCV infection and
24 trafficking. Deleting the OCLN cytosolic domain led to protein instability and
25 intracellular retention. The first 15 residues (OCLN-C15 mutant) of the cytosolic
26 domain were sufficient for OCLN stability, but led to its accumulation in the trans
27 Golgi network (TGN) due to a deficient cell surface export after synthesis. In contrast,
28 OCLN-C18 mutant, containing the first 18 residues of the cytosolic domain, was
29 expressed at the cell surface and could mediate HCV infection. Point mutations in the
30 context of C18 showed that I279 and W281 are crucial residues for cell surface
31 expression of OCLN-C18. However, in the context of full-length OCLN, mutation of
32 these residues only partially affected infection and cell surface localization.
33 Importantly, the characterization of OCLN-C18 in human polarized hepatocytes

1 revealed a defect in its TJ localization without affecting HCV infection. These data
2 suggest that TJ localization of OCLN is not a prerequisite for HCV infection in
3 polarized hepatocytes.

4

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11

12

13 **Keywords**

14 Hepatitis C virus, virus entry, virus-host interaction, occludin, tight junctions,
15 trafficking

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Introduction

With 70 million people infected, hepatitis C virus is a major health burden. In most cases, HCV establishes chronic infection that eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC). This positive single-stranded RNA virus infects almost exclusively human hepatocytes. Restriction of HCV infection to hepatocytes is partially due to the specific entry of the virus into these cells. Several lines of evidence show that HCV entry into cells is a complex multistep process that involves a series of specific cellular entry factors. Among them, scavenger receptor BI (SR-BI), tetraspanin CD81 and tight-junction (TJ) proteins claudin-1 (CLDN1) and occludin (OCLN) constitute the four major factors essential for HCV entry¹⁻³. SR-BI and CD81 have been shown to interact with the E2 HCV envelope protein and their role in HCV entry has been well documented. On the contrary, no direct interaction between HCV envelope proteins and the TJ proteins CLDN1 and OCLN was reported. CLDN1 contributes to a post-binding step of HCV entry by interacting with CD81 thus facilitating virus internalization^{4,5}.

OCLN seems to play a role at a late entry step. However, its precise role in the HCV life cycle remains poorly understood. Noteworthy, CD81 and OCLN determine the tropism of HCV for human cells. Since CLDN1 and OCLN are TJ proteins, it was initially thought that HCV virions would migrate to TJs for internalization; however, experimental data could not support this hypothesis until recently⁵⁻¹⁰. Indeed, imaging of HCV entry in a three-dimensional polarized hepatoma system revealed a sequential engagement of the entry factors with an initial localization of HCV with SR-BI and CD81 at the basolateral membrane and a subsequent accumulation of the virus at the TJs in an actin-dependent manner¹¹.

TJs are specialized sites of cell-cell contacts involved in signalling and formation of the paracellular barrier between epithelial cells and endothelial cells. OCLN was the first integral membrane protein to be identified at TJs. Its tetraspan topology orients two loops in the extracellular space (EC1 and EC2) with the N- and C-termini within the cell¹². OCLN functions at TJs are poorly understood. It is involved in the regulation of paracellular permeability and cell adhesiveness. The 4 transmembrane segments of OCLN constitute the MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain that is common to two additional junctional

1 proteins (tricellulin and MARVEL-D3) and is involved in membrane apposition¹³. The
2 extended (259aa) C-terminal cytosolic domain is endowed with signaling functions. It
3 mediates interaction with scaffolding proteins and subcellular trafficking of the protein
4 and is subjected to numerous post-translational modifications, suggesting it has an
5 important functional role^{12,14}. In this context, we sought to determine the role of the
6 OCLN C-terminal cytosolic domain during HCV infection. For this purpose, we
7 expressed a series of C-terminal deletion and point mutants in Huh-7 cells KO for
8 OCLN and characterized their functionality towards HCV infection and cellular
9 trafficking.

10 Our data showed that deletion of the OCLN cytosolic domain leads to protein
11 instability and intracellular retention. The first 15 residues of the cytosolic domain
12 (C15, aa281-522 deleted) were sufficient to restore protein stability but not cell
13 surface export nor HCV infection. Moreover, OCLN-C15 was retained in the trans
14 Golgi network (TGN) most probably due to a defect in the transport of the protein to
15 the cell surface after its biosynthesis. In contrast, the first 18 residues of the cytosolic
16 domain (C18, aa284-522 deleted) led to cell surface expression and HCV infection.
17 Among these residues I279 and W281 were crucial for the cell surface export of
18 OCLN-C18 and its ability to mediate infection. In OCLN full-length background,
19 mutation of I279 and W281 modulated HCV infectivity with no striking effect on its
20 subcellular localization. Finally, in polarized hepatocytes, OCLN-C18 did not localize
21 at TJs but was still functional for infection.

22

23 **Results**

24

25 **The first 18 cytosolic residues of the OCLN C-terminal tail are important for** 26 **HCV infection and OCLN cell surface localization**

27 In order to determine the potential role of the cytosolic C-terminal tail of OCLN in
28 HCV infection, we generated a series of C-terminal deletion mutants. The first
29 deletion corresponded to the removal of the C-terminal distal domain that contains 3
30 α -helices (aa416-522) involved in interactions with partner proteins^{15,16}. We also
31 generated 10 additional mutants by serially deleting 15 C-terminal residues per
32 construct (Figure 1). The different OCLN mutants carried an N-terminal HA epitope

1 tag and were cloned in the pTRIP lentiviral expression plasmid. A Huh-7-derived cell
2 clone knockout for OCLN (OKH4 cells)¹⁷ was used to characterize the ability of the
3 different constructs to mediate HCVcc entry. OKH4 cells were transfected with the
4 different constructs and infected with HCVcc virus at 48h post-transfection. Infectivity
5 levels were determined at 30-32h post-infection by immunofluorescence using an
6 antibody directed against the E1 viral protein. Expression levels of the mutants were
7 analyzed at the time of infection by Western blotting (Figure 2A). For most of the
8 deletion mutants, besides the band of predicted size, we could also detect a second
9 band migrating closely to the major band that could correspond to the mono-
10 ubiquitinated form of the corresponding protein. In agreement with this hypothesis,
11 several lysines of the C-terminus part of OCLN (K276, K283, K299) have been
12 reported to be ubiquitinated in 293T cells¹⁸. However, we failed to detect the
13 presence of ubiquitin by Western blotting after precipitation of HA-tagged OCLN
14 (data not shown). Alternatively, these secondary products could correspond to
15 truncated forms of OCLN as proposed previously by Liu *et al.* or to an
16 uncharacterized post-translational modification¹⁹. As expected, the expression of an
17 OCLN mutant lacking the second extracellular loop (Δ EC2) did not allow HCVcc to
18 infect OKH4 cells, whereas the complementation of OKH4 cells with the full length
19 OCLN protein led to the infection of OKH4 cells by HCVcc (Figure 2B). Surprisingly,
20 most of the deletion mutants were functional for HCVcc infection reaching levels of
21 infectivity ranging from 50% up to 100% of the level obtained with wild-type OCLN.
22 Interestingly, OCLN lacking the entire cytosolic domain failed to complement OKH4
23 cells for HCV infection. Moreover, this construct accumulated at lower level in the
24 cells as shown by the Western blot analysis. This result suggests that the C-terminal
25 cytosolic tail of OCLN is important for the stability of the protein. While OCLN mutant
26 harboring the first 15 cytosolic residues after the last transmembrane segment (C15,
27 aa281-522 deletion) accumulated at the same level as wild-type OCLN in OKH4 after
28 transfection, this truncated protein did not confer susceptibility to HCVcc infection in
29 OKH4. In contrast, the complementation with OCLN mutant harboring the first 30
30 cytosolic residues of the C-terminal tail (C30, aa296-522 deletion) restored HCV
31 infection, indicating that important residues for HCV infection were located between
32 residues 281 and 296 of OCLN C-terminal cytosolic domain. To further identify the
33 functional residues located in this region, four additional deletion mutants carrying
34 shorter deletions at aa284-522 (C18), aa287-522 (C21), aa290-522 (C24), aa293-

1 522 (C27) were generated (Figure 2C, 2D). It is worth noting that the C18 mutant that
2 contains three additional residues downstream of the OCLN-C15 C-terminal
3 sequence was able to restore HCV infection suggesting that residues 281, 282, 283
4 constituted or were part of a crucial motif for OCLN to mediate HCV entry.

6 **OCLN mutant C15 accumulates in the TGN compartment**

7 The C-terminal tail of OCLN is known to be important for OCLN subcellular
8 localization^{20,21}. Thus, the lack of infection observed for the C15 mutant could be due
9 to a defect in cell surface localization of this protein. To test this hypothesis, we
10 analyzed the cell surface expression of OCLN mutants in a biotinylation assay
11 (Figure 3A). We used sulfo-NHS-SS-biotin to specifically label plasma membrane
12 bound HA-tagged OCLN constructs. Biotinylated cell surface proteins were
13 subsequently precipitated with streptavidin-conjugated agarose beads. OCLN
14 present at the cell surface was then analyzed by Western blotting using an anti-HA
15 antibody. Whereas similar levels of biotinylated OCLN could be detected for wild-type
16 OCLN and OCLN-C18, a weaker signal (corresponding to 24% of the intensity of
17 wild-type OCLN) was obtained for OCLN-C15 showing that this mutant was less
18 efficiently exported to the cell surface.

19 To further characterize the subcellular localization of OCLN-C15 mutant, transfected
20 cells were processed for immunofluorescence detection of HA-labeled wild-type and
21 mutant OCLN (Figure 3B). Whereas wild-type OCLN and OCLN-C18 were mainly
22 observed at the plasma membrane of OKH4 cells, OCLN-C15 and OCLN Δ C
23 accumulated intracellularly. Altogether, these results suggested that the first 18
24 residues (aa266 to 283) of the C-terminal tail of OCLN are required for OCLN cell
25 surface localization.

26 The analysis of OCLN-C15 mutant subcellular localization by confocal microscopy
27 revealed a perinuclear staining, suggesting an endoplasmic reticulum (ER) or a trans
28 Golgi network (TGN) localization of the protein in transfected OKH4 cells. To further
29 characterize the subcellular localization of this mutant, we performed colocalization
30 experiments using different subcellular compartment markers. As shown in Figure 4A,
31 OCLN-C15 colocalized with the TGN marker, TGN46 with a Pearson correlation
32 coefficient value of 0.6, indicating a substantial overlap. In contrast, no significant
33 colocalization was observed with the calreticulin (CRT), an ER resident protein
34 (Pearson correlation coefficient: 0.2) (Figure 4B). On the contrary, wild-type OCLN

1 localized mainly at the plasma membrane and did not overlap with the TGN and ER
2 markers (Pearson correlation coefficient: 0.15 and 0.2 respectively). This result
3 indicates that deletion of the residues 281-522 of the C-terminal cytosolic part of
4 OCLN leads to an accumulation of the protein in the TGN.

5 6 **Deletion of residues 281-522 of OCLN does not affect the fate of cell surface** 7 **expressed OCLN**

8 Intracellular accumulation of OCLN-C15 could be due to a defect in the targeting of
9 the protein to the plasma membrane along the secretory pathway or to an impaired
10 recycling to the cell surface after internalization. To distinguish between these two
11 hypotheses, we characterized the trafficking of the fraction of OCLN-C15 that
12 reached the cell surface using a biotinylation assay (Figure 5). In a first step, we
13 evaluated the ability of the protein to internalize at 37°C. For this purpose, cell
14 surface proteins were biotinylated at 4°C and the cells were subsequently incubated
15 at 37°C for different periods of time, allowing their internalization. Thereafter, the
16 biotin that remained at the cell surface was cleaved. This way, biotinylated proteins
17 corresponded to internalized proteins. Biotinylated proteins were then precipitated
18 with streptavidin-agarose beads and OCLN detected by Western blotting. As a
19 control experiment, we monitored the endocytosis of the transferrin receptor whose
20 cellular trafficking is well documented (Figure 5C). As shown in Figure 5, similar
21 fractions of cell surface wild-type OCLN (Figure 5A) and OCLN-C15 (Figure 5B)
22 internalized with close kinetics (Figure 5D).

23 A fraction of OCLN has been previously shown to recycle back to the plasma
24 membrane²². Since OCLN-C15 was able to internalize, we tested its ability to recycle
25 back to the plasma membrane. To do so, cell surface proteins were biotinylated and
26 cells incubated at 37°C to induce their internalization. After this step, cell surface
27 biotin was removed by cleavage, which resulted in a selective biotinylation of
28 internalized proteins. A second step of incubation at 37°C during different periods of
29 time allowed internalized protein to potentially recycle back to the plasma membrane.
30 For each time point, one sample was then treated with reduced glutathione to
31 remove the biotin from the proteins that recycled back to the cell surface (internalized
32 biotinylated proteins, Figure 6 biotin cleavage +), whereas another sample was left
33 untreated (total biotinylated proteins, Figure 6 biotin cleavage -). In this experimental
34 setting, the amount of OCLN proteins that recycled to the cell surface corresponds to

1 the difference between untreated and treated samples. Calculation of the recycling
2 rate for wild-type OCLN and OCLN-C15 did not reveal any significant difference
3 between these two proteins (Figure 6A and 6B). In both cases, the proportion of
4 recycled OCLN reached a maximum of 25% (Figure 6D), whereas 90% of the TfR
5 was found to recycle back to the cell surface in the same conditions (Figure 6C and
6 D). This result is in agreement with a previous report showing that 20% of
7 internalized OCLN is transported back to the cell surface²². Altogether these findings
8 did not show major differences between OCLN-C15 mutant and wild-type OCLN for
9 their trafficking in the endocytic pathway after their internalization from the plasma
10 membrane.

11 To further confirm that the accumulation of OCLN-C15 in the TGN was not due to a
12 defect of trafficking of OCLN-C15 in the endocytic pathway, we performed antibody
13 uptake experiments. This approach requires antibodies recognizing the extracellular
14 region of the protein. To our knowledge all currently commercially available OCLN-
15 specific antibodies recognize intracellular epitopes of the protein. To overcome this
16 limitation, we introduced a Flag epitope in the second extracellular loop (EC2) of
17 OCLN at a position (between I212 and Y213) that was previously reported to be
18 neutral for HCV infection²³. As expected, the introduction of a Flag epitope in wild-
19 type OCLN and OCLN-C15 EC2 did not affect HCV infectivity levels obtained in
20 transfected OKH4 cells (data not shown). 48 hours after transfection of Flag-tagged
21 wild-type OCLN and OCLN-C15 constructs, OKH4 cells were incubated with anti-
22 Flag antibodies for 30 min to label endocytosed OCLN. The cells were then fixed and
23 processed for immunofluorescent detection of anti-Flag antibody. As shown in Figure
24 7, intracellular staining was observed for wild-type OCLN and OCLN-C15, which
25 corresponds to the detection of the internalized OCLN. In order to further
26 characterize the intracellular trafficking of the internalized OCLN constructs, we
27 performed colocalization studies using markers of the TGN (TGN46), of the recycling
28 endosomes (transferrin receptor) and of the lysosomes/ late endosomes (Lamp1).
29 Although we had previously observed an accumulation of OCLN-C15 in the TGN of
30 transfected cells, no significant colocalization of internalized OCLN with the TGN
31 marker was observed (Figure 7A and D). On the contrary, endocytosed OCLN
32 predominantly colocalized with the TfR receptor (Figure 7B and D). No significant
33 colocalization was observed with the lysosomal marker, Lamp1 (Figure 7C and D).
34 Moreover, OCLN-C15 and wild-type OCLN exhibited the same staining pattern in all

1 co-labelling immunofluorescence experiments, confirming that the deletion carried by
2 OCLN-C15 had no impact on the OCLN endocytic pathway.
3 Collectively, these findings indicate that OCLN-C15 accumulation in the TGN does
4 not result from an alteration of trafficking in the endocytic pathway. Consequently,
5 they indicate that it is due to a defect of transport of the newly synthesized protein
6 from the TGN to the cell surface in the secretory pathway.

