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The use of anthrolysin O and ostreolysin A to study cholesterol in cell membranes

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Abstract

Cholesterol is a major component of the plasma membranes (PMs) of animal cells, comprising 35–40 mol% of total PM lipids. Recent studies using cholesterol-binding bacterial toxins such as domain 4 of Anthrolysin O (ALOD4) and fungal toxins such as Ostreolysin A (OlyA) have revealed new insights into the organization of PM cholesterol. These studies have defined three distinct pools of PM cholesterol—a fixed pool that is essential for membrane integrity, a sphingomyelin (SM)-sequestered pool that can be detected by OlyA, and a third pool that is accessible and can be detected by ALOD4. Accessible cholesterol is available to interact with proteins and transport to the endoplasmic reticulum (ER), and controls many cellular signaling processes including cholesterol homeostasis, Hedgehog signaling, and bacterial and viral infection. Here, we provide detailed descriptions for the use of ALOD4 and OlyA, both of which are soluble and non-lytic proteins, to study cholesterol organization in the PMs of animal cells. Furthermore, we describe two new versions of ALOD4 that we have developed to increase the versatility of this probe in cellular studies. One is a dual His₆ and FLAG epitope-tagged version and the other is a fluorescent version where ALOD4 is fused to Neon, a monomeric fluorescent protein. These new forms of ALOD4 together with previously described OlyA provide an expanded collection of tools to sense, visualize, and modulate levels of accessible and SM-sequestered cholesterol on PMs and study the role of these cholesterol pools in diverse membrane signaling events.

1. Introduction

Cholesterol is an essential and abundant component of animal cell membranes. The concentration of this lipid varies by as much as 10-fold in membranes of various cellular organelles. In the plasma membrane (PM), cholesterol comprises 35–40 mol% of total PM lipids whereas in the endoplasmic reticulum (ER) membrane, cholesterol comprises only ~5 mol % of total ER lipids (Das, Goldstein, Anderson, Brown, & Radhakrishnan, 2013; Radhakrishnan, Goldstein, McDonald, & Brown, 2008). In contrast to eukaryotes, cholesterol is extremely rare in prokaryotic organisms (Ourisson, Rohmer, & Poralla, 1987). Several Gram-positive bacteria exploit this difference to target eukaryotic membranes with soluble toxin proteins known as cholesterol dependent cytolysins (CDCs), which bind to eukaryotic cholesterol-containing membranes and form pores (Heuck, Moe, & Johnson,

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2010; Ohno-Iwashita et al., 2010; Tweten, 2005). Bacteria are not alone in their lipid-dependent, pore-forming toxin strategy. Other organisms, including mushrooms, worms, and sea anemones, target the sphingomyelin (SM) lipid component of eukaryotic cell membranes (Anderluh & Macek, 2002; Bernheimer & Avigad, 1979; Bhat et al., 2013; Makino et al., 2017; Skocaj et al., 2014; Tomita et al., 2004; Yamaji et al., 1998). In some cases, special combinations of SM and cholesterol are targeted (Endapally, Frias, et al., 2019; Makino et al., 2017; Skocaj et al., 2014).

These diverse toxins use at least two different mechanisms to form pores. Some pore forming toxins contain both lipid binding and pore formation activities in a single protein. Examples of such toxins are Perfringolysin O (PFO) from *Clostridium perfringens* or Anthrolysin O (ALO) from *Bacillus anthracis* which bind to accessible cholesterol with one region of the protein (Domain 4, D4) and use a different region of the protein (Domains 1–3, D1–3) to homo-oligomerize and form a transmembrane pore (see Fig. 1) (Bourdeau et al., 2009; Gay, Rye, & Radhakrishnan, 2015; Heuck et al., 2010). Other pore forming toxins require activities of two proteins—one to bind to membrane lipids and another to drive oligomerization and pore formation. An example of such a two-protein system is provided by *Pleurotus ostreatus*. This fungus produces two proteins, Ostreolysin A (OlyA) and Pleurotolysin B (PlyB). OlyA binds to SM-sequestered cholesterol in membranes (see Fig. 1) and PlyB binds to the membrane-bound OlyA to promote oligomerization and pore formation (Endapally, Frias, et al., 2019; Lukoyanova et al., 2015; Skocaj et al., 2014).

In this chapter, we will review the uses of ALO and OlyA, both of which are soluble proteins, to detect two distinct pools of cholesterol—accessible and SM-sequestered—in the PMs of animal cells. Assessing the PM without interference from membranes of other cellular organelles requires elimination of the pore-forming properties of these toxins. Fortunately, after significant protein engineering, we generated a soluble form of domain 4 of ALO (ALOD4) that does not form pores in membranes (Gay et al., 2015; Infante & Radhakrishnan, 2017) (Fig. 1). In the case of OlyA, no protein engineering was required since OlyA is non-lytic on its own as described above. These non-lytic tools have been used to resolve how cholesterol is organized in PM (He et al., 2017; Johnson & Radhakrishnan, 2020), how cholesterol organization in PM influences its accessibility for intracellular transport and controls cholesterol synthesis (Das, Brown, Anderson, Goldstein, & Radhakrishnan, 2014; Infante & Radhakrishnan, 2017; Johnson, Endapally, Vazquez, Infante, & Radhakrishnan, 2019), how accessibility of cholesterol controls Hedgehog signaling at the ciliary membrane (Kinnebrew et al., 2019), and how immune signals mobilize accessible cholesterol away from the PM during bacterial and viral infection (Abrams et al., 2020; Wang et al., 2020; Zhou et al., 2020). To expand the versatility and potential uses of ALOD4, we have developed two new versions of this probe, a dual epitope-tagged version (His₆-FLAG-ALOD4) and a fluorescent version (His₆-Neon-FLAG-ALOD4) (Fig. 1). Here, we describe how to purify these new versions of ALOD4 (Section 2), how to use ALOD4 and OlyA as sensors for accessible and SM-sequestered cholesterol on live cells (Section 3), how to visualize accessible cholesterol on PM with His₆-Neon-Flag-ALOD4 (Section 4), and how to assay for ALOD4 inhibition of accessible cholesterol transport from PM to ER (Section 5).