7 8 **Identification of the residues involved in the targeting of OCLN to the cell** 9 **surface**

10 Whereas OCLN-C15 mutant was affected in its ability to reach the cell surface,
11 addition of the next three residues downstream C15 cytosolic region in the OCLN C-
12 terminal cytosolic sequence restored OCLN cell surface expression and as a
13 consequence HCV entry. This suggested that these three residues (W281, D282,
14 K283) were part of a crucial motif for OCLN export to the cell surface. In order to
15 identify residues that are important for OCLN cell surface expression, we created
16 point mutations in the context of OCLN-C18 mutant sequence background.
17 Interestingly, the C-terminal sequence of OCLN-C18 contains an Isoleucine-Leucine
18 sequence that could function as a di-leucine motif. Di-leucine motifs have been
19 shown to constitute Golgi sorting as well as internalization signals ²⁴. Moreover,
20 OCLN-C18 C-terminal sequence contains an aspartic acid (D275) that could
21 constitute, together with the isoleucine (I279) and leucine (L280) residues, an
22 internalization signal (Figure 8A).

23 We thus mutated the C-terminal residues of OCLN-C18 starting from D275. Indeed,
24 D275, K276, S277, N278, I279, L280, W281, D282 and K283 were individually
25 replaced by an alanine residue in OCLN-C18 background and cloned in the pTRIP
26 lentiviral vector. After having checked that all the constructs were expressed at
27 similar levels in transfected OKH4 cells (Figure 8B), we evaluated their ability to
28 complement OKH4 cells for HCV infection (Figure 8C). Interestingly, the mutation of
29 residues I279 and W281 led to a 70% decrease of infectivity while for L280A and
30 D282A mutants, a decrease of infectivity of 35% was observed. Unexpectedly,
31 mutation K283A led to an increase of infectivity (Figure 8C). The remaining mutations
32 had no major impact on HCV infection levels. Cumulative mutations of I279 and
33 W281 (IW mutant) and I279 L280 W281 and D282 (ILWD mutant) did not result in
34 any decrease of infectivity compared to that observed for the single I279A and

1 W281A mutants suggesting that these residues were concomitantly required for
2 OCLN-C18 functionality (Figure 8C). We then tested the importance of these
3 residues in the context of full-length OCLN. As shown in Figure 8C, single mutations
4 in the I279 or W281 residues led to a decrease of infection of approximately 50%, in
5 the full-length OCLN background. As observed for the OCLN-C18 mutants, the
6 cumulative IW and ILWD mutations did not significantly increase the deleterious
7 effect of individual mutations on HCV infection (Figure 8C). Together, these results
8 show that I279 and W281 constitute key residues for HCV infection in the context of
9 OCLN-C18 and modulate the ability of full-length OCLN to mediate infection.

10 As observed for OCLN-C15 mutant, the effect of IW mutations on HCV infection
11 could be the consequence of a defect in cell surface expression of OCLN. Moreover,
12 the weaker effect of IW and ILWD mutations on the ability of full-length OCLN to
13 mediate HCV infection could be due to the presence of additional cell surface export
14 signal or sequences compensating the function of IW during infection in the C-
15 terminal part of the protein. To test this hypothesis, we introduced IW and ILWD
16 mutations in OCLN C-75 and OCLN-150 mutants that harbour shorter deletion of the
17 C-terminal tail than OCLN-C18 mutant (Figure 8D). Noteworthy, OCLN C-terminus
18 contains a tyrosine-based motif at position 481-484 (YNRL) and a site (FYXXXXY
19 residues 336-342) potentially involved in caveolar endocytosis^{14,25,26}. To determine
20 whether these motifs contributed to the ability of the IW and ILWD full-length OCLN
21 mutants to mediate infection, we cumulated I279A and W281A mutations with that of
22 residues F336, Y337, Y342, Y481 and L484 in the OCLN-TYR-IW mutant. OCLN-
23 TYR mutant that only contained mutations of the potential tyrosine based motifs as
24 well as OCLN-C150 mutant were fully competent for HCV infection in OKH4 cells
25 (Figure 8D). As observed in the context of full-length OCLN, introduction of I279A
26 and W281A mutations in OCLN-C150 and OCLN-TYR mutants led to a 30 to 40%
27 decrease of the infectivity levels obtained for wild-type OCLN in OKH4 cells. OCLN-
28 C75 mutant was associated with a 30% decrease of the infectivity level and
29 introduction of IW mutations in that mutant further impacted its ability to mediate
30 infection by 13% (corresponding to a residual infectivity of 50 %). As observed in full-
31 length OCLN, no significant differences of phenotypes were observed when IW or
32 ILWD residues were mutated in the different OCLN mutants' context (data not
33 shown). The weak impact of IW mutations in OCLN-C150, OCLN-TYR and OCLN-

1 C75 mutant backgrounds suggests that these mutants still contain signals
2 compensating the absence of I279 and W281 residues for HCV infection.
3 We then sought to determine if, as found for OCLN-C15, the decreased ability of the
4 OCLN C18I, C18L, C18W, C18D, C18IW, C18ILWD, OCLN_IW, and OCLN_ILWD
5 mutants to mediate infection was correlated to a defect in cell surface expression.
6 For this purpose, we performed cell surface protein biotinylation experiments. While
7 similar levels of HA-tagged wild-type and OCLN mutants were observed in the
8 lysates (Figure 9A), significant differences in the amounts of biotinylated wild-type
9 and mutant OCLN were observed. Thus, as for OCLN-C15, weaker signals were
10 observed for the OCLN-C18I, C18W, C18IW, and C18ILWD mutants, compared to
11 wild-type OCLN and OCLN-C18 (Figure 9A). This result shows that the OCLN C18I,
12 C18W, C18IW, and C18ILWD mutants are less efficiently exported to the cell surface.
13 Conversely, the C18L and C18D mutants were not affected in their cell surface
14 expression, which is in agreement with the weaker impact of these mutations on HCV
15 infection. Furthermore, no significant differences in the amount of biotinylated OCLN
16 were observed for the OCLN_IW and OCLN_ILWD mutants, indicating that these
17 mutations have no major impact on the cell surface expression in the context of the
18 full-length OCLN.

19 Next, we tested the effect of the mutations on the subcellular localization of OCLN. In
20 agreement with the strong effect of I279A and W281A mutations on the ability of
21 OCLN-C18 to mediate HCV infection, we observed an intracellular accumulation of
22 both mutants as described for the OCLN-C15 mutant. The results of OCLN co-
23 labeling with TGN46-specific antibody in immunofluorescence experiments indicated
24 that similarly to what was observed for OCLN-C15, the mutants C18I and C18W
25 were mainly localized in the TGN (Figure 9 B and C).

26 In contrast, confocal microscopy analysis of the subcellular localization of the
27 OCLN_IW, OCLN_ILWD, OCLN-C75, OCLN-C75IW, OCLN-C150IW and OCLN-TYR-
28 IW mutants did not show any significant change in the protein localization as
29 compared to the wild-type OCLN protein. Indeed, in all cases, the proteins were
30 detected at the plasma membrane (Figure 9B and C and Supplementary Figure 1).
31 This result is in line with the weaker effect observed for these mutants regarding HCV
32 infection (Figure 8C and 8D).

33 Mutation of I279 and W281 in the context of full-length OCLN affected HCV infection
34 with no significant effect on the cell surface expression of the protein (Figure 8C and

9). Since I279 and L280 residues could potentially constitute an internalization signal, the decrease in infectivity observed for OCLN_IW mutant could be due to a defect in the internalization of the protein. To test this hypothesis, we performed internalization assays after cell surface biotinylation. As shown in Figure 10, no significant difference between the internalization kinetics of wild-type OCLN and OCLN_IW could be observed, excluding any effect of the mutations on OCLN internalization.

N-linked carbohydrates can restore cell surface transport of TGN-retained OCLN mutants

N-glycans have been shown to constitute apical sorting signals²⁷ and to promote cell surface expression of membrane proteins deleted from their basolateral sorting signals in non-polarized cells²⁸. Thus, Gut and collaborators have shown that the addition of N-glycosylation sites in the extracellular loops of OCLN deleted from its C-terminal cytosolic domain allowed the restoration of the cell surface expression of the protein in CHO cells²⁸. In a similar manner, we tested the effect of the insertion of N-glycosylation sites in the extracellular loops of OCLN deletion mutants that exhibited a TGN intracellular retention. As previously reported in Gut *et al.* 1998, the introduction of N-glycosylation sites in OCLN-C18 resulted in a shift of the bands corresponding to the OCLN protein in anti-HA Western blotting (Figure 11A). Several bands of different molecular weights could be detected for the C15 and C18IW mutants that could represent high mannose and complex glycosylated forms of the protein. Treatment of the transfected OKH4 cell lysates with the PNGase F amidase, which cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid and complex oligosaccharides, led to the recovery of the non-glycosylated OCLN proteins (Figure 11A). Interestingly, the addition of N-glycosylation sites in the OCLN sequence restored the cell surface expression of OCLN-C15 and C18IW mutants in OKH4 cells (Figure 11B). However, despite the recovery of the cell surface expression of these OCLN mutants, the insertion of N-glycosylation sites did not restore the functionality of these mutants towards HCV infection (data not shown). This result might be due to the masking of OCLN regions involved in HCV infection by the N-glycans. We thus tested the effect of the addition of a single N-glycosylation site in the first extracellular loop (EC1) of OCLN. This modification was sufficient to restore the cell surface expression of the C15 and C18IW mutants but did not allow the protein to mediate HCV infection (data not

1 shown). However, again, we cannot exclude that the presence of this glycan
2 interferes with the role of OCLN during HCV infection. In agreement with this
3 hypothesis, addition of a N-glycosylation site in EC1 of wild-type OCLN and OCLN-
4 C18 compromised the ability of the proteins to mediate HCV infection (data not
5 shown).

6

7 **Impact of C-terminal mutations on the addressing of OCLN to the tight** 8 **junctions and on HCV infection in polarized Huh-7 cells**

9 Our data show that the deletion of the OCLN C-terminus from aa284 to aa522 (C18
10 mutant) has no impact on the cell surface expression of OCLN and on its role during
11 HCV infection. However, the deletion of the C-terminal domain might potentially
12 affect its ability to associate with TJs and modulate their functions. In order to
13 determine the functionality of the different OCLN mutants in a more physiologically
14 relevant system, we used polarized Huh-7 derived cells⁹. Thus, Huh-7 clone 1sc3
15 cells that polarize in the presence of DMSO were transduced by lentiviral vectors
16 expressing wild-type or mutants OCLN. At two days post-transduction, the
17 localization of the different mutants and wild-type OCLN was determined by
18 immunofluorescence. In parallel, the TJ protein ZO1 was labeled to control the
19 polarization level of the cells. As expected, we observed a colocalization of wild-type
20 OCLN and ZO1 in the area of cell-to-cell contact suggesting a targeting of OCLN at
21 the TJs in Huh-7 clone 1sc3 cells (Figure 12A, B and C). Whereas a labeling pattern
22 similar to that of wild-type OCLN was observed for the OCLN_IW, C15 and C18IW
23 mutants accumulated intracellularly in a similar manner to what was observed in
24 transfected OKH4 cells. Surprisingly, OCLN-C18 mutant that was expressed at the
25 cell surface of transfected OKH4 cells did not present any colocalization with ZO1 but
26 appeared to be rather scattered at the cell surface of polarized 1sc3 clone cells. This
27 result suggests that the deletion of the 284-522 residues leads to a loss of OCLN
28 targeting to TJs in 1sc3 polarized cells.

29 We then sought to determine if the defect in the addressing of OCLN-C18 to the TJs
30 was affecting its ability to mediate HCV infection. For this purpose, we generated
31 OCLN-knockout 1sc3 cells using the clustered regularly interspaced short
32 palindromic repeat (CRISPR)/CRISPR-associated protein 9 system. As shown in
33 panel A of Figure 13, no expression of OCLN was detected by Western blotting
34 analysis using an anti-OCLN mAb in the OCLN-KO 1sc3 cells, whereas a product of

1 the expected size could be detected in the parental cells.

2 In order to test the functionality of OCLN mutants regarding HCV infection, OCLN-KO
3 1sc3 cells were transduced with lentiviral vectors expressing the different OCLN
4 mutants or the wild-type protein. We first characterized the subcellular localization of
5 wild-type and mutant OCLN in complemented cells (Figure 13 panel B and C).
6 Analysis of the cell surface expression of OCLN mutants by biotinylation assay
7 revealed a decrease in the cell surface expression of C15 and C18IW mutants,
8 whereas similar fractions of OCLN_IW and wild-type OCLN were exported to the cell
9 surface (Figure 13B). For the C18 mutant, we observed an intermediate phenotype
10 with a slightly lower fraction than that of wild-type OCLN reaching the cell surface.
11 These results are in agreement with the immunofluorescence analysis (Figure 13C)
12 that showed similar localization patterns to those previously observed in parental
13 clone 1sc3 cells. Thus, OCLN_IW and wild-type OCLN localized at TJs, whereas
14 C15 and C18IW mutants were retained intracellularly and C18 was partially
15 expressed at the cell surface.

16 In order to further characterize the subcellular localization of OCLN-C18 mutant, we
17 co-transduced OCLN-KO 1sc3 cells with lentiviral expression vectors for OCLN
18 mutants and with lentiviral expression vectors for CD81 or CLDN1 fused to a
19 fluorescent protein (Green Fluorescent Protein and cerulean respectively). Whereas
20 CLDN1 localizes at TJ, CD81 has been shown to be only present at the basolateral
21 pole of 1sc3 cells⁹. In agreement with the data obtained in ZO-1 co-labeling assay,
22 wild-type OCLN colocalized with CLDN1 in the areas of cell-to-cell contact while
23 OCLN-C15 accumulated in the cytosol. OCLN-C18 mutant labelling did not overlap
24 with CLDN-1, confirming the previously observed loss of TJ association of this
25 mutant (Figure 13 D and E). Interestingly, as shown in Figure 13D and E, OCLN-C18
26 mutant seemed to distribute to the same cell surface areas as CD81 (z section 1 and
27 2) with no perfect colocalization of both proteins, suggesting that they belong to
28 different domains of the basolateral membrane.

29 In a second step, we characterized the susceptibility of complemented OCLN-KO
30 clone 1sc3 cells to HCV infection. For this purpose, cells were infected with HCVcc
31 two days after transduction. Similar levels of expression for wild-type and mutant
32 OCLN were observed in transduced cells at the time of infection (Figure 14A).

1 Infection levels were determined by immunofluorescence. As shown in Figure 14
2 (panel B), HCV infection profiles in wild-type and mutant OCLN rescued 1sc3 cells
3 were similar to those found in OKH4 cells. Indeed, C15, C18IW and OCLN_IW
4 mutants were affected in their ability to mediate infection, whereas OCLN-C18 was
5 fully competent for infection. In order to confirm this result in fully polarized cells,
6 transduced clone 1sc3 cells were treated with DMSO to reinforce polarization in
7 transwell dishes. This experimental setting allows the specific infection of cells on
8 their basolateral pole. In this context, the impairment of C15 and C18IW mutants for
9 HCV infection was confirmed and C18 mutant was fully functional for infection (data
10 not shown). However, no significant decrease in infection was observed for OCLN-IW
11 mutant.

12 Finally, despite its inability to be located at TJs, the OCLN-C18 mutant remains
13 competent for HCV infection in polarized cells. This finding strongly suggests that at
14 least in the polarized clone 1sc3 model, the localization of OCLN to TJs is not
15 absolutely required for HCV entry.

16

17 **Discussion**

18 The role of OCLN in HCV entry remains poorly understood while the C-terminal part
19 of OCLN has been shown to be a key component for the protein functions. Overall,
20 this study aimed at investigating the role of the OCLN C-terminal cytosolic tail in HCV
21 infection. For this purpose, a series of C-terminal deletion mutants were expressed in
22 Huh-7 cells KO for OCLN and their functionality in HCV infection and trafficking were
23 characterized. This approach revealed that the deletion of the OCLN C-terminal
24 cytosolic domain (Δ C) leads to protein instability and intracellular retention. The first
25 15 residues (C15) of the cytosolic domain were sufficient for protein stability but not
26 for cell surface expression nor HCV infection. Furthermore, the characterization of
27 the subcellular localization of the C15 mutant revealed an accumulation of the protein
28 in the TGN. In contrast, the first 18 residues (C18) of the C-terminal cytosolic domain
29 led to cell surface expression and HCV infection. Among these amino acids,
30 isoleucine 279 and tryptophan 281 of OCLN-C18 were key residues for both cell
31 surface expression and HCV infection. Moreover, mutation of residues I279 and
32 W281 led to the accumulation of OCLN-C18 in the TGN as found for OCLN-C15. In
33 the context of the full-length OCLN, mutation of these residues partially affected the

1 ability of OCLN to restore the permissivity of OCLN-KO Huh-7 cells to HCV infection
2 but had no significant effect on the cell surface localization of the protein. Finally, the
3 assessment of OCLN-C18 functionality in polarized human hepatoma-derived cells
4 revealed that despite a defect in TJ targeting, this mutant could mediate HCV
5 infection. This result suggests that OCLN localization at TJs is not required for entry
6 of HCV into polarized hepatocytes.

7 Deletion of the C-terminal cytosolic tail of OCLN led to a decrease in OCLN stability
8 and to an intracellular retention of the protein. The C-terminal part of OCLN, which
9 represents half of the protein length, has been shown to be the target of extensive
10 post-translational modifications that modulate OCLN function in TJs ¹². Moreover, it
11 has been reported to mediate the interaction with scaffolding proteins and the
12 subcellular trafficking of OCLN ²⁰. Thus, the C-terminal cytoplasmic domain of OCLN
13 is sufficient to mediate efficient basolateral transport of a reporter protein, indicating
14 that it contains basolateral-targeting determinants ²⁰. In agreement with our data,
15 deletion of the C-terminal domain of OCLN had been previously shown to lead to the
16 accumulation of the protein in the Golgi complex when expressed in polarized Madin-
17 Darby canine kidney cells (MDCK) as well as in non-polarized Chinese hamster
18 ovary cells (CHO) ²⁸. However, no effect of the deletion on the stability of OCLN was
19 reported in the study of Gut *et al.* In contradiction with our findings, Liu and
20 collaborators found that a Flag-tagged OCLN deleted of its C-terminal tail (Flag
21 OCLN Δ C) could confer susceptibility to retroviral particles pseudotyped with HCV
22 envelope proteins (HCVpp) when this mutant OCLN protein was expressed in 786-O
23 renal carcinoma cells that express low level of OCLN ¹⁹. However, they also
24 observed an intracellular retention of the protein when expressed in 293T cells as a
25 GFP-tagged protein. Furthermore, biotinylation analysis of the cell surface
26 expression of the Flag OCLN Δ C construct revealed lower levels of protein expression
27 at the cell surface, in agreement with our findings. The different effects of the OCLN
28 C-terminal tail deletion on HCV infection observed in Liu's study as compared to our
29 work could be due to the fact that two distinct infection systems were used to
30 characterize OCLN deletion mutants. Indeed, Liu and colleagues performed their
31 infection experiments with HCVpp and their OCLN mutants were expressed in 786-O
32 carcinoma renal cells, whereas we used HCVcc virus and Huh-7 hepatoma cells KO
33 for OCLN. Since transfected OCLN has been shown to be able to dimerise with

1 endogenous OCLN, residual expression of low level of OCLN in 786-O cells might
2 have an impact on the functional characterization of OCLN mutants ²⁰.

3 Our finding that cells complemented with OCLN-C18 displayed phenotypes that were
4 similar to those of the wild-type protein in non-polarized hepatoma-derived cells was
5 surprising. The C-terminal domain of OCLN is 259 aa long and corresponds to half of
6 the protein length, but the presence of only 18 residues of the C-terminal domain was
7 sufficient to restore the ability to mediate HCV infection, as well as the localization of
8 OCLN at the cell surface. This raised the question of the role of the remaining part of
9 the C-terminal domain. However, the use of polarized hepatoma-derived cells
10 showed the importance of the remaining part of the cytosolic tail of OCLN for its TJ
11 association. Our results are in agreement with the findings showing that the distal C-
12 terminal part of OCLN mediates the interaction of the protein with scaffolding proteins
13 such as ZO1 ¹⁵ and is required for the localization of OCLN at the site of cell-to-cell
14 junctions. However, despite its mislocalization, OCLN-C18 could still render OCLN
15 KO polarized hepatoma-derived cells competent for HCV infection. This result
16 suggests that the localization of OCLN to the TJs is not an absolute prerequisite for
17 infection. In line with this hypothesis, a fraction of the CLDN1 pool was found to be
18 expressed together with CD81 and SR-BI at the basolateral membrane of clone 1sc3
19 polarized cells ⁹. Moreover, in this model, HCV entry has been shown to occur
20 preferentially from the basolateral pole of the cell. Thus, the presence of OCLN-C18
21 at the basolateral membrane would be sufficient to promote HCV infection in
22 polarized cells. Nevertheless, the importance of TJs for HCV infection might depend
23 on the cell culture system used. Indeed, the recent imaging of HCV entry in a three-
24 dimensional polarized hepatoma system revealed an initial colocalization of HCV with
25 basolateral entry factors SR-BI, CD81 and EGFR. This step was followed by an
26 actin-mediated accumulation of the virus at the TJ and its association with OCLN and
27 CLDN1 prior to cell entry ¹¹.

28 The characterization of the internalization and recycling of the OCLN-C15 mutant did
29 not show any difference with wild-type OCLN. In agreement with the results obtained
30 by Fletcher *et al.* in MDCK cells ²², we found that approximately 20% of internalized
31 OCLN was recycled back to the plasma membrane. This result indicates that the
32 accumulation of the C15 mutant in the TGN is not due to a defect in OCLN recycling
33 but rather to a defect of transport of the protein to the cell surface after its
34 biosynthesis. In agreement with this hypothesis, antibody uptake experiments

1 performed to characterize the fate of the cell surface expressed C15 mutant did not
2 reveal any difference in the localization of internalized C15 and wild-type OCLN.
3 Indeed, both of them colocalized with the transferrin receptor that follows the fast
4 recycling route from early endosomes as well as the slow recycling route via the
5 endocytic recycling compartment ²⁹.

6 Our study identified I279 and W281 as crucial residues for the cell surface
7 expression and the functionality in HCV infection of the OCLN-C18 mutant.
8 Moreover, L280 and D282 residues seemed to modulate the function of the protein in
9 HCV infection, most likely in the entry step. The mild effect of L280A and D282A
10 mutations on infection and the absence of effect on cell surface expression suggest
11 that these residues play an indirect role in the trafficking of the protein. Thus, their
12 replacement by alanine induces a local change of the environment of I279 and W281
13 that might impact their functionality.

14 As it is the case for the exit from the ER, it is now admitted that linear sorting signals
15 also mediate the targeting of the membrane proteins from the Golgi complex to their
16 assigned membrane compartment ³⁰. Thus, di-leucine motifs ([DE]XXXL[LI] or
17 DXXLL), tyrosine-based motifs (NPXY or YXX ϕ , where X is any amino acid and
18 ϕ is an amino acid with bulky hydrophobic side chain) as well as non-canonical motifs
19 have been shown to be recognized by adaptor protein complexes or adaptor proteins
20 ³¹ that sort Golgi cargo to different vesicle carriers. In the case of OCLN, a di-leucine
21 motif (D275XXXI279L280) present in the functional OCLN-C18 mutant did not seem
22 to be involved in the trafficking of this mutant since replacing L280 and D275 with
23 alanine had no effect on its cell surface localization. Instead, I279 and W281 residues
24 were crucial for the cell surface export of the OCLN-C18 mutant and their mutation
25 resulted in TGN accumulation of the protein. This result suggested that these
26 residues constitute a TGN export signal in the OCLN-C18 context and further
27 illustrate the diversity of Golgi export signals. While in general the deletion of these
28 signals leads to the apical or non-polarized distribution of the protein, it has also been
29 reported to induce the retention of the protein in the Golgi apparatus in several cases
30 ^{32,33}. Thus, the mutation of a membrane proximal tribasic motif in the reovirus p14
31 fusion-associated small transmembrane (FAST) protein was associated with the
32 accumulation of the protein in the TGN ³⁴. Similarly, the mutation of the Golgi export
33 signals of the potassium channel Kir2.1 that is formed by a patch of residues present

1 in the tertiary structure of the protein ³⁵ leads to the intracellular accumulation of the
2 protein in the Golgi.

3 Whereas I279A and W281A mutations clearly affected OCLN-C18 cell surface
4 localization and HCV infection in complemented OKH-4 cells, they only partially
5 affected the functionality of full-length OCLN for HCV infection. Moreover, they had
6 no significant effect on the subcellular localization of the protein or on its
7 internalization. This result suggests that the cytosolic domain of OCLN contains
8 additional independent cell surface export and internalization signals. In agreement
9 with this hypothesis, the OCLN C-terminus contains a tyrosine-based motif at
10 position 481-484 (YNRL) that is associated with clathrin-mediated endocytosis ^{14,25}.
11 Moreover, it contains a site with high homology with known caveolin-1 binding
12 sequences (FYXXXXY residues 336-342) ²⁶, suggesting that OCLN internalization
13 also involves caveolar endocytosis. However, the OCLN mutant harbouring
14 mutations in these tyrosine-based motifs as well as I279A and W281A mutations was
15 only partially affected in its ability to mediate HCV infection, and no significant effect
16 on the subcellular localization of the protein was observed. These results suggest
17 that these motifs are not crucial for the cell surface expression of OCLN.
18 Alternatively, OCLN trafficking has also been reported to be regulated by post-
19 translational modification of its C-terminal tail ¹³. Although the ILWD motif seems to
20 be non-essential to the cell surface localization of the full-length protein, this motif
21 was found to be highly conserved in OCLN among species.

22 Interestingly, the addition of 2 glycosylation sites in the extracellular loop of the C15
23 and C18IW mutants restored the cell surface expression of the mutants. Similarly,
24 addition of two N-glycosylation sites in the extracellular loop of the C-terminus
25 deletion mutant of OCLN led to the export of the protein to the apical surface of
26 MDCK cells and the cell surface of non-polarized CHO cells ²⁸. N-glycans present in
27 the extracytoplasmic domain of proteins have been shown to constitute apical sorting
28 signals ³⁶⁻³⁸. The contribution of N-glycans to apical sorting has been shown by the
29 retention of deglycosylated protein at the level of the Golgi complex ²⁸. The
30 carbohydrate-binding proteins galectins constitute key regulators of cargo molecules
31 bearing N- or O-glycans ³⁹. Thus galectins 3,4,9 have been implicated in apical
32 sorting in kidney and intestinal cells ⁴⁰. Galectins possess multivalent carbohydrate
33 binding sites that allow the clustering of glycoproteins and their associated lipid rafts
34 may participate to the sorting process ⁴¹. However, some galectins may mediate

1 apical targeting in a lipid rafts independent way^{42,43}.

2 Overall, our study allowed the definition of the minimal region of the OCLN C-terminal
3 cytosolic domain required for HCV infection and OCLN cell surface expression.
4 Moreover, it emphasizes the specific involvement of the distal C-terminus domain of
5 OCLN in its TJ association and indicates that OCLN can mediate HCV entry in a TJ-
6 independent manner.

7

8

9 **Material and methods**

10

11 **Construction of OCLN mutants**

12 All the OCLN mutants were cloned into the pTRIP vector kindly provided by C. M.
13 Rice (The Rockefeller University, New York, USA). pTRIP vector is a self-inactivating
14 lentiviral vector that express no HIV proteins but instead contains the CMV promoter
15 that controls the expression of cloned genes. The parental pTRIP-OCLN-GFP
16 previously described⁴⁴ was modified to generate a construct harboring an N-terminal
17 HA-tagged full-length OCLN. A series of C-terminal OCLN deletion mutants was
18 generated by PCR using pTRIP-OCLN-GFP as template with the forward primer FW-
19 HA-XbaI-OCLN 5'-CCGACTCTAGAGATGTACCCATACGATGTTCCAGAT
20 TACGCTTCATCCAGGCCTCTTGAAAGT-3' containing the HA tag sequence and a
21 series of reverse primers annealing at different positions of the OCLN sequence. For
22 example, the smallest deletion (C150 OCLN Δ 416-522) in the cytosolic part of OCLN
23 was obtained using the reverse primer 5'-
24 ATATAGCTCGAGCTAGTCCTCCTCCAGCTC ATCACA-3'. For generation of the 15
25 residues deletion mutant series, each next reverse primers annealed 45 bases
26 upstream of the 3' end sequence of the downstream primer. PCR products were
27 cloned between the XbaI and XhoI restriction sites of the pTRIP vector.