2. Purification of recombinant cholesterol-binding toxin proteins

Procedures for the overexpression and purification of His₆-tagged versions of ALOD4 and OlyA have been described in detail (Endapally, Frias, et al., 2019; Endapally, Infante, & Radhakrishnan, 2019). Recently, our lab has generated additional ALOD4 constructs, His₆-Flag-ALOD4, His₆-Flag-ALOD4 (Mut) (GTTLYP→AAAAA, non-binding mutant), His₆-Neon-Flag-ALOD4, and His₆-Neon-Flag-ALOD4 (Mut) (GTTLYP→AAAAA, non-binding mutant). His₆-Flag-ALOD4 and His₆-Flag-ALOD4 (Mut) were overexpressed and purified using a previously described protocol (Endapally, Infante, et al., 2019). Unfortunately, when we used this same protocol to purify His₆-Neon-Flag-ALOD4, the resultant purified protein was prone to degradation. Therefore, we developed an alternate purification scheme which is described below.

2.1 Materials

1. Expression plasmids
 - a. pHis₆-ALOD4, in the pRSET B expression vector, encodes, in sequential order from the NH₂-terminus, a 10-aa linker that includes a His₆ epitope tag (MRGSHHHHHH), a 23 aa-linker that includes the epitope for the anti-Xpress monoclonal antibody and an entero-kinase cleavage site (GMASMTGGQQMGRDLYDDDDDKDP), followed by domain 4 of ALO (aa 404–512). The sole native cysteine in ALOD4 (C472) was mutated to alanine and S404 was mutated to cysteine to allow for maleimide labeling at a position far from the cholesterol binding site in ALOD4. This plasmid is available from Addgene (Cat # 111026) (Gay et al., 2015).
 - b. pHis₆-Flag-ALOD4, also in the pRSET B expression vector, encodes, in sequential order from the NH₂-terminus, a 10-aa linker that includes a His₆ epitope tag (MRGSHHHHHH), a 23 aa-linker that includes a FLAG epitope tag (GMASMTGGQQMGRDYKDDDDDKDP), and domain 4 of ALO (aa 404–512). The ALOD4 portion of the plasmid contains the same two mutations (S404C and C472A) described above for pHis₆-ALOD4.
 - c. pHis₆-Flag-ALOD4 (Mut) is the same as pHis₆-Flag-ALOD4 described above, except that it contains six mutations (G501A, T502A, T503A, L504A, Y505A, and P506A) that disrupt cholesterol binding.
 - d. pHis₆-Neon-Flag-ALOD4, in the pRSET B expression vector, encodes, in sequential order from the NH₂-terminus, a 13-aa linker that includes a His₆ epitope tag (MRGSHHHHHHGMMA), the monomeric fluorescent protein Neon (aa 1–236) (Shaner et al., 2013), a 21 aa-linker that includes a FLAG epitope tag (ASMTGGQQMGRDYKDDDDDKDP), and domain 4 of ALO (aa 404–512). The ALOD4 portion of the plasmid contains the same two mutations (S404C and C472A) described above for pHis₆-ALOD4.

- e. pHis₆-Neon-Flag-ALOD4 (Mut) is the same as pHis₆-Neon-Flag-ALOD4 described above, except that it contains six mutations (G501A, T502A, T503A, L504A, Y505A, and P506A) that disrupt cholesterol binding.
- f. pOlyA-His₆, in the pET21C+ expression vector, encodes, in sequential order from the NH₂-terminus, OlyA (aa 1–138) and a 21-aa linker that includes a His₆ epitope tag (LVPRGSEGKSGCGLEHHHHHH). The two native cysteines in OlyA (C62 and C94) was mutated to serine and a new cysteine was introduced near the COOH-terminus (S151C) to allow for maleimide labeling at a position far from the binding site in OlyA for sphingomyelin/cholesterol complexes. This plasmid is available from Addgene (Cat # 132391) (Endapally, Frias, et al., 2019).
2. BL21 (DE3) pLysS *Escherichia coli* competent cells, Invitrogen, Cat # C6060
 3. Luria Broth (LB) capsules, Research Products International, Cat # L24041
 4. Ampicillin, 1000 × (100 mg/mL) stock solution, powder available from RPI Cat # A40040
 5. Isopropyl β-D-thiogalactoside (IPTG), 1000 × (1 M) stock solution in water, powder available from Goldbio, Cat # I2481C300
 6. Buffer A, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM tris(2-carboxyethyl)phosphine (TCEP)
 7. Buffer B, buffer A supplemented with 500 mM Imidazole (pH 7.5)
 8. Phenyl methanesulfonyl fluoride (PMSF), 1000 × (20 mg/mL) stock solution in ethanol, powder available from Sigma-Aldrich, Cat # P7626
 9. Protease inhibitor tablets, use 1 per 20 mL buffer, Thermo Fisher scientific, Cat # 88266
 10. Lysozyme from chicken egg white 10 × (10 mg/mL) stock in water, Sigma-Aldrich, Cat # L6876
 11. 100-mL all glass Dounce tissue grinder, Kimble though Fisher Scientific, Cat # K885303
 12. Branson Digital Sonifier, Fisher Scientific
 13. Ni-NTA agarose beads, Qiagen, Cat # 30250
 14. Econo-column chromatography column, 1.5 × 20 cm, BioRad, Cat # 7371522
 15. Amicon Ultra-4 10-kDa cutoff centrifugal filters, Millipore, Cat # UFC801024
 16. Column for size-exclusion chromatography (SEC) such as Superdex 200 column, GE Healthcare, Cat # 28990944
 17. Fast protein liquid chromatography (FPLC) system for SEC such as AKTA purifier, GE Healthcare

18. 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) gels
19. Quick Coomassie stain, Anatrace, Cat # GEN-QC-STAIN
20. 5 × SDS loading buffer, 10% (w/v) SDS, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2 M Tris–HCl, pH6.8, 0.05% (w/v) bromophenol blue
21. Spectrophotometer such as the NanoDrop, Thermo Fisher Scientific, to determine protein concentration
22. Imager to visualize fluorescent protein bands on SDS/PAGE, such as the LICOR Odyssey, LICOR

2.2 Protocols

2.2.1 Expression protocol for His₆-Neon-Flag-ALOD4

1. Transform His₆-Neon-Flag-ALOD4 plasmid into BL21 (DE3) pLysS *Escherichia coli* competent cells according to the manufacturers protocol and plate transformed cells on LB-Agar plates with ampicillin (100 µg/mL).
2. After incubation of plates for 16h at 37°C, pick a single colony and inoculate 160 mL LB containing 100 µg/mL ampicillin. Incubate this starter culture in a shaker-incubator (250rpm) for 12–16h at 30°C or until OD₆₀₀ is between 0.8 and 1.0.
3. Inoculate 1L LB containing 100 µg/mL ampicillin with 20 mL of the starter culture. Incubate at 37°C in a shaker-incubator (250rpm) until OD₆₀₀ is 0.4–0.6. Reduce shaker temperature to 18°C, add IPTG to a final concentration of 1 mM to induce protein expression and continue shaking at 18°C for 18–24h. Harvest the cells by centrifugation at 3220 × *g* for 10 min at 4°C, and immediately use cell pellet for protein purification or flash-freeze bacterial pellets in liquid nitrogen and store at –80°C for up to 6 months.