28 Point mutants were generated by fusion PCR using forward and reverse overlapping
29 primers containing the codon of the replacement alanine residue.

30 OCLN Δ EC2 mutant carrying the deletion of aa200-238 was generated as described
31 in¹⁹ and¹⁷ using fusion PCR with FW-XbaI-HA-OCLN and Rev-XhoI-OCLN 5'-
32 ATATAGCTCGAGCTATGTTTTCTGTCTATCATA-3' external primers and FW- Δ EC2

1 5'- ATGGGAGTGAACCCAGTGGATCCCCAGGAGGCC-3' and Rev-ΔEC2 5'-
2 CTCCTGGGGATCCACTGGGTTCCTCCATTAT-3' internal primers.

3 For the antibody uptake experiments, a Flag tag was introduced in OCLN EC2 at the
4 F5 position as described in ²³. This was done by fusion PCR using external primers
5 Fw-HA-XbaI-OCLN and Rev-XhoI-OCLN and internal primers Rev-Flag-OCLN 5'-
6 CTTGTCGTCGTCGTCCTTGTAGTCTATTTGTGAACCATATAGAG-3' and FW-Flag-
7 OCLN 5'- GAC TAC AAG GAC GAC GAC GAC AAG TAT GCC CTC TGC AAC CAA
8 TT-3' that contains the Flag sequence insert. For introduction of the Flag in the C15
9 OCLN mutant EC2, the external primer REV-XhoI-C15 5'-
10 ATATAGCTCGAGCTACAAAATATTGGACTTGTCATA-3' was used.

11 To replace residues F336, Y337, Y342, Y481 and Y484 of OCLN C-terminus by
12 alanine, the following internal primers were used in fusion PCR: FW-FYY 5'-
13 GACAAGCGGGCAGCACCAGAGTCTTCCGCAAATCCACG-3', REV-FYY 5'-
14 CGTGGATTTTGC GGAAGACTCTGGTGCTGCCCGCTTGTC-3', FW-YNRL 5'-
15 GCTGATGAAGCAAATAGAGCAAAGCAAGTG-3', REV-YNRL 5'-
16 CACTTGCTTTGCTCTATTTGCTTCATCAGC-3'.

17 N-glycosylation C15 and C18IW mutants were generated by inserting a N-
18 glycosylation site in EC1 and EC2 as previously described ²⁰. Briefly, for introduction
19 of a N-glycosylation site in EC1, G119 Y120 G121 residues were replaced by an
20 asparagine-alanine-serine sequon. In EC2, a N-glycosylation site was introduced by
21 replacing the L215 residue by an asparagine and N217 by a serine. These
22 modifications were done by fusion PCR approaches.

23 24 **Cell culture**

25 HEK293T/17 cells (ATCC), OCLN-knockout human hepatic Hu7.5.1-8-derived OKH4
26 cells ¹⁷, Huh-7.5 ⁴⁵, Huh-7 ⁴⁶, Huh-7 clone 1sc3 cells ⁹ were cultured in Dulbecco's
27 modified eagle medium (DMEM) (Life Technologies) supplemented with 10% fetal
28 bovine serum, 2mM GlutaMax and non-essential amino acids (Life Technologies).

29 30 **Antibodies**

31 Mouse anti-HCV E1 mAbs A4 ⁴⁷ was generated *in vitro* from hybridoma culture using
32 Miniperm apparatus (Heraeus). Rat Anti-HA mAb 3F10 was from Roche, rabbit anti-
33 ZO1 was from Zymed (Life Technologies), mouse anti-tubulin was from Sigma,

1 sheep anti-TGN46 was from Biorad, rabbit anti-calreticulin was from Stressgen and
2 rat anti-Flag was from Agilent Technologies, mouse anti-Lamp1 was from BD
3 Bioscience, mouse anti-TfR CD71 was from Santa-Cruz, mouse anti-human OCLN
4 mAb (OC-3F10) was purchased from Life Technologies. Secondary antibodies used
5 for immunofluorescence were from Jackson ImmunoResearch.

6 7 **Virus production**

8 The virus used in this study was based on the JFH1 strain engineered to reconstitute
9 the A4 epitope in E1⁴⁸ with titer-enhancing mutations⁴⁹. Viral RNAs were produced
10 from the plasmid carrying the full-length JFH1 genome by *in vitro* transcription.
11 Viruses were produced by electroporation of viral RNA into Huh-7 cells as previously
12 described⁵⁰.

13 14 **Immunofluorescence**

15 For immunofluorescence microscopy, cells were grown on glass coverslips and fixed
16 with either cold methanol or 3% paraformaldehyde (PFA, Sigma-Aldrich) for 20 min.
17 In the case of PFA fixation, the cells were permeabilized for 3 min in PBS 0.1% Triton
18 X-100. Cells were then incubated in 10% goat serum PBS blocking buffer for 10 min.
19 Both primary and secondary antibody incubations were carried out in blocking buffer
20 for 30 min at room temperature. Nuclei were stained by a 5-min incubation in PBS
21 containing 1 µg/mL DAPI. Coverslips were mounted on glass slides using 4-88
22 Mowiol-based mounting medium. Images were acquired using an inverted laser
23 scanning confocal microscope (LSM880, Zeiss) using a x63 (1.4 numerical aperture)
24 oil immersion lens. Double-label immunofluorescence signals were sequentially
25 collected by using single fluorescence excitation and acquisition setting to avoid
26 crossover. Images were processed with Image J software. Colocalization was
27 evaluated using the plugin JACoP of ImageJ⁵¹. The extent of colocalization of HA-
28 OCLN and different subcellular compartment markers was measured with the
29 Pearson's correlation coefficient, which is a correlation coefficient describing the
30 relationship between the intensities in two images.

31 For the acquisition of Z-stacks of xy sections, 7 to 10 confocal planes separated by
32 0.4 µm were acquired. The image stacks were processed using Image J.

33 34 **Western blotting**

1 Cells were rinsed with cold PBS and lysed at 4°C for 20 min in PBS lysis buffer (1%
2 Triton X-100 and mix of protease inhibitors (Roche)). Insoluble material was removed
3 by centrifugation at 4°C. Protein samples were heated for 7 min at 70°C in Laemmli
4 sample buffer and resolved by SDS-PAGE. The proteins were then transferred onto
5 nitrocellulose membranes (Hybond-ECL; Amersham) using a Trans-Blot apparatus
6 (BioRad). Proteins of interest were revealed upon incubation of the membrane with
7 specific primary antibodies followed by incubation with species-specific secondary
8 antibodies conjugated to peroxidase. Detection of proteins was done using enhanced
9 chemiluminescence (ECL Plus, GE Healthcare). Quantification of unsaturated signals
10 was carried out using the gel quantification function of Image J.

11

12 **Assays based on cell surface biotinylation**

13

14 **Endocytosis**

15 OKH4 cells expressing HA-OCLN constructs were incubated with cleavable EZ-link
16 Sulfo-NHS-SS-Biotin (0.3 mg/mL)(Pierce) in PBS for 30 min at 4°C to label cell
17 surface proteins. Unreacted biotin was quenched with 50 mM glycine in PBS buffer.
18 After washing, cells were incubated at 37°C in DMEM for increasing periods of time.
19 Cells were then rinsed with cold PBS and reduced glutathione (50 mM) containing
20 buffer (75 mM NaCl, 75 mM NaOH, 10% FBS) to cleave biotin bound to non-
21 endocytosed proteins. Cells were then washed with PBS supplemented with 1%
22 bovine serum albumin and 50mM iodoacetamide to quench free SH groups. The cells
23 were then lysed in lysis buffer and biotinylated proteins were recovered using
24 streptavidin-agarose beads (Amersham Biosciences). After washing, the bound
25 proteins were detected by immunoblotting.

26

27 **Recycling**

28 The procedure used to monitor the recycling of OCLN to the cell surface by a
29 biotinylation approach has been described in detail in Fletcher et al. 2014 ²². Briefly,
30 cells were labeled with biotin as described above and incubated at 37°C for a 30-min
31 internalization period. This step was followed by removal of biotin from cell surface
32 proteins with reduced glutathione followed by quenching with iodoacetamide, for all
33 except one sample. The non-treated sample and one treated sample were lysed at
34 this stage to quantify the starting pool of internalized OCLN. Then, the remaining
35 samples were incubated a second time at 37°C for increasing periods of time. This

1 step allowed the restoration of the intracellular trafficking of the proteins. After each
2 time point, one sample was treated with reduced glutathione and quenched, to
3 measure the intracellular biotinylated OCLN pool and a second sample was
4 quenched only, which allowed the measurement of the total biotinylated OCLN pool.
5 Thus, the recycled OCLN pool corresponded to the difference between the total
6 biotinylated OCLN pool and the intracellular biotinylated OCLN pool. Cells were then
7 lysed and lysates were further processed for quantification of biotinylated OCLN as
8 described above.

9 10 11 **Endocytosis assay by antibody uptake**

12 HA-OCLN constructs were expressed by transient transfection in OKH4 cells. 48h
13 post-transfection, cells were incubated with rat anti-Flag mAb for 30 min at 37°C.
14 Cells were then rinsed with PBS and fixed with 3% PFA. After permeabilization with
15 PBS 0.1% Triton X-100 the internalized antibody was revealed with either a cy3-
16 conjugated goat anti-rat or an Alexa 488-conjugated donkey anti-rat (Santa Cruz).
17 Subcellular compartment markers were co-labeled using either sheep anti-TGN46,
18 mouse anti-Lamp1 or mouse anti-TfR antibody. Secondary antibodies used were
19 cy3-conjugated donkey anti-sheep or Alexa-488-conjugated goat anti-mouse
20 respectively.

21 22 **Plasmid transfection**

23 OKH4 cells were seeded in 24-well plates and transfected with 250 ng of plasmid
24 DNA using Trans-IT LT1 transfection reagent (Mirus Bio) according to the
25 manufacturer's instructions. Further analyses were performed at 48h post-
26 transfection.

27 28 **Lentivirus transduction**

29 pTRIP-CD81-GFP and pTRIP-cerulean-CLDN1 were kindly provided by C. M. Rice
30 (Rockefeller University)².

31 To produce lentivirus stocks, HEK293T/17 cells were seeded in 6-well dishes. The
32 next day, cells were transfected with 500 ng pTRIP vector, 400 ng of HIV gag-pol
33 expressing vector and 100 ng of vesicular stomatitis virus G protein expressing
34 vector with Turbofect (Thermo Fisher) according to the manufacturer's instructions.
35 Virus stocks were collected at 48h post-transfection and filtered.

1 Clone 1sc3 cells were transduced with lentivirus containing supernatants for 48h
2 before being further analyzed for transgene expression or infected with HCV.

3 4 **Cell polarization and infection**

5 To increase the level of cell polarization clone 1sc3 Huh-7 cells were seeded on
6 transwell permeable supports (polyester membranes with 3- μ m pores; Corning).
7 When cells reached confluency the medium was replaced with William's medium
8 (Life Technologies) supplemented with 10% FBS, 2mM Glutamax, 10 ng/mL
9 gentamycin and 1% DMSO. After 5 days of culture in the presence of DMSO, cells
10 were infected by HCV on the basolateral chamber of the transwell. Cells were lysed
11 at 30h post-infection, and RNAs were extracted with NucleoSpin RNA II kit
12 (Macherey Nagel) according to the manufacturer's instructions. HCV RNA was
13 quantified by quantitative real-time RT PCR assay as described previously⁵².

14 15 **Generation of OCLN-knock out clone 1sc3 Huh-7 cells**

16
17 To generate OCLN knockout cells, the pSpCas9(BB)-2A-Puro (pX459)(Addgene)
18 was used. Two single-guide RNAs (site A and B) specific for the human OCLN gene
19 described by Shirasago and collaborators¹⁷ were cloned in the pX459 vector. Clone
20 1sc3 Huh-7 cells were seeded in 6-well plates and transfected with 500 ng of pX459-
21 site A and pX459-site B plasmid using Trans-IT LT1 transfection reagent according to
22 the manufacturer's protocol. Two days post-transfection, the cells having
23 incorporated the plasmids were selected with DMEM containing 2.5 μ g/mL puromycin.
24 Four to five days later, the puromycin selection was removed and OCLN knockout
25 was checked by Western blotting.

26 27 **Acknowledgements**

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39

1 **Figure Legends**

2

3 Figure 1: Schematic description of the series of C-terminal OCLN deletion mutants.

4 The first deletion corresponds to the removal of the C-terminal distal domain that

5 contains 3 α -helices (aa416-522) involved in interactions with partner proteins (C150

6 Δ 416-522 deletion). Ten additional mutants were generated by serially deleting 15

7 more C-terminal residues per constructs. The shortest construct carries the deletion

8 of the complete C-terminal cytosolic domain (Δ C, Δ 266-522 deletion). Δ EC2 mutant

9 was generated by deletion of residues 200 to 238 of the second extracellular loop.

10 The different mutants are designed by C followed by the number of cytosolic residues

11 present downstream the fourth transmembrane domain of OCLN. The different

12 OCLN mutants carry a N-terminal HA epitope tag and are cloned in the pTRIP

13 lentiviral expression plasmid. A second set of 4 mutants (C18, C21, C24, C27) was

14 generated by removing 3 residues per construct starting from the C30 mutant

15 sequence. Transmembrane domains are represented in red, extracellular loops in

16 blue, intracellular loop in green and cytosolic tails in violet. The distal coiled-coil of

17 OCLN that contains three alpha-helices¹⁻³ is shown in yellow. The dotted line stands

18 for the non-represented intermediate deletion mutants.

19

20 Figure 2: Characterization of OCLN C-terminal deletion mutants.

21 OKH4 cells were transiently transfected with plasmids encoding the different HA-

22 tagged OCLN deletion mutants and wild-type OCLN (wt), or left untransfected (NT)

23 and infected at 48h post-transfection with HCVcc virus. Expression levels of the

24 mutants after transfection were analyzed at the time of infection by Western blotting

25 using an anti-HA mAb. The equivalent loading of cell lysates was confirmed with an

26 anti- β -tubulin antibody. The positions of the molecular weight ladder (kDa) are shown

27 on the left side of the Western blots (A and C). Infectivity levels were determined at

28 30-32h post-infection by immunofluorescence assay using an antibody directed

29 against the E1 viral protein (B and D). Infectivity is expressed as the percentage of

30 the infection level obtained in OKH4 cells expressing wt OCLN. Error bars indicate

31 standard errors of at least three independent experiments (SD). * $P < 0.05$; ** $P < 0.01$;

32 *** $P < 0.001$ in the paired t-test.

33

34 Figure 3: Cell surface expression of OCLN deletion mutants.

1 A) Cell surface biotinylation of OCLN mutants. OKH4 cells were transfected with
2 plasmids encoding HA-tagged OCLN deletion mutants (C15, C18) and wild-type
3 OCLN (wt). At 48 hours post-transfection, cell surface proteins were biotinylated at
4 4°C. HA-tagged proteins from the cell lysates were analyzed by Western blotting with
5 an anti-HA mAb (Total). The biotinylated cell surface expressed proteins were pulled
6 down with streptavidin agarose beads. OCLN abundance in these samples was also
7 determined by Western blot analysis using an anti-HA mAb.

8 B) Subcellular localization of OCLN mutants. OKH4 cells were transfected with
9 plasmids encoding HA-tagged OCLN deletion mutants (Δ C, C15, C18) and wild-type
10 OCLN (wt). At 48 hours post-transfection, the cells were fixed and processed for
11 immunofluorescence detection of HA-tagged wt and mutant OCLN. HA staining is
12 shown in red and nuclei are shown in blue. Bars, 10 μ m.

13

14 Figure 4: Characterization of the subcellular localization of the OCLN-C15 mutant.
15 OKH4 cells were transfected with plasmids encoding HA-tagged OCLN-C15 mutant
16 and wild-type OCLN (wt). At 48 hours post-transfection, the cells were fixed and
17 processed for double-label immunofluorescence detection of HA-tagged wt OCLN or
18 C15 mutant and the TGN marker, TGN46 (A) or the ER marker, calreticulin (CRT, B).
19 Pearson correlation coefficients calculated for at least 10 cells in each condition are
20 shown. The error bars represent the SD. * $P < 0.05$ in the Wilcoxon signed-rank test.
21 Bars, 10 μ m.

22

23 Figure 5: Endocytosis kinetics of wild-type OCLN and OCLN-C15 mutant. OKH4 cells
24 were transfected with plasmids encoding HA-tagged wild-type OCLN (wt, A) or C15
25 mutant (B). At 48h post-transfection, the cells were biotinylated at 4°C and incubated
26 for increasing periods of time at 37°C to allow internalization. The biotin present on
27 cell surface proteins was then removed with reduced glutathione; thus, only
28 internalized proteins remained biotinylated. After cell lysis, the HA-tagged proteins
29 were analyzed by Western blotting with an anti-HA mAb allowing the quantification of
30 HA-OCLN abundance in the lysates of biotinylated cells (A, B, Total). Internalized
31 biotinylated proteins present in the lysates were pulled down with streptavidin
32 agarose beads. Internalized biotinylated OCLN abundance was determined via
33 Western blot analysis using an anti-HA mAb (A, B, Biotinylated). The kinetic of

1 internalization of the transferrin receptor (TfR, C) was used as the positive control.
2 The signal intensity obtained in the Western blot (biotinylated blot) was quantified for
3 each time point and expressed as the % of the intensity obtained without cleavage of
4 biotin and 37°C incubation (Ctrl). The averages of the values obtained in three
5 repeats are shown. The error bars represent the standard error of the mean.

6
7 Figure 6: Kinetics of cell surface recycling of wild-type OCLN and OCLN-C15 mutant.
8 OKH4 cells were transfected with plasmids encoding HA-tagged wild-type OCLN (wt,
9 A) and C15 mutant (B). At 48h post-transfection, the cells were biotinylated at 4°C
10 and incubated for 30 min at 37°C to allow cell surface protein internalization. Biotin
11 was removed from cell surface proteins with reduced glutathione; thus, only
12 internalized proteins remained biotinylated. Cells were then incubated at 37°C for
13 increasing periods of time, thus allowing the recycling of the internalized biotinylated
14 protein to the cell surface. For each time point, two samples were used. The first
15 sample was treated with reduced glutathione (+) to remove biotin while the second
16 was left untreated (-). This way, the glutathione treated sample allowed the
17 quantification of the amount of intracellular protein, while the non-treated sample
18 gave access to the amount of total biotinylated proteins, which is composed, of the
19 intracellular biotinylated proteins and the cell surface recycled proteins. Thereafter
20 cells were lysed and the HA-tagged proteins were analyzed by Western blotting with
21 an anti-HA mAb allowing the quantification of HA-OCLN abundance in the lysates of
22 biotinylated cells (A, B, Total). Biotinylated proteins present in the lysates were pulled
23 down with streptavidin agarose beads and their abundance was determined via
24 Western blot analysis using an anti-HA mAb (A, B, Biotinylated). As an experimental
25 control, the kinetics of the recycling of the transferrin receptor (TfR, C) was
26 determined in parallel. The percentage of recycled proteins at each time point was
27 determined by subtracting the signal intensity obtained after glutathione treatment
28 (biotin cleavage +: intracellular protein) from the signal obtained for the untreated
29 sample (biotin cleavage -: intracellular + recycled biotinylated protein). The difference
30 was then expressed as a percentage of the signal obtained with no incubation at
31 37°C and no GSH treatment (0 min -, D).

32
33 Figure 7: Subcellular localization of internalized wild-type OCLN and OCLN-C15
34 mutant. OKH4 cells were transfected with plasmids encoding HA-tagged wild-type

1 OCLN (wt) or OCLN-C15 mutant carrying a Flag tag in the second extracellular loop
2 of the protein. At 48h post-transfection, cells were incubated for 30 min at 37°C with
3 an anti-Flag mAb. Cells were then fixed with PFA and processed for double-label
4 immunofluorescent detection of internalized mAbs together with different intracellular
5 organelle markers (TGN46, A; TfR, B; Lamp1, C). Pearson correlation coefficients
6 calculated for at least 10 cells of each condition are shown. The error bars represent
7 the SD (D). Bars, 10 μ m

8

9 Figure 8: A) Schematic representation of OCLN-C15 and OCLN-C18 mutants. The
10 amino acid sequence of the cytosolic C-terminal part of each mutant is indicated
11 below OCLN drawings. Residues differing between the two mutants are shown in red.
12 The residues that have been mutated into alanine are depicted in bold characters.

13 B) Effect of point mutations on the expression of OCLN-C18 and full-length OCLN in
14 complemented OKH4 cells. OKH4 cells were transfected with plasmids encoding the
15 different HA-tagged OCLN mutants or wild-type (wt) OCLN. At 48h post-transfection,
16 the cells were lysed. Loading of equal amounts of proteins was verified by Western
17 blotting using an anti- β -tubulin antibody. The HA-tagged proteins were analyzed by
18 Western blotting with an anti-HA mAb.

19 C) Effect of point mutations in OCLN-C18 and full-length OCLN on HCV infection of
20 complemented OKH4 cells.

21 D) Effect of point mutations in OCLN-C75, OCLN-C150 and OCLN-TYR mutants on
22 HCV infection of complemented OKH4 cells. (C and D) OKH4 cells were transfected
23 with plasmids encoding the different HA-tagged OCLN mutants or wt OCLN. At 48h
24 post-transfection, the cells were infected with HCVcc. At 30h post-infection, infection
25 was quantified via immunofluorescence using the A4 anti-E1 mAb and expressed as
26 the percentage of the infection level obtained in OKH4 cells expressing wt OCLN.
27 Mean values and standard deviations from at least three independent experiments
28 are shown. * $P < 0.05$ in the Wilcoxon signed-rank test.

29

30 Figure 9: A) Cell surface expression of OCLN-C18 and full-length OCLN point
31 mutants. OKH4 cells were transfected with plasmids encoding the different HA-
32 tagged OCLN mutants, or wild-type OCLN (wt). At 48h post-transfection, the cells
33 were biotinylated at 4°C or left untreated (wt Ctrl) and subsequently lysed. Cell
34 surface biotinylated proteins present in the lysates were pulled down with streptavidin

1 agarose beads. OCLN abundance was determined by Western blot analysis using an
2 anti-HA mAb (Biotinylated). The total amount of HA-OCLN present in the lysate was
3 determined by Western blotting using an anti-HA mAb (Total). B-C) Characterization
4 of the subcellular localization of OCLN-C18 and full-length OCLN point mutants. B)
5 OKH4 cells were transfected with plasmids encoding the different HA-tagged mutant
6 or wild-type OCLN (wt). At 48h post-transfection, the cells were fixed and processed
7 for double-label immunofluorescence detection of TGN46 and HA-tagged wild-type
8 and mutant OCLN. Bars, 10 μ m. C) Pearson correlation coefficients calculated for at
9 least 10 cells of each condition are shown. The error bars represent the SD. * $P <$
10 0.05; ** $P <$ 0.01; *** $P <$ 0.001 in the Wilcoxon signed-rank test.

11
12 Figure 10: Kinetics of internalization of wild-type and OCLN_IW mutant. OKH4 cells
13 were transfected with plasmids encoding HA-tagged wild-type OCLN (wt) or
14 OCLN_IW mutant (OCLN_IW). At 48h post-transfection, the cells were biotinylated
15 at 4°C and incubated for increasing periods of time at 37°C to allow internalization.
16 Then, biotin was removed from cell surface proteins with reduced glutathione; thus,
17 only internalized proteins remained biotinylated. After cell lysis, the HA-tagged
18 proteins were analyzed by Western blotting with an anti-HA mAb allowing the
19 quantification of HA-OCLN abundance of biotinylated cells in the lysates (Total).
20 Internalized biotinylated proteins present in the lysates were pulled down with
21 streptavidin agarose beads. Internalized biotinylated OCLN abundance was then
22 determined via Western blot analysis using an anti-HA mAb (Biotinylated).

23
24 Figure 11: Rescuing of cell surface expression of OCLN-C15 and OCLN-C18IW
25 mutants by N-glycosylation. OKH4 cells were transfected with plasmids expressing
26 OCLN-C15 and OCLN-C18IW mutants carrying (NC15 and NC18IW) or not carrying
27 (C15 and C18IW) two glycosylation sites in the extracellular loops of the protein. At
28 48h post-transfection the cells were either lysed for protein glycosylation analysis (A)
29 or fixed with PFA to determine the subcellular localization of the proteins (B). A) The
30 cell lysates were digested (+) or not (-) with PNGase F amidase which cleaves the
31 glycans from N-glycosylated proteins and the OCLN mutants were analyzed by
32 Western blotting using an anti-HA mAb. B) In parallel, PFA fixed cells were
33 processed for double-label immunofluorescence detection of TGN46 and HA-tagged
34 glycosylated (N-C15, N-C18IW) or non-glycosylated C15 and C18IW OCLN mutants.

1 Pearson correlation coefficients calculated for at least 10 cells of each condition are
2 shown. The error bars represent the SD. * $P < 0.05$ in the Wilcoxon signed rank test.
3 Bars, 10 μm .

4
5 Figure 12: Subcellular localization of OCLN mutants in polarized clone 1sc3 cells. A)
6 Clone 1sc3 cells were transduced with lentiviral vectors to express wild-type OCLN
7 (wt) or the different mutants. At 48h post transduction, cells were fixed and
8 processed for double label staining of ZO1 and HA-tagged wt and mutant OCLN.
9 Nuclei were stained with DAPI. z stacks of xy sections of the cells were acquired by
10 confocal microscopy and three different z sections are presented. Bars, 10 μm . B)
11 Schema showing the relative position of the shown sections.. C) y-z projections of
12 the cells stained for ZO1 and HA-tagged OCLN.

13
14 Figure 13: Rescuing of OCLN-KO clone 1sc3 cells with OCLN mutants.

15 A) Establishment of OCLN-knockout clone 1sc3 cells. OCLN-knockout cells were
16 generated from the Huh-7 clone 1sc3^{4,5} by the CRISPR/Cas9 system. OCLN
17 expression in the lysates of parental clone1sc3 cells (Ctrl), OCLN-KO clone 1sc3
18 cells (OCLN-KO), OKH4 and Huh-7.5 cells were analyzed by Western blotting using
19 anti-OCLN mAb.

20 B) Cell surface expression of OCLN mutants in complemented OCLN-KO clone1sc3
21 cells. OCLN-KO clone 1sc3 cells were transduced with lentiviral expression vectors
22 for wild-type (wt) and mutant OCLN. At 48h post-transduction, the cells were
23 biotinylated at 4°C or let untreated (Ctrl) and subsequently lysed. Cell surface
24 biotinylated proteins present in the lysates were pulled down with streptavidin
25 agarose beads. OCLN abundance was determined by Western blot analysis using an
26 anti-HA mAb (Biotinylated). The total amount of HA-OCLN present in the lysate was
27 determined by Western blotting using an anti-HA mAb (Total).

28 C to E) Intracellular localization of OCLN mutants in complemented OCLN-KO
29 clone1sc3 cells. C) OCLN-KO clone 1sc3 cells were transduced with lentiviral
30 expression vectors for wt and mutants OCLN constructs or not (Ctrl). At 48h post-
31 transduction, cells were fixed with PFA and processed for double label staining of
32 HA-tagged OCLN and ZO1. D) OCLN-KO clone 1sc3 cells were co-transduced with
33 lentiviral expression vectors for wt OCLN, OCLN-C15 or OCLN-C18 and with
34 lentiviral expression vectors for CD81-GFP or CLDN1-Cer. The cells were fixed and

1 stained for HA-tagged OCLN. Z stacks of xy sections of the cells were acquired by
2 confocal microscopy and 3 different z sections are presented. Bars, 10 μ m. E) y-z
3 projection of the cells stained for HA-tagged OCLN and expressing CD81-GFP or
4 CLDN1-Cer are shown. CD81-GFP, CLDN1-Cer are depicted in green, HA-tagged
5 OCLN in red, and nuclei in blue.

6

7 Figure 14: Ability of OCLN mutants to mediate HCV infection in complemented
8 OCLN-KO clone 1sc3 cells. OCLN knockout clone 1sc3 cells were transduced with
9 lentiviral expression vectors for the different HA-tagged OCLN mutants and wt OCLN.
10 At 48h post-transduction, expression levels of the mutants in the transduced cells
11 were analyzed by Western blotting using an anti-HA monoclonal antibody (A). In
12 parallel, transduced cells were infected with HCVcc. At 30h post-infection, infectivity
13 levels were determined via immunofluorescence using the A4 anti-E1 mAb. Infectivity
14 is expressed as the percentage of the infection level obtained in OCLN-KO 1sc3 cells
15 complemented with wild-type OCLN (B). Mean values and standard deviations from
16 at least three independent experiments are shown. * $P < 0.05$ in the Wilcoxon signed-
17 rank test.