2.2.2 Purification protocol for His₆-Neon-Flag-ALOD4

1. Resuspend cell pellet from 1L of bacterial culture in 20 mL buffer A supplemented with PMSF, 1 protease inhibitor tablet, and lysozyme (1 mg/mL).
2. Homogenize the cell suspension using 12 strokes in a Dounce homogenizer, incubate at 4°C while rotating for 2h, then repeat Dounce homogenization step.
3. Further lyse the lysozyme-disrupted cells using a tip sonicator: 40% amplitude, 3 min cycle (repeating sequences of 3s on, 3s off) followed by 6 min cool down; repeat cycle two additional times.
4. Subject cell lysate to centrifugation (4°C, 30 min, 25,000 × *g*) to eliminate insoluble cell debris.
5. Incubate supernatant with 10 mL buffer A-equilibrated Ni-NTA beads on an orbital shaker at 4°C for 30 min.
6. Pour protein-Ni-NTA mixture into an empty chromatography column.

7. After beads have settled, wash column with 50 mL buffer A, then with 50 mL buffer A containing 25 mM imidazole and protease inhibitor (5% buffer B), and finally with 50 mL buffer A containing 50 mM imidazole and protease inhibitor (10% buffer B).
8. Elute bound protein from column with 20 mL buffer A containing 250 mM imidazole and protease inhibitor (50% buffer B).
9. Concentrate eluate to 4 mL with Amicon Ultra-4 10-kDa cutoff centrifugal filters ($3220 \times g$, 4°C).
10. Subject concentrated eluate to centrifugation (4°C, 30 min, $20,000 \times g$) to remove aggregate protein (if any) to prepare for SEC.
11. Inject 2 mL at a time onto Superdex 200 column equilibrated in buffer A connected to a FPLC system such as AKTA purifier. Collect protein-rich fractions and determine protein concentration with a bicinchoninic acid (BCA) assay kit or Spectrometer such as a NanoDrop according to manufacturer instructions.
12. See SEC profiles in Fig. 2A for examples of the monodisperse nature of His₆-Neon-Flag-ALOD4 (*dashed line*) purified as described above and His₆-Flag-ALOD4 (*solid line*) purified as described previously (Endapally, Infante, et al., 2019). Maximum values of absorbance at 280 nm for each protein were normalized to 1.
13. An aliquot (2 µg) of either His₆-Flag-ALOD4 (Fig. 2B, *lane 1*) or His₆-Neon-Flag-ALOD4 (Fig. 2B, *lane 2*), was subjected to 15% SDS/PAGE and stained with Coomassie (*left panel*) or imaged with the 600 nm filter on a Licor Imager to detect Neon fluorescence (*right panel*). Coomassie staining shows that purified His₆-Flag-ALOD4 migrates as a single band whereas purified His₆-Neon-Flag-ALOD4 migrates as a doublet. Only one of the two Coomassie-stained bands observed for His₆-Neon-Flag-ALOD4 showed a fluorescent signal. We did not conduct further analysis of the second Coomassie-stained band.
14. An aliquot (20 ng) of either His₆-Flag-ALOD4 (Fig. 2C, *lanes 1 and 3*) or His₆-Neon-Flag-ALOD4 (Fig. 2C, *lanes 2 and 4*), was subjected to 15% SDS/PAGE followed by immunoblot analysis with an anti-His (Fig. 2C, *left panel*) or anti-Flag (Fig. 2C, *right panel*) antibody. The immunoblot procedure used here is described in Section 3.3.3.
15. Store purified proteins for up to 2 months at 4°C in buffer A or 6 months at -80°C in buffer A supplemented with 20% (v/v) glycerol.
 - a. His₆-Neon-Flag-ALOD4 is prone to degradation. Storage at -80°C in buffer A supplemented with 20% (v/v) glycerol is recommended. Thaw protein aliquots on ice before use.
 - b. See Section 5 for the SREBP2 processing assay, which is performed with every batch of purified ALOD4 proteins to determine protein activity.

3. Using toxins as cholesterol sensors on live cells

3.1 Background

Most of the total cellular cholesterol is localized in the PM. Within the PM, cholesterol is organized into three distinct pools (Das et al., 2014). Two of these pools of cholesterol are inaccessible due to (i) interactions with SM, or (ii) interactions with other membrane factors including gly-cerophospholipids containing phosphatidylcholine headgroups (Moe & Heuck, 2010). PM cholesterol in excess of these two pools constitutes a third pool that is accessible and can be transported to the ER to shut down cholesterol synthesis and uptake (see Section 5). In this section, methods to detect changes in accessible cholesterol with ALOD4 and SM-sequestered cholesterol with OlyA are described (Fig. 3). Degradation of SM in the PM using a bacterial sphingomyelinase (SMase) liberates cholesterol from the SM-sequestered pool, leading to an increase in accessible cholesterol levels as detected by ALOD4 and a decrease in SM-sequestered cholesterol levels as detected by OlyA (Fig. 3A). Accessible cholesterol can be depleted from PMs using low concentrations of hydroxypropyl-beta cyclodextrin (HPCD), SM-sequestered cholesterol can also be depleted, but require higher HPCD concentrations (Fig. 3A).

3.2 Materials

1. Purified ALOD4 and OlyA proteins (see Section 2 and Endapally, Frias, et al., 2019; Endapally, Infante, et al., 2019). Similar results are obtained with all versions of ALOD4 described in Section 2.1, list 1 (also see Section 5).
2. Medium A, 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin sulfate (1% (v/v) P/S), and 5% (v/v) fetal calf serum (FCS). Warm to 37°C prior to use.
3. Medium B, sphingomyelin-depleting medium, medium A supplemented with 100 mU/mL SMase (SMase, Sigma, Cat # S8633). Warm to 37°C prior to use.
4. Medium C, cholesterol-depleting medium, medium A supplemented with 2% (w/v) HPCD. Warm to 37°C prior to use.
5. Dulbecco's phosphate-buffered saline (PBS)
6. Buffer C, 10 mM Tris-HCl (pH 6.8), 100 mM NaCl, 1% (w/v) SDS, 1 mM, EDTA, 1 mM EGTA, 20 µg/mL PMSF and protease inhibitors (1 tablet per 20 mL; Roche). Warm to room temperature (RT, 20–22°C) before use.
7. 5 × SDS loading buffer, 10% (w/v) SDS, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2 M Tris-HCl, pH6.8, 0.05% (w/v) bromophenol blue
8. 15% SDS/PAGE gels
9. 1 × SDS/PAGE running buffer, Tris/Glycine/SDS buffer (TGS), BioRad Cat #1610772
10. 1 × Transfer buffer (4°C) from Trans-Blot Turbo 5 × transfer buffer, BioRad, Cat #10026938

11. Phosphate buffered saline (pH 7.4) supplemented with 0.05% (w/v) TWEEN 20 (PBST), Millipore Sigma, Cat #P3563
12. 5% (w/v) milk in PBST
13. Anti-His antibody, Millipore, cat # 05-949, use 1:1000 dilution
14. Anti-mouse IgG, JacksonResearch, cat # 715-035-150, use 1:5000 dilution
15. SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo-Fisher Scientific, cat # 34580

3.3 Protocols

3.3.1 Sphingomyelin depletion protocol

1. On day 0, CHOK1 cells were set up in medium A at a density of 6×10^4 cells per well of a 48 well plate.
2. On day 1, media was removed, cells were washed twice with 500 μ L PBS, then treated with 200 μ L of either medium A (without SMase) or sphingomyelin-depleting medium B (with SMase).
3. After incubation for 30 min at 37°C, cells were washed twice with PBS, then treated with 200 μ L medium A containing either 3 μ M ALOD4 or 3 μ M OlyA.
4. After incubation for 1h at 37°C, cells were washed twice with 500 μ L PBS, then harvested with 200 μ L buffer C. Cell lysates were mixed with SDS loading buffer, 10% of each cell lysate was subjected to 15% SDS/PAGE followed by immunoblot analysis as described below.
5. Fig. 3A shows that SMase treatment of CHOK1 cells increases PM binding of ALOD4 (*lanes 1, 2*) while decreasing the PM binding of OlyA (*lanes 3, 4*).

3.3.2 Cholesterol depletion protocol

1. On day 0, CHOK1 cells were set up in medium A at a density of 6×10^4 cells per well of a 48-well plate.
2. On day 1, media was removed, cells were washed twice with 500 μ L PBS, then treated for 1h with either 200 μ L cholesterol-depleting medium C or a mixture of medium A and C to achieve the indicated concentration of HPCD (0%, 0.1%, 0.3%, 1%, 1.5%, or 2% (w/v)). This HPCD treatment did not result in any significant cell death, as judged by normal cell morphology and the lack of any significant rounding up or loss of cells from the plate.
3. After incubation for 1h at 37°C as described above, cells were washed twice with 500 μ L PBS and then treated with 200 μ L medium A containing either 3 μ M ALOD4 or 3 μ M OlyA.
4. After incubation for 1h at 37°C, cells were washed twice with PBS then lysed with 200 μ L buffer C. Cell lysates were mixed with SDS loading buffer, 10% of each cell lysate was subjected to 15% SDS/PAGE followed by immunoblot analysis as described below.

5. Fig. 3B shows that HPCD treatment of CHOK1 cells decreases PM binding of both ALOD4 (*lanes 1–6*) and OlyA (*lanes 7–12*), although with different sensitivities.

3.3.3 Immunoblot protocol

1. Proteins were transferred from electrophoresed gels onto nitrocellulose membranes using a BioRad Trans-Blot Turbo apparatus (2.5 A, 25V for 10 min).
2. Membranes were blocked for 1h at RT in 5% (w/v) milk in PBST.
3. To detect bound ALOD4 or OlyA, membranes were incubated with primary antibody (anti-His, 1:1000 dilution) in 5% (w/v) milk in PBST either overnight at 4°C on an orbital shaker or for 1h at room temperature on an orbital shaker.
4. Primary antibody was removed, and membranes were washed with PBST for 8 min. The wash step was repeated two additional times.
5. Membranes were then incubated with secondary antibody (anti-mouse, 1:5000 dilution) in 5% (w/v) milk in PBST.
6. After incubation for 30 min at RT, secondary antibody was removed and membranes were washed with PBST for 8 min, three times as described above.
7. Bound antibodies were visualized by chemiluminescence. The signal was developed by addition of SuperSignal Chemiluminescent substrate for 5 min. Membranes were then exposed to film for 1–30s, a Western blot imager such as a LICOR could also be used.

4. Using toxins to visualize cholesterol accessibility on PM

4.1 Background

While the analysis described in Section 3 captures overall changes in cholesterol pools, recent work has illuminated differences in the distribution of accessible and SM-sequestered cholesterol within PM. When fluorescently-labeled ALOD4 and OlyA were added to live cells, the overall binding patterns were largely distinct but also showed some regions of overlap (Johnson et al., 2019; Johnson & Radhakrishnan, 2020). Over the course of a few minutes, some of the bound OlyA was internalized while ALOD4 remained bound to the surface. In contrast to SM-sequestered cholesterol, which may recycle through endocytic pathways, accessible cholesterol relies on transport proteins to move from PM to ER where it performs regulatory signaling duties. Cholesterol transport proteins such as GramD/AsterB are enriched at PM-ER contact sites and are required for accessible cholesterol transport from PM to ER (Naito et al., 2019; Sandhu et al., 2018). Visualization of accessible and SM-sequestered pools of cholesterol could also shed light on how these cholesterol pools regulate Hedgehog signaling (Kinnebrew et al., 2019) and allow for cell-to-cell spread of pathogenic bacteria such as *Listeria monocytogenes* (Abrams, Johnson, Perelman, et al., 2020; Abrams, Johnson, Radhakrishnan, & Alto, 2020). In this section, we describe the use of a new fluorescent version of ALOD4, His₆-Neon-Flag-ALOD4, to visualize accessible cholesterol on PM of cells.