18

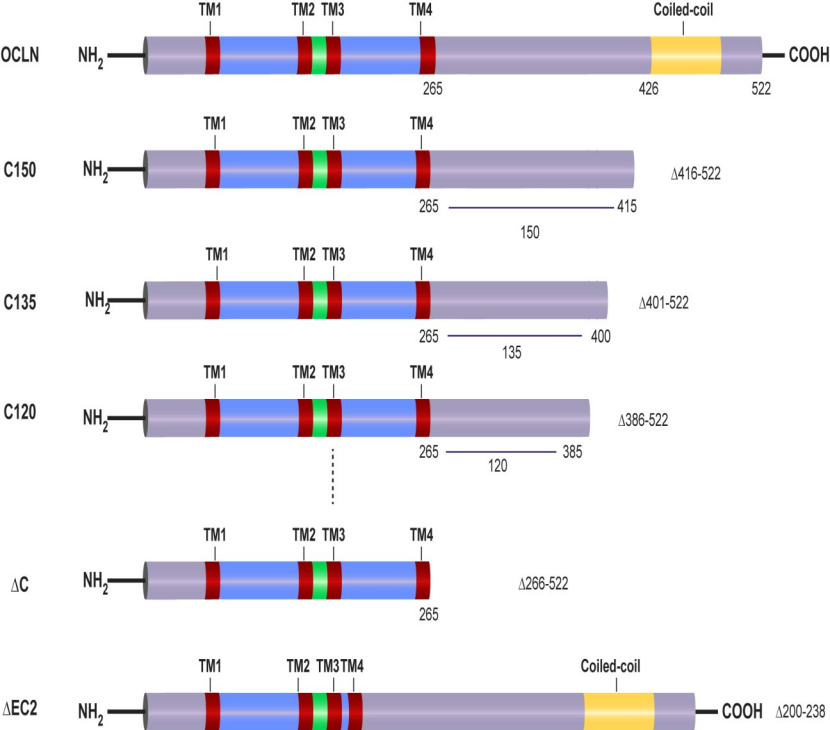


Figure 1

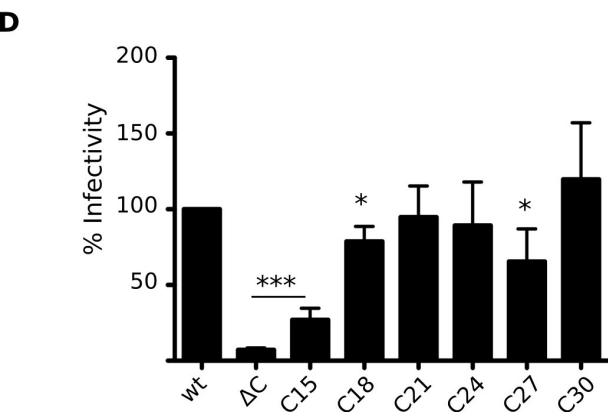
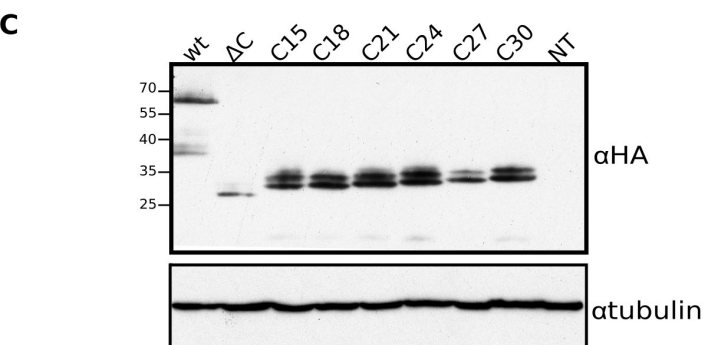
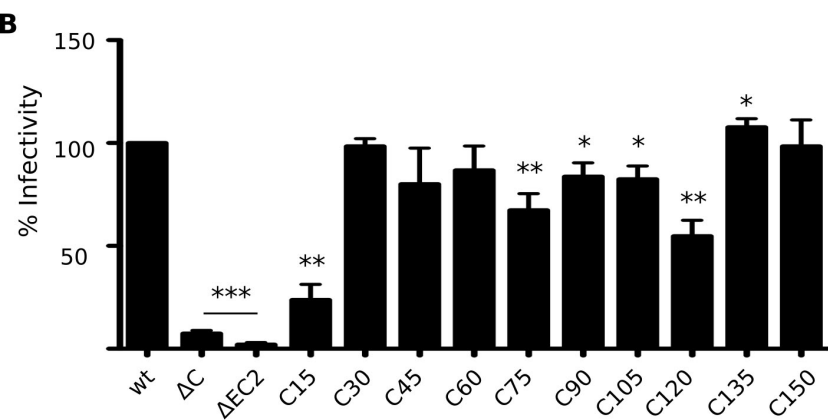
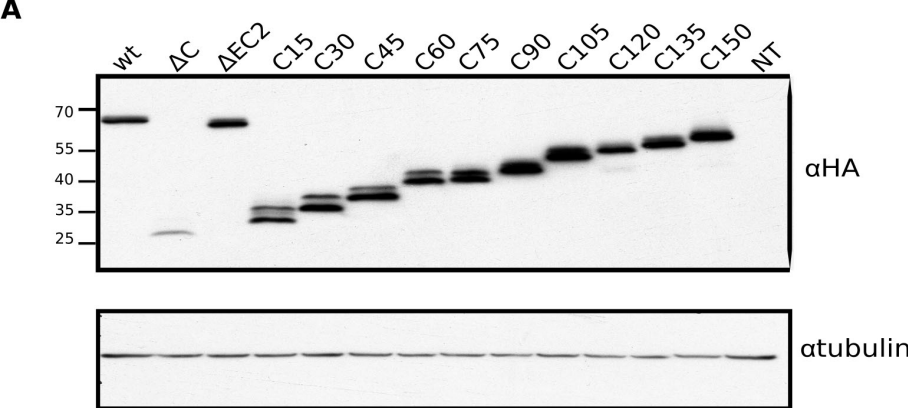
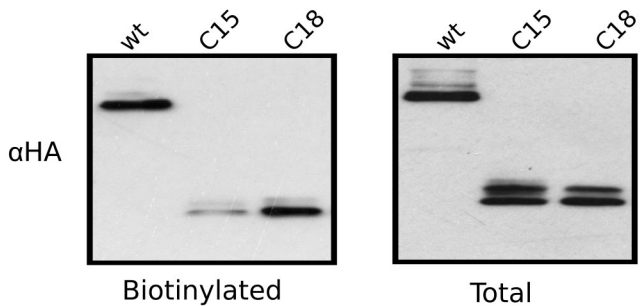
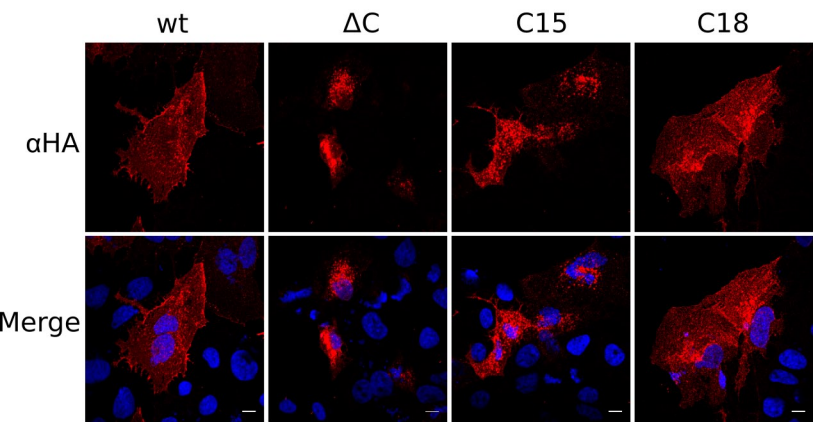


Fig 2

A**B****Fig 3**

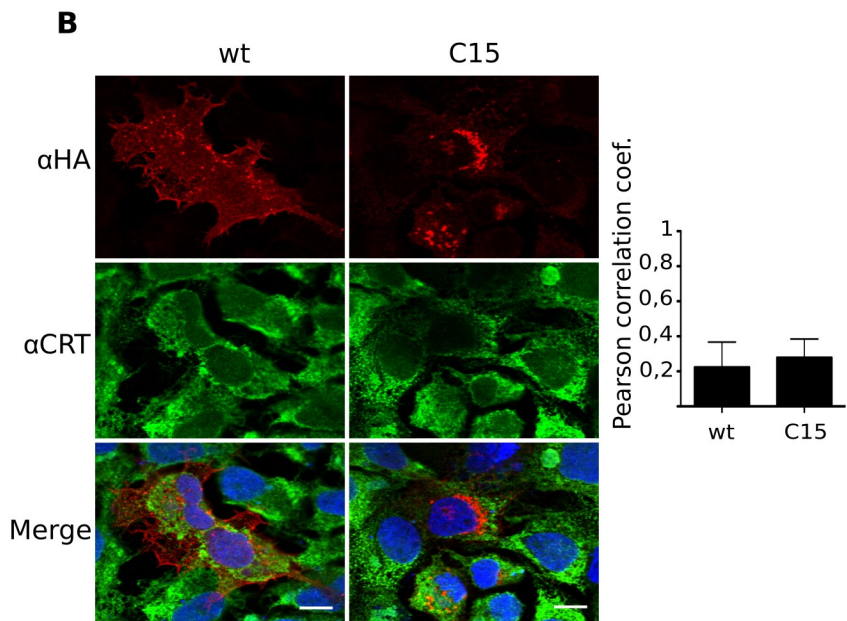
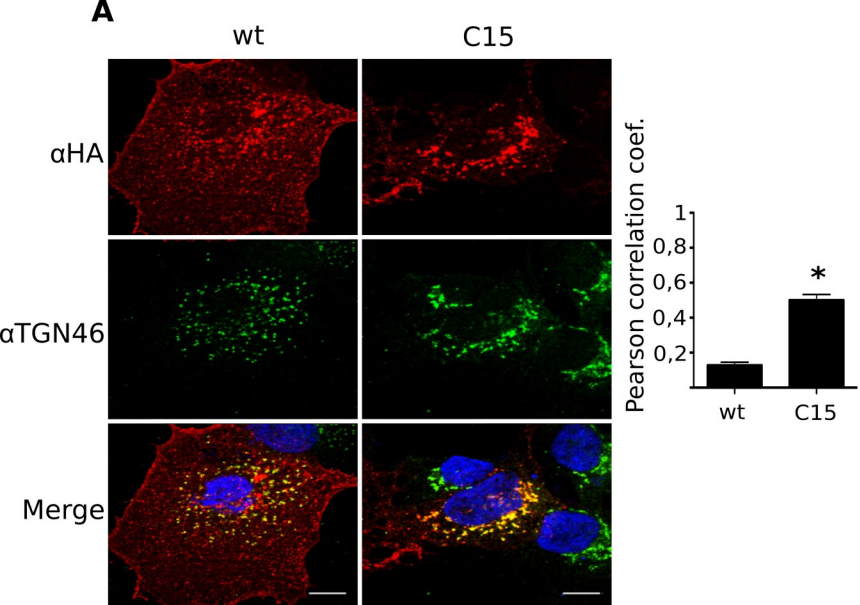