4.2 Materials

1. Fluorescent versions of ALOD4 where Neon, a monomeric fluorescent protein, is fused to ALOD4 (His₆-Neon-Flag-ALOD4) or where the lone cysteine of ALOD4 (His₆-ALOD4 or His₆-Flag-ALOD4) is labeled with Alexa Fluor or equivalent fluorescent maleimide reagents and non-fluorescent versions of ALOD4 (His₆-ALOD4 or His₆-Flag-ALOD4) (see Section 2 for details of protein constructs). Fluorescent versions of OlyA-His₆ can also be generated by labeling its lone cysteine with an Alexa Fluor or equivalent fluorescent maleimide reagent. The various fluorescent versions of ALOD4 and OlyA can be used individually or in combination.
2. Nunc Lab-Tek II chambered cover glass 8-well dish No. 1.5 glass (8-well imaging plate), Thermo Fisher Scientific, Cat # 12-565-338, or equivalent glass bottom dish suitable for imaging.
3. Medium A, 1:1 mixture of DMEM and Ham's F12 supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin sulfate and 5% (v/v) fetal calf serum (FCS). Warm to 37°C prior to use.
4. Medium B, medium A supplemented with 2% (w/v) HPCD. Warm to 37°C prior to use.
5. Dulbecco's phosphate-buffered saline (PBS), Sigma, Cat # D8537
6. Buffer D, 4% paraformaldehyde solution in PBS, Alfa Aesar through Fisher Scientific, Cat # AAJ61899AK
7. Buffer E, 2% (w/v) bovine serum albumin (BSA) in PBS solution, sterile filtered through 0.22 µm filter before use. Lyophilized BSA is available from Sigma-Aldrich, Cat # A6003.
8. Anti-His antibody, Millipore, Cat # 05-949, use 1:500 dilution
9. Anti-Flag M2 antibody, Sigma-Aldrich, Cat # F1804, use 1:500 dilution
10. Goat anti-mouse-Alexa Fluor 594 secondary antibody, Thermo Fisher, Cat # A-21235, use 1:400 dilution
11. 4',6-diamidino-2-phenylindole (DAPI), 5000 × (5 mg/mL) stock solution in water, treat with 1 µg/mL, Millipore Sigma, Cat # 5087410001
12. Microscope for visualization such as a Nikon Eclipse *Ti*, Nikon

4.3 Protocols

4.3.1 Protocol using His₆-Neon-Flag-ALOD4 to visualize accessible cholesterol on PM

1. On day 0, CHOK1 cells were set up in medium A at a density of 2×10^4 cells/well of an 8-well imaging plate.

2. On day 1, media was removed, cells were washed twice with 500 μ L PBS, then treated with 200 μ L lipoprotein-rich medium A or 200 μ L cholesterol-depleting medium B.
3. After incubation for 1h, cells were washed twice with 500 μ L PBS, then treated with 200 μ L medium A containing either 3 μ M His₆-Neon-Flag-ALOD4 or 3 μ M His₆-Neon-Flag-ALOD4 (Mut).
4. After incubation for 15 min, cells were washed twice with PBS and fixed by addition of 200 μ L buffer D for 15 min.
5. After fixation, cells were washed 5 times with 500 μ L PBS then treated with 500 μ L buffer E on an orbital shaker.
6. After 30 min, blocking buffer was replaced with 500 μ L buffer E containing 1 μ g/mL DAPI.
7. After 1h, cells were washed 5 times with PBS, postfixed with buffer D, then imaged to visualize bound His₆-Neon-Flag-ALOD4.
 - a. Representative images are shown for His₆-Neon-Flag-ALOD4 binding to CHOK1 cells (Fig. 4A). When cells are incubated with lipoprotein-rich FCS, His₆-Neon-Flag-ALOD4 binds robustly to the PM of cells. When cellular cholesterol is depleted with HPCD, binding is lost. His₆-Neon-Flag-ALOD4 (Mut) does not bind to cells under either condition (Fig. 4A).
 - b. Additional information on preparing and using Alexa Fluor labeled ALOD4 and OlyA is available in previous publications (Endapally, Infante, et al., 2019; Johnson et al., 2019; Kinnebrew et al., 2019).

4.3.2 Immunofluorescence protocol to visualize binding of fluorescent and non-fluorescent versions of ALOD4 to PMs

1. On day 0, CHOK1 cells were set up in medium A at a density of 2×10^4 cells/well of an 8-well imaging plate.
2. On day 1, media was removed, and cells were washed twice with 500 μ L PBS, then treated with 200 μ L medium A containing 3 μ M of His₆-ALOD4, His₆-Flag-ALOD4, or His₆-Neon-Flag-ALOD4.
3. After incubation for 15 min, cells were washed twice with 500 μ L PBS and fixed by addition of 200 μ L buffer D for 15 min.
4. After fixation, cells were washed 5 times with 500 μ L PBS then treated with 500 μ L buffer E and placed on an orbital shaker (50rpm).
5. After 30 min, buffer E was removed and replaced with 200 μ L buffer E supplemented with either anti-His (1:1000 dilution) or anti-Flag (1:1000 dilution).
6. After incubation for 1h, cells were washed by incubation with 500 μ L PBS for 5 min. This wash step was repeated 5 additional times.

7. After washing, cells were treated with 200 μ L buffer E supplemented with 1:400 goat anti-mouse-Alexa Fluor 647 secondary antibody and 1 μ g/mL DAPI.
8. After incubation for 1h, cells were washed by incubation with 500 μ L PBS for 5 min. The wash step was repeated 5 additional times, and the cells were then post-fixed with 200 μ L buffer D for 10 m, followed by 5 washes with PBS.
9. Cells were imaged to visualize bound ALOD4 as shown in Fig. 4B and C. When using immunofluorescence, it is important to include untreated samples to determine background signal of the antibodies used. Fig. 4B shows CHOK1 cells in the absence of ALOD4 addition after the complete immunofluorescence protocol with either anti-His (*left panel*) or anti-Flag (*right panel*) to confirm specificity of signal observed. Fig. 4C shows Neon (*left panel*), anti-His (*middle panel*), or anti-Flag (*right panel*) fluorescence signal after treatment with 3 μ M His₆-ALOD4 (*top row*), His₆-Flag-ALOD4 (*center row*), or His₆-Neon-Flag-ALOD4 (*bottom row*).
 - a. This protocol can also be used to detect OlyA-His₆ bound to cells.