Fig4

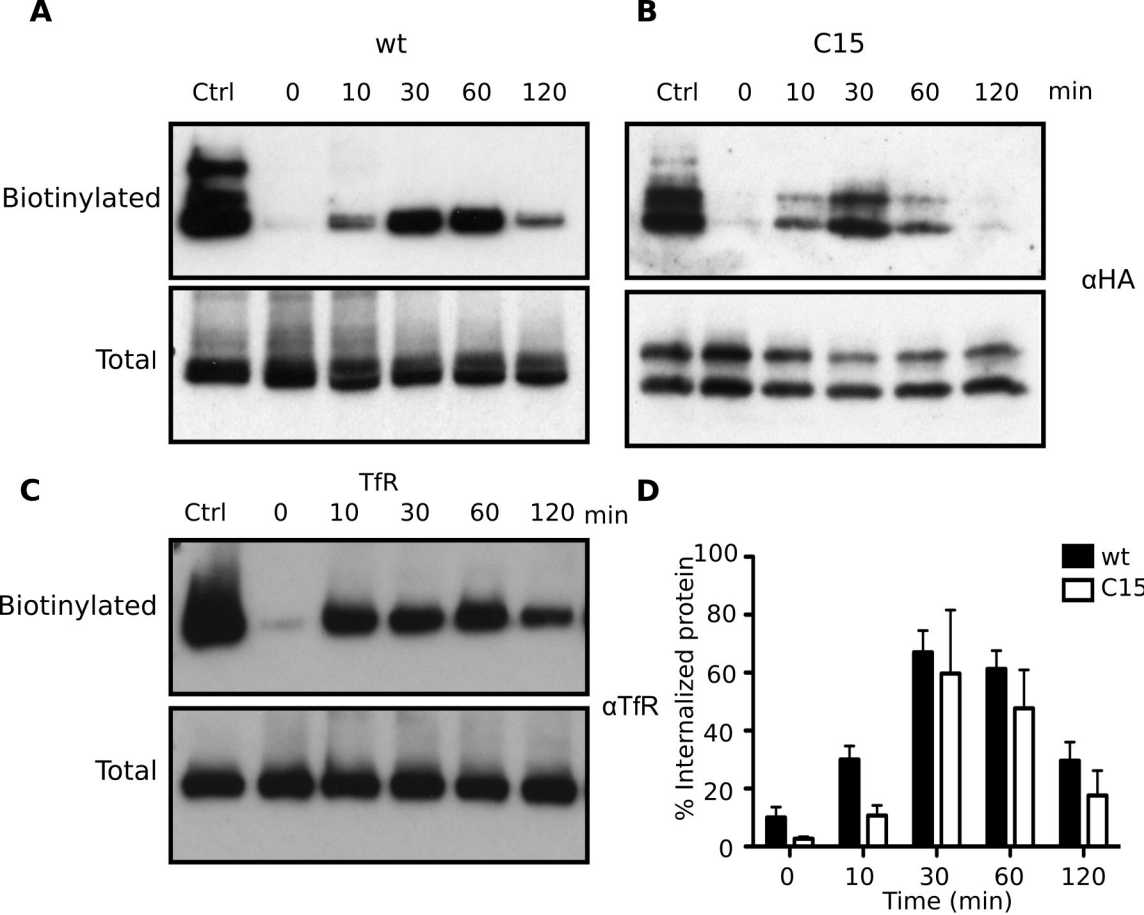


Figure 5

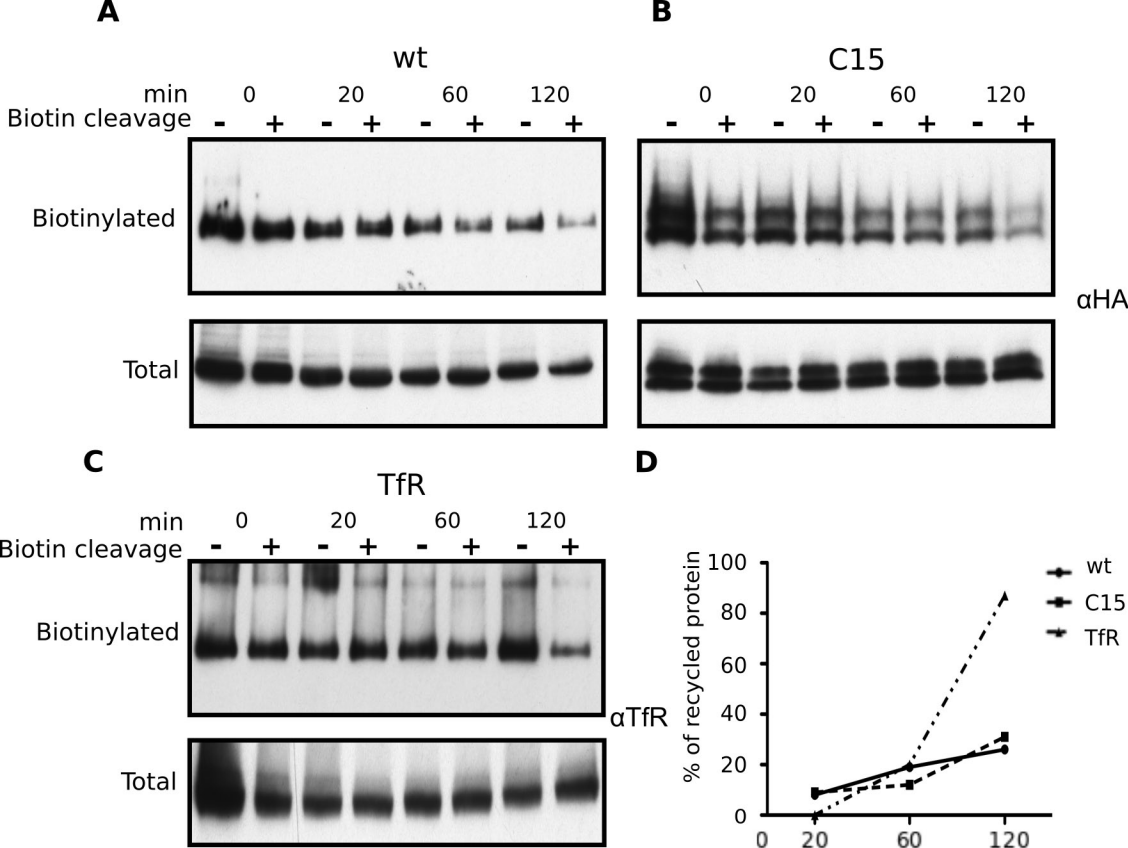


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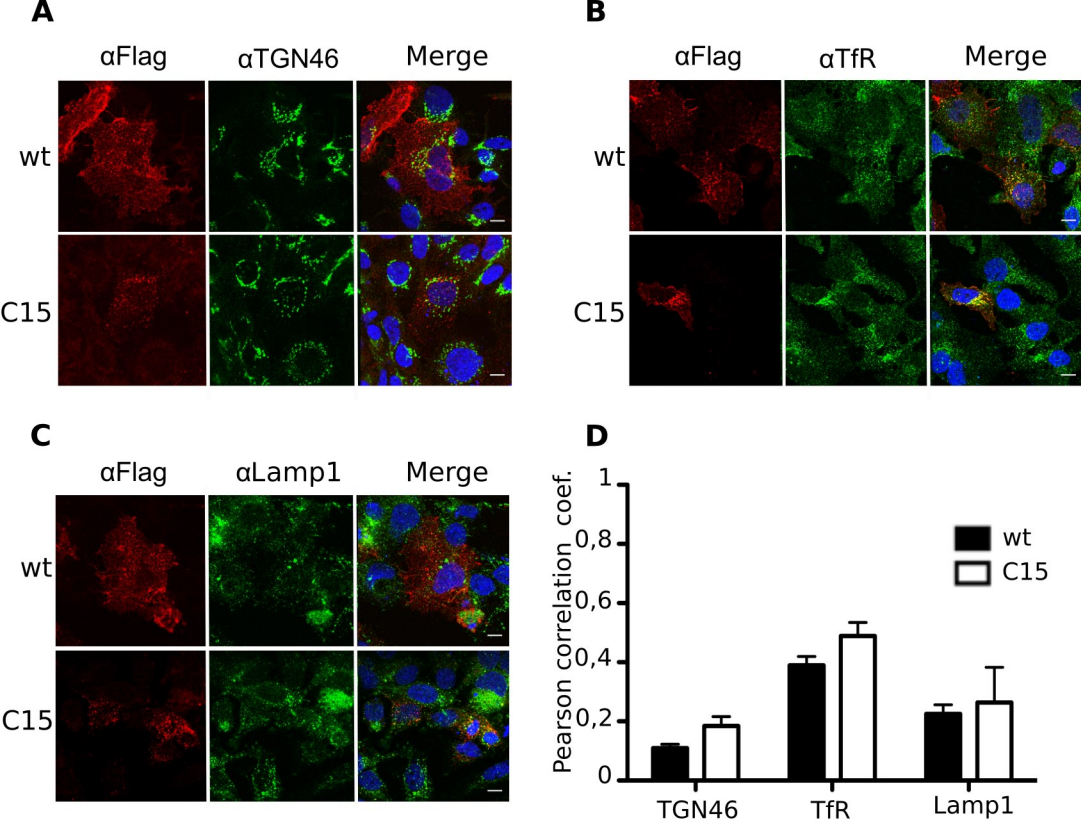
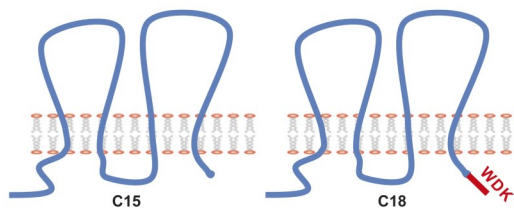
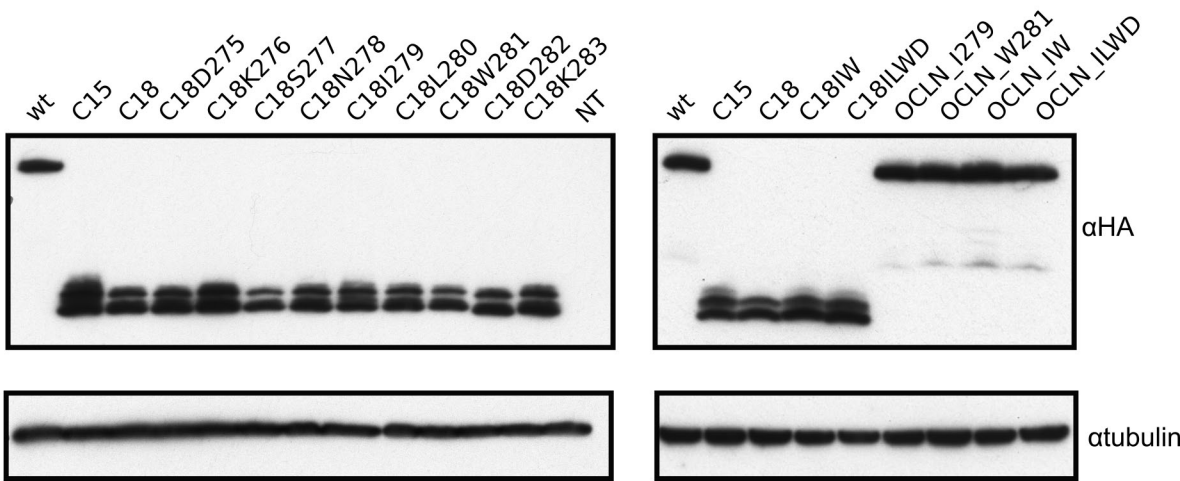


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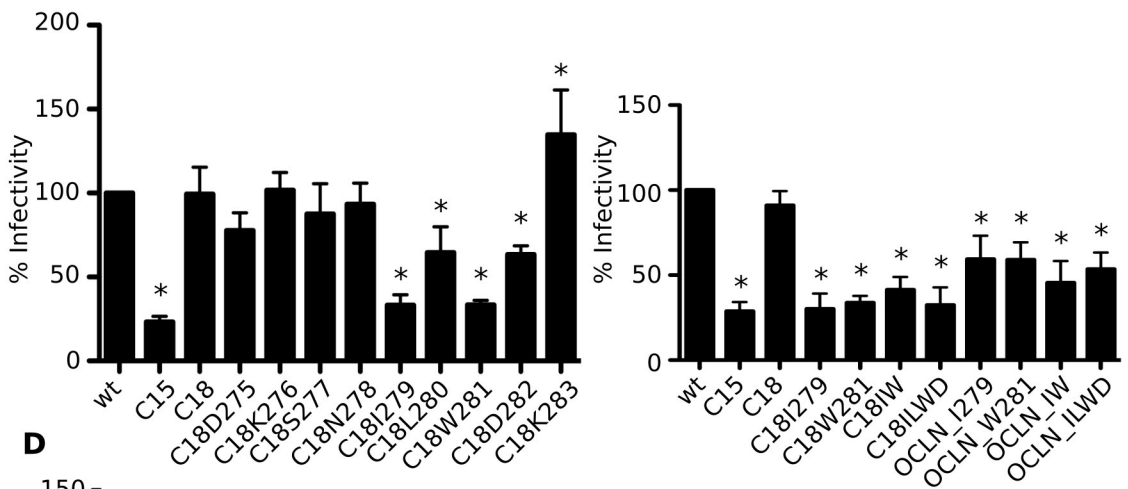


Intracellular C-terminus sequence **TM-KTRRKMDRYDKSNIL** **TM-KTRRKMDRYDKSNILWDK**

B



C



D

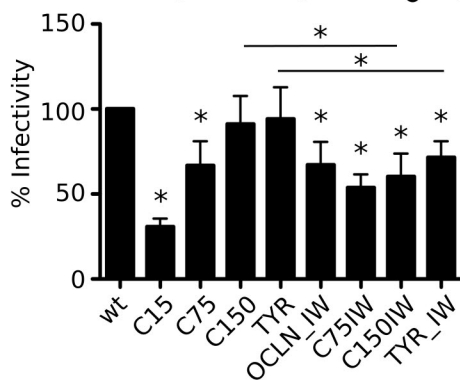


Figure 8

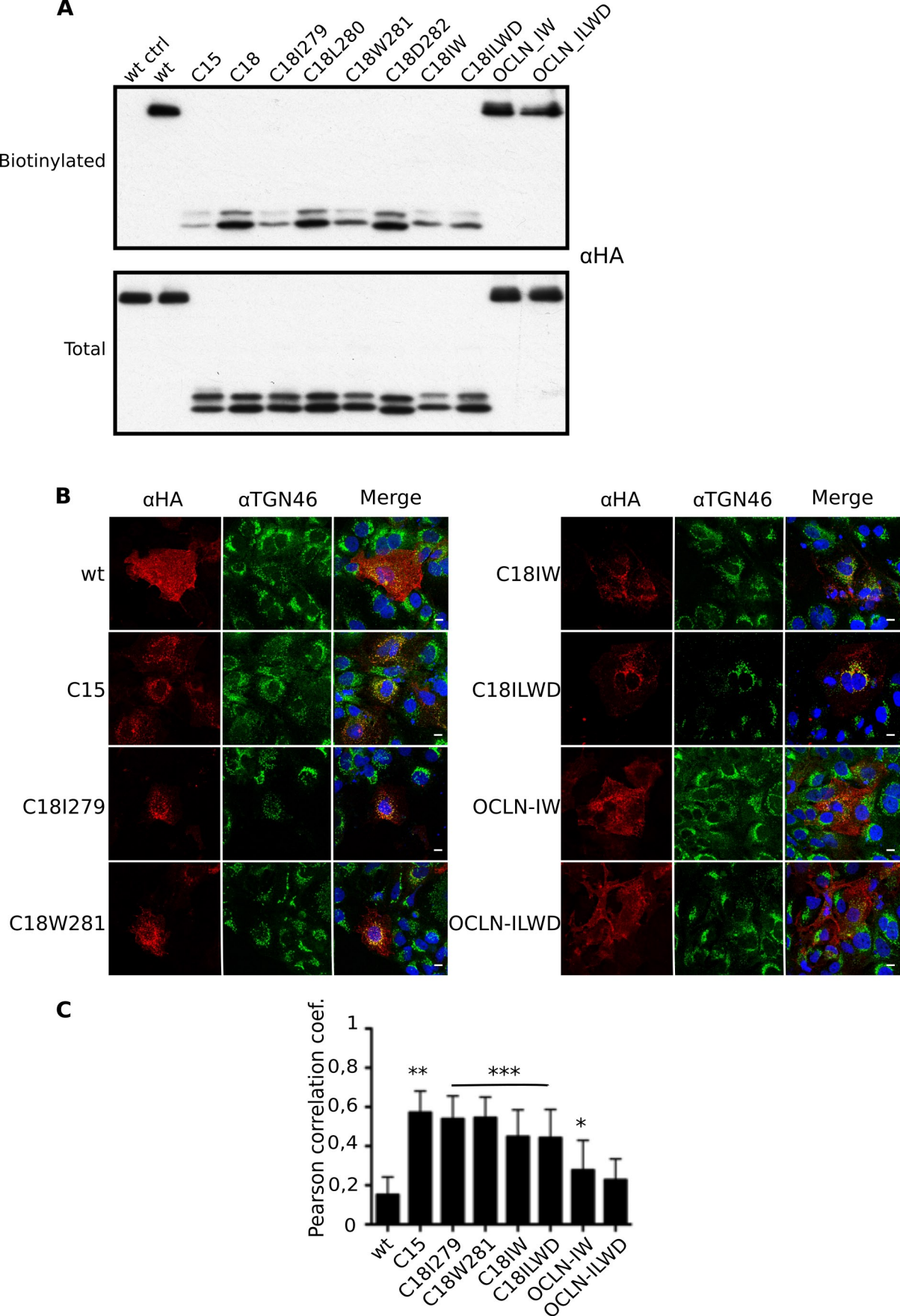


Figure 9

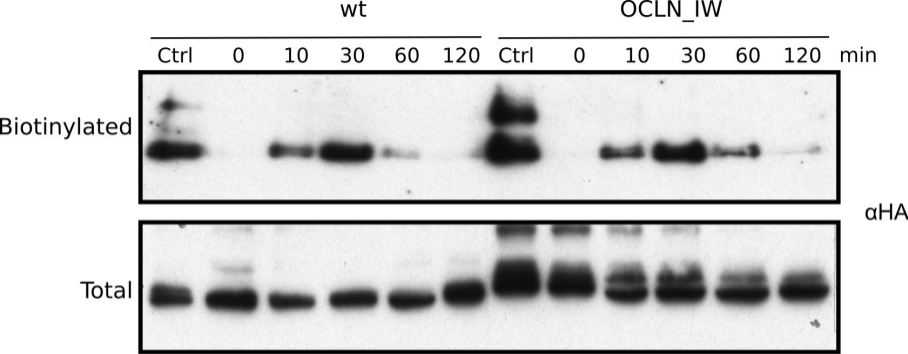


Figure 10

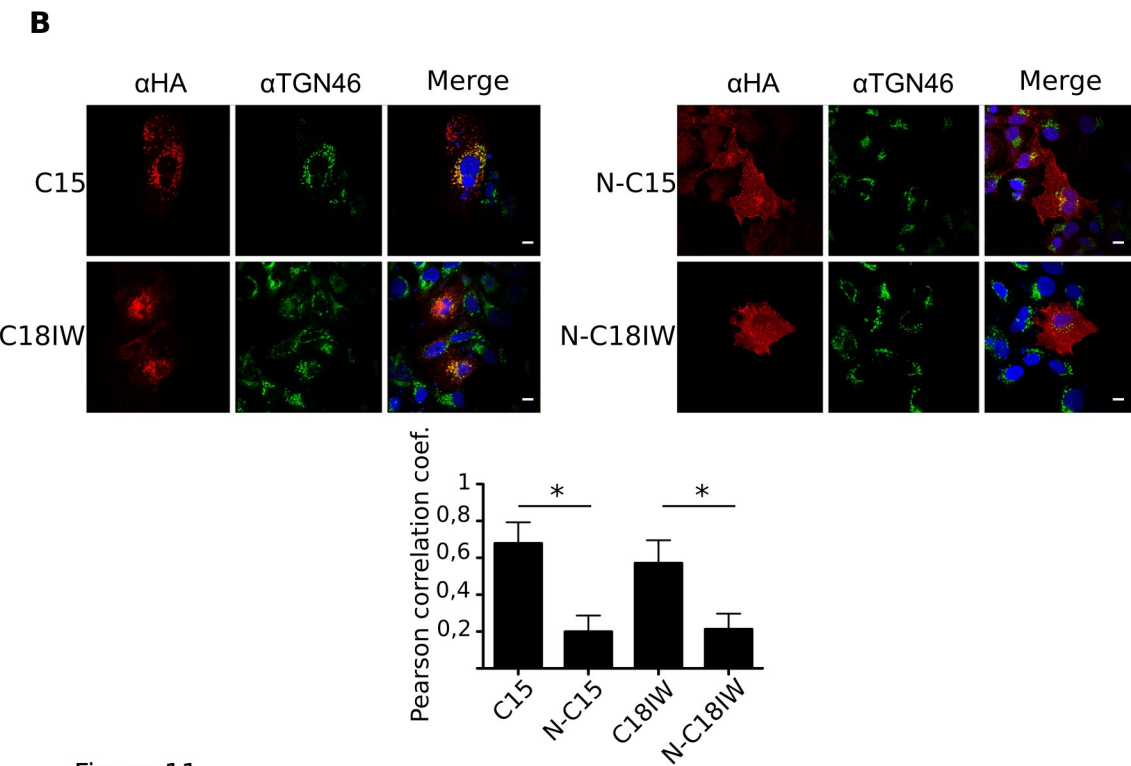
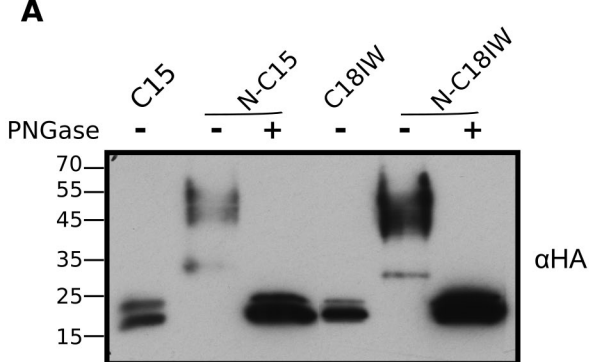


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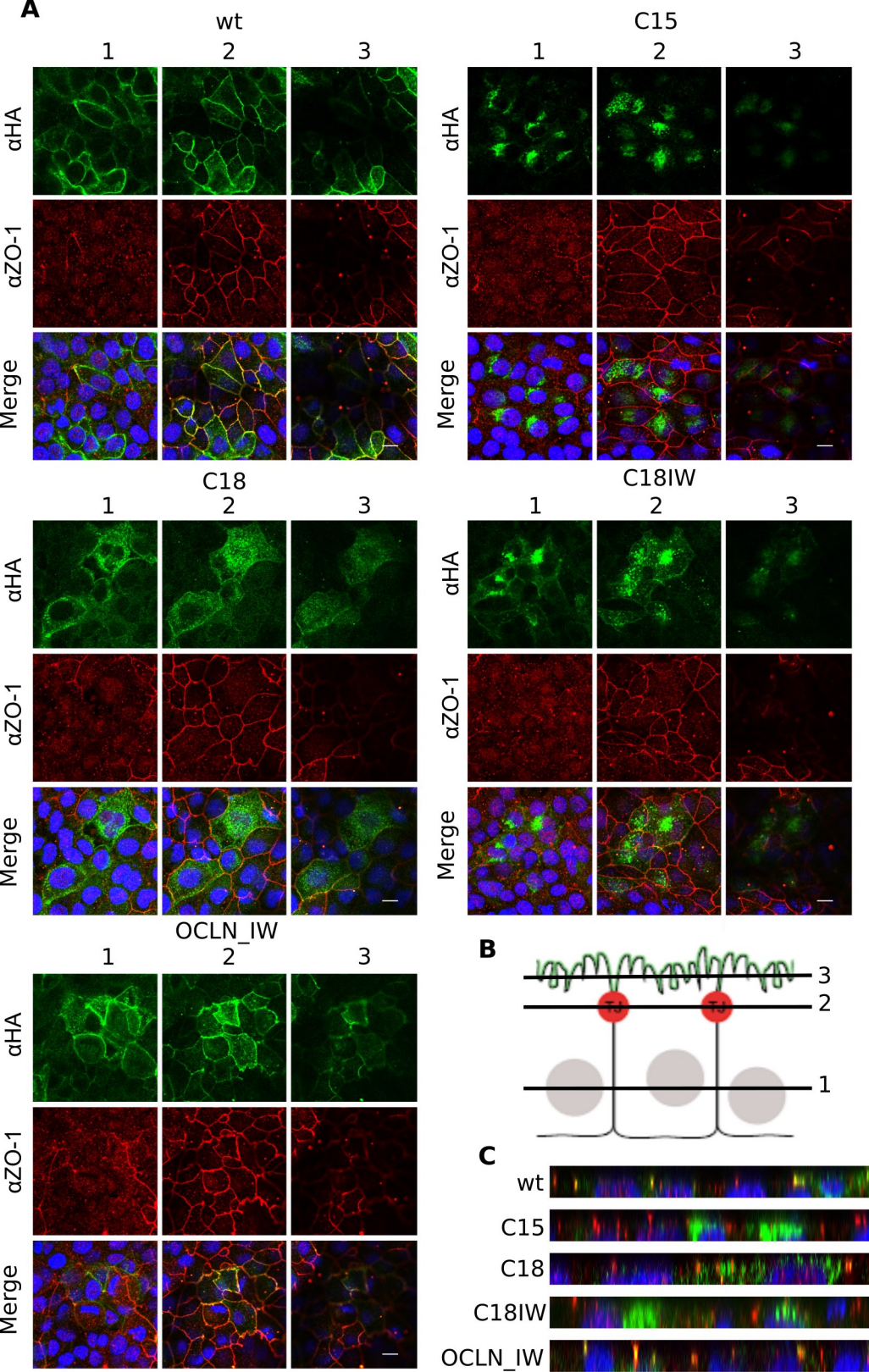


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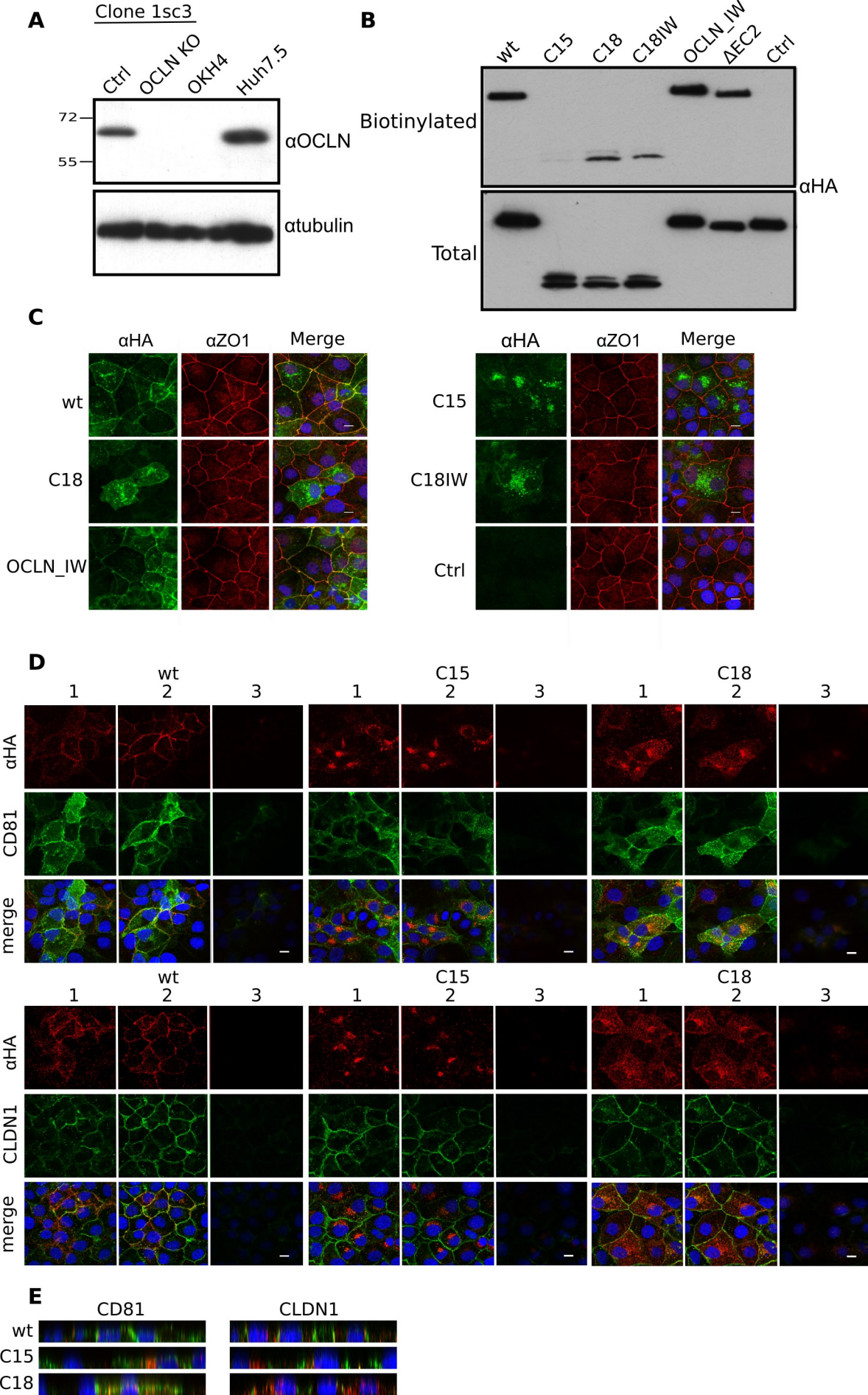


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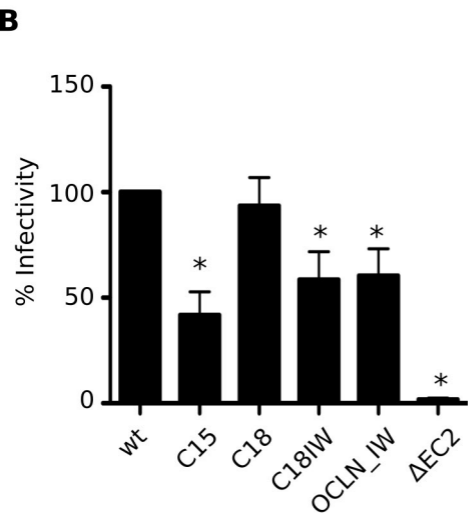
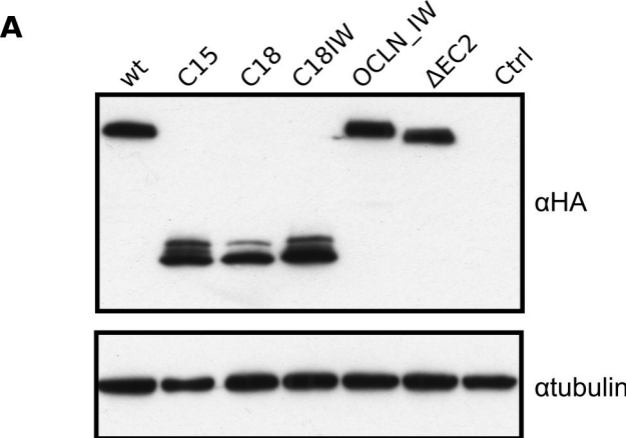
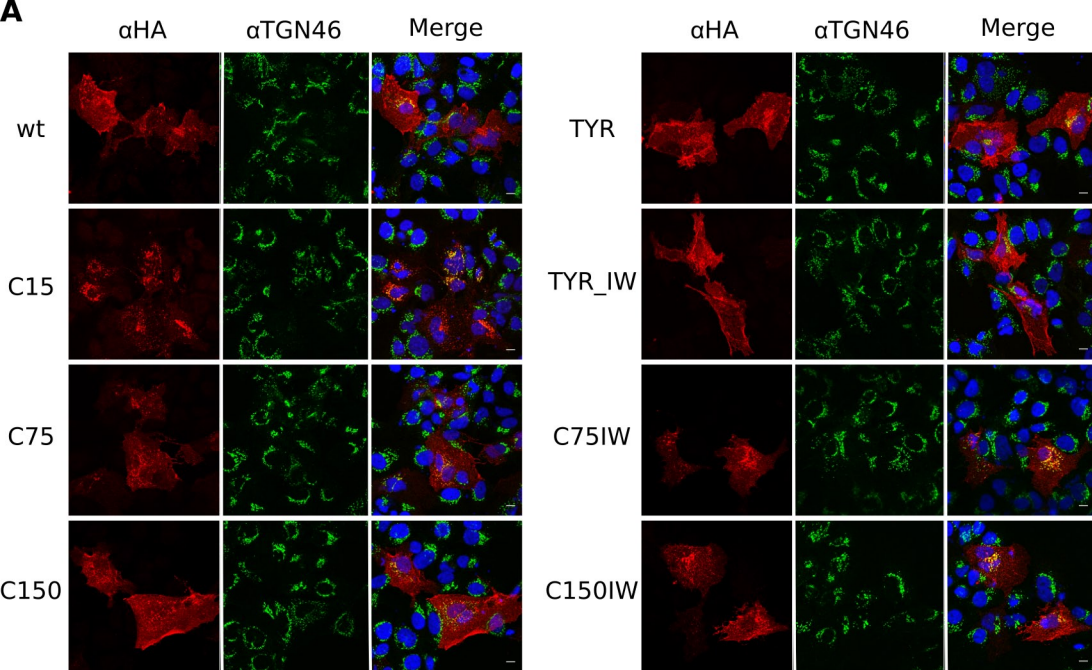


Figure 14



B