5. Using toxins to study cholesterol sensing and transport

5.1 Background

Animal cells carefully regulate their cholesterol content. To enable such regulation, cells must measure cholesterol levels in their membranes. If the level is too low, cholesterol synthesis and uptake are initiated. If the level is too high, synthesis and uptake are terminated. These opposite outcomes are triggered by the actions of a sensor protein called Scap that resides in the ER membrane (Brown, Radhakrishnan, & Goldstein, 2018). Scap forms complexes with ER membrane-bound transcription factors called sterol regulatory element-binding proteins (SREBPs), which activate all the genes involved in synthesis of cholesterol and the LDL receptor which acquires exogenous cholesterol from LDL particles. Activation of SREBPs requires their transport by Scap to the Golgi where two proteases release their transcription factor domain that then enter the nucleus for target gene activation. When cholesterol in the ER rises and binds to Scap, it traps SREBPs in the ER, thus preventing proteolytic cleavage and nuclear entry. However, most of the cell's cholesterol is located in the PM (Das et al., 2014; Lange, Ye, & Steck, 2004). How does the Scap/SREBP regulatory machine in the ER detect changes in PM cholesterol? A solution to this problem lies in the organization of cholesterol in PMs. As described in Section 3, PM cholesterol is organized into three distinct pools (Das et al., 2014). Two of these pools of cholesterol are inaccessible due to interactions with SM or interactions with other membrane factors. PM cholesterol in excess of these two pools constitutes a third pool that is accessible and is transported to the ER to terminate SREBP activation.

While the cholesterol sensing machinery in ER has been well characterized, how the various cholesterol sensing and transport machineries work in concert to maintain cholesterol homeostasis remains unclear. ALOD4 and OlyA are useful tools to study the connection between cholesterol levels in PM and the response of the cholesterol sensing machinery in ER. When ALOD4 binds accessible cholesterol on the outer leaflet of PM, cholesterol

transport from PM-to-ER is blocked and ER cholesterol drops below a threshold concentration of 5 mol% of total ER lipids, triggering activation of SREBP transcription factors even though cellular cholesterol has not been diminished (Infante & Radhakrishnan, 2017). In this section, we describe assays to confirm functionality of our new ALOD4 protein constructs by testing whether they trigger SREBP processing.

5.2 Materials

1. Purified ALOD4 proteins (see Section 2 and Endapally, Infante, et al., 2019)
2. Medium A, 1:1 mixture of DMEM and Ham's F12 supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin sulfate (1% (v/v) P/S) and 5% (v/v) fetal calf serum (FCS). Warm to 37°C prior to use.
3. Dulbecco's phosphate-buffered saline (PBS)
4. Buffer C, 10 mM Tris-HCl (pH 6.8), 100 mM NaCl, 1% (w/v) SDS, 1 mM EDTA, 1 mM EGTA, 20 µg/mL PMSF, and protease inhibitors (1 tablet (Roche) per 20 mL). Warm to RT before use.
5. 5 × SDS loading buffer, 10% (w/v) SDS, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 0.2 M Tris-HCl, pH6.8, 0.05% (w/v) bromophenol blue
6. 15% SDS/PAGE gel and 10% SDS/PAGE gel
7. 1 × SDS/PAGE running buffer, Tris/Glycine/SDS buffer (TGS), BioRad Cat # 1610772
8. 1 × Transfer buffer (4°C) from Trans-Blot Turbo 5 × transfer buffer, BioRad, Cat # 10026938
9. Phosphate buffered saline (pH 7.4) supplemented with 0.05% (w/v) TWEEN 20 (PBST), Millipore Sigma, Cat # P3563
10. 5% Milk (w/v) in PBST
11. Anti-His antibody, Millipore, Cat # 05-949, use 1:1000 dilution
12. Anti-Flag M2 antibody, Sigma-Aldrich, Cat # F1804, use 1:1000 dilution
13. Anti- β-actin to detect actin, Cell Signaling Technology, Cat# 4970S, 13E5, use 1:1000 dilution
14. IgG-7D4, antibody to detect SREBP2, one of the three isoforms of SREBP, commercially available from ATCC CRL-2198, use 10 µg/mL dilution
15. Anti-rabbit IgG, JacksonResearch, Cat # 111-035-003, use 1:5000 dilution
16. Anti-mouse IgG, JacksonResearch, Cat # 715-035-150, use 1:5000 dilution
17. SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo-Fisher Scientific, Cat # 34580

5.3 Protocol

1. On day 0, CHOK1 cells were set up in medium A at a density of 6×10^4 cells per well of a 48 well plate.
2. On day 1, cells were washed twice with PBS then treated with the indicated concentration of ALOD4 in medium A.
3. After incubation for 1h at 37°C, media was removed, cells were washed twice with 500 μ L PBS, and harvested by addition of 200 μ L buffer C. Cell lysates were mixed with SDS loading buffer, 10% of each cell lysate was subjected to 15% SDS/PAGE followed by immunoblot analysis described below.
4. 10% of each cell lysate sample was subjected to either 10% SDS/PAGE or 15% SDS/PAGE followed by immunoblot analysis for SREBP2 and bound ALOD4, respectively.
5. The immunoblot procedure described in Section 3.3.3 was followed.
 - a. Use IgG 7D4 (10 μ g/mL) to detect SREBP2, anti-His (1:1000 dilution) or anti-Flag M2 (1:1000 dilution) to detect bound ALOD4, and anti- β -Actin (1:1000 dilution) to detect actin.
6. Fig. 5 shows results of experiments assaying for the effects of various versions of ALOD4 on trapping accessible cholesterol on PMs and triggering SREBP2 processing. As shown in Fig. 5A, incubation with either His₆-ALOD4 (*lanes 1–4*) or His₆-Flag-ALOD4 (*lanes 5–8*) results in binding of these proteins to the PM and processing of SREBP2 to its activated nuclear form. Fig. 5B shows that His₆-Neon-Flag-ALOD4 also binds to the PM and triggers the processing of SREBP2 (*lanes 1–4*) whereas His₆-Neon-Flag-ALOD4 (Mut) shows no binding to PMs and does not trigger the processing of SREBP2 (*lanes 5–8*).

6. Summary

Cholesterol dependent cytolysins such as ALOD4 and fungal toxins such as OlyA have been engineered to serve as laboratory tools to detect the level and distribution of cholesterol in animal membranes. These probes have allowed for the determination of three distinct pools of cholesterol in PMs and for elucidating the role of these pools in controlling various signaling processes in cells. While these tools are easy to work with and work robustly in a variety of animal cell types, caution must be used when interpreting results. For example, while ALOD4 and OlyA are excellent sensors for accessible and inaccessible cholesterol (see Section 3), these probes also rapidly inhibit transport of accessible cholesterol from PM to ER, triggering cholesterol uptake and synthesis pathways even though cellular cholesterol levels have not changed (see Section 5 and Infante & Radhakrishnan, 2017; Johnson et al., 2019). This feature of ALOD4 and OlyA binding to PMs can be useful in studying intracellular cholesterol transport; however, it should also not be ignored when using ALOD4 or OlyA as live-cell sensors.

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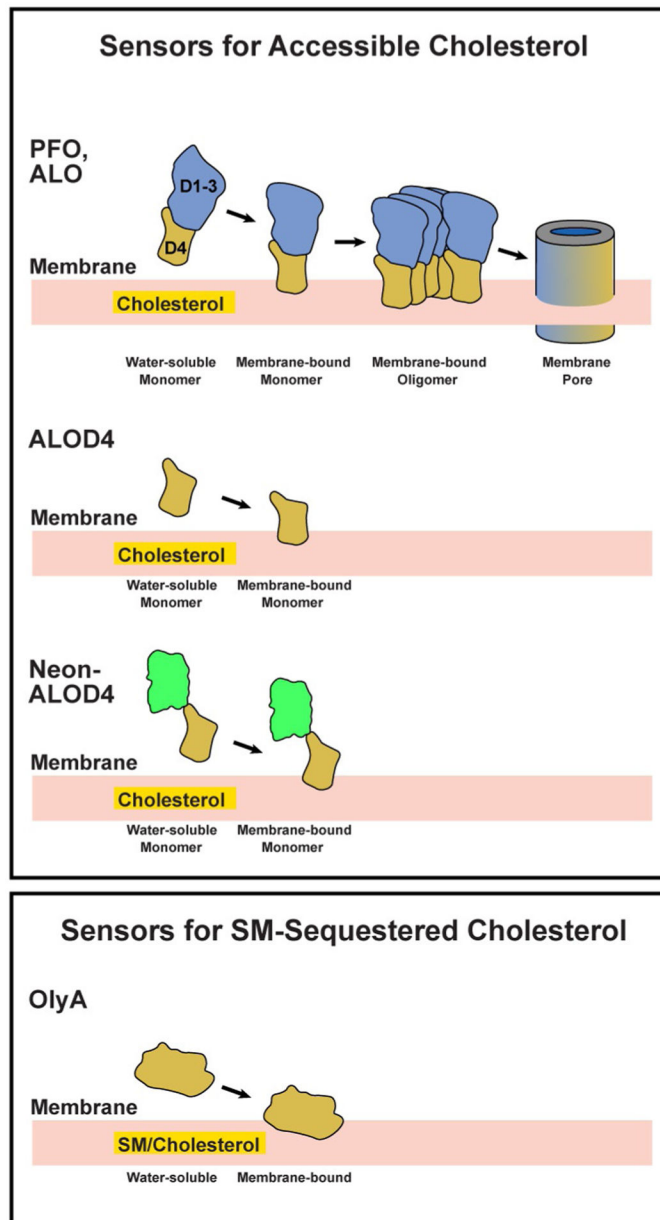


Fig. 1. Schematic representation of sensors for accessible cholesterol and SM-sequestered cholesterol that are described in this study.

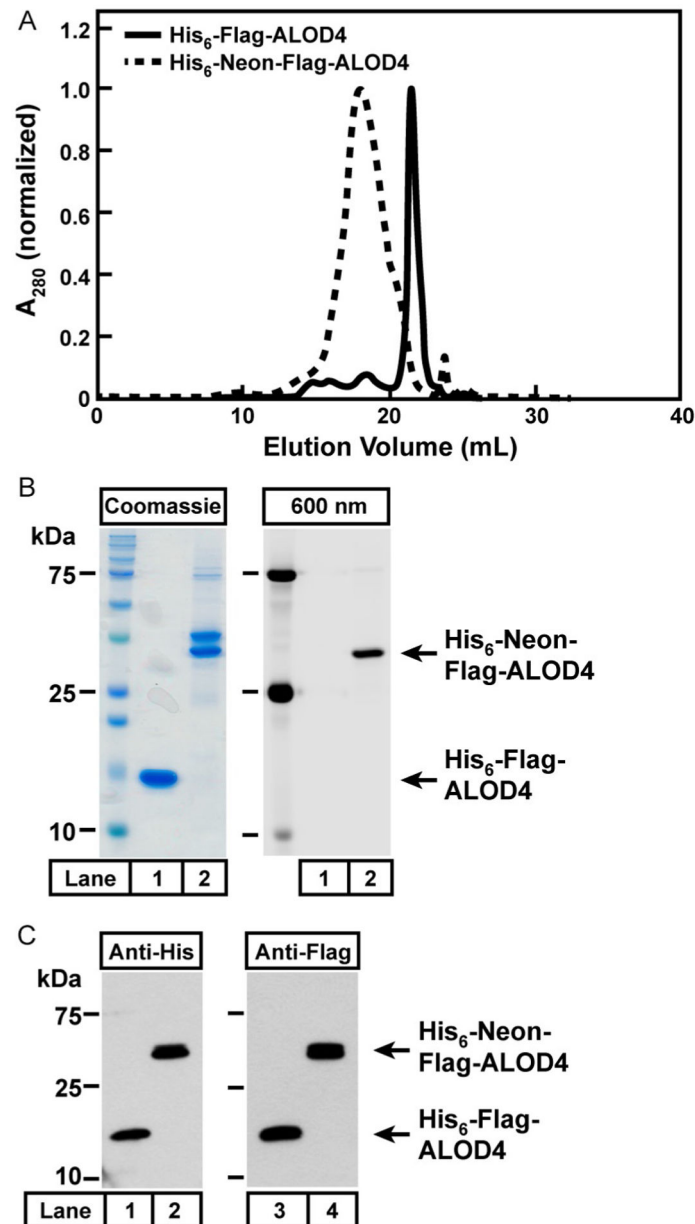
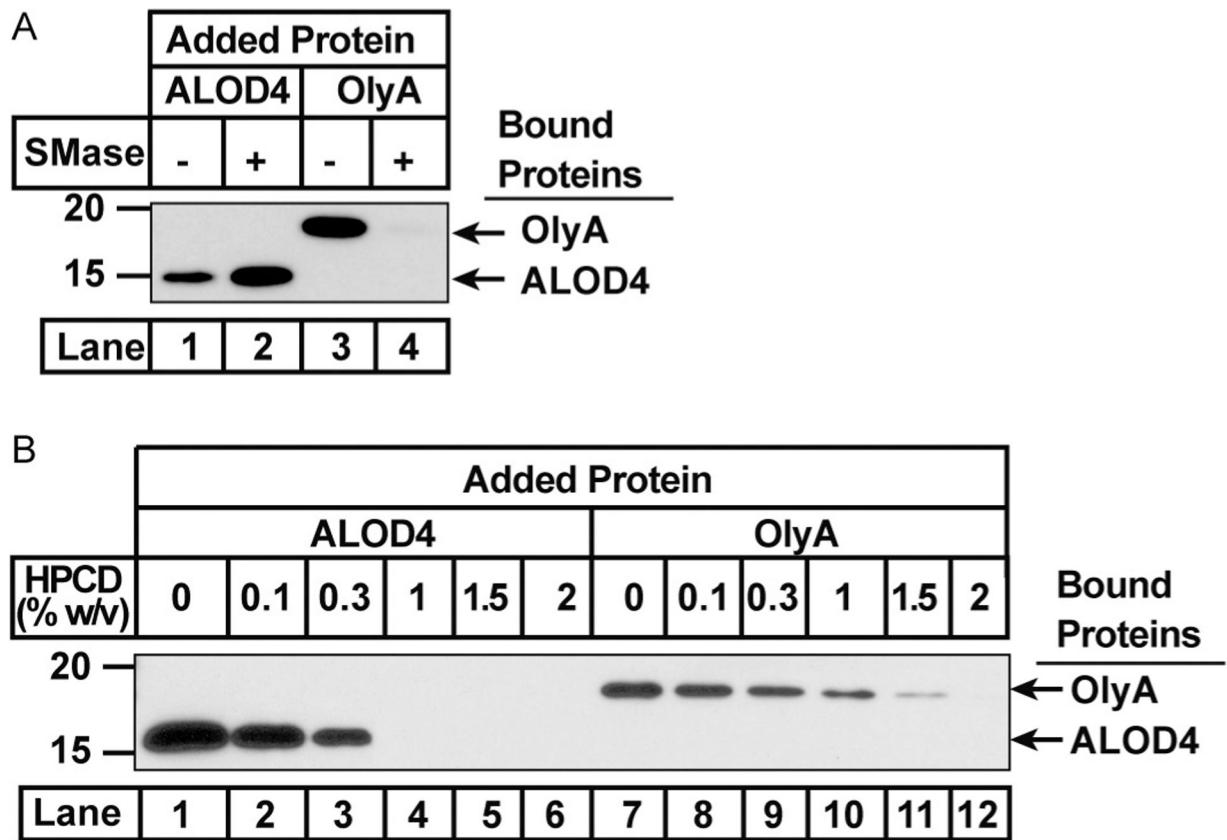


Fig. 2. Purification of recombinant cholesterol binding toxins. (A) Representative Superdex 200 SEC profiles of purified recombinant His₆-Flag-ALOD4 (*solid line*) and His₆-Neon-Flag-ALOD4 (*dashed line*). The maximum values for the absorbance at 280 nm for each protein are normalized to 1 to allow for comparison of the two profiles. (B) 2 μ g of purified His₆-Flag-ALOD4 (*lane 1*) or His₆-Neon-Flag-ALOD4 (*lane 2*) were subjected to 15% SDS PAGE, followed by Coomassie staining (*left panel*) or fluorescence imaging with a 600 nm filter on a LICOR imager (*right panel*). (C) 20 ng of purified His₆-Flag-ALOD4 (*lane 1*) and His₆-Neon-Flag-ALOD4 (*lane 2*) were subjected to 15% SDS PAGE, followed by immunoblot analysis with anti-His (*left panel*) or anti-Flag (*right panel*) antibodies.

**Fig. 3.**

ALOD4 and OlyA detect different cholesterol pools in PM. On day 0, CHOK1 were set up in medium A at a density of 6×10^4 cells per well of a 48 well plate. (A) On day 1, cells were washed twice with 500 μ L PBS, then treated with 200 μ L of medium A (without SMase) or sphingomyelin-depleting medium B (with SMase). After incubation for 30 min at 37°C, media was removed, cells were washed twice with PBS, then treated with 200 μ L medium A containing either 3 μ M His₆-ALOD4 (*lanes 1, 2*) or 3 μ M OlyA-His₆ (*lanes 3, 4*). (B) On day 1, cells were washed twice with 500 μ L PBS then treated with either 200 μ L medium A or medium A supplemented with cholesterol-depleting medium C to produce the indicated concentration of HPCD. After incubation for 1h at 37°C, media was removed, cells were washed twice with 500 μ L PBS then treated with 200 μ L medium A containing either 3 μ M His₆-ALOD4 (*lanes 1–6*) or 3 μ M OlyA-His₆ (*lanes 7–12*). (A and B) After incubation with ALOD4 or OlyA for 1h at 37°C, cells were washed twice with 500 μ L PBS, then harvested in 200 μ L buffer C. Equal aliquots of each cell lysate (10% of total) was subjected to immunoblot analysis as described in Section 3.3.

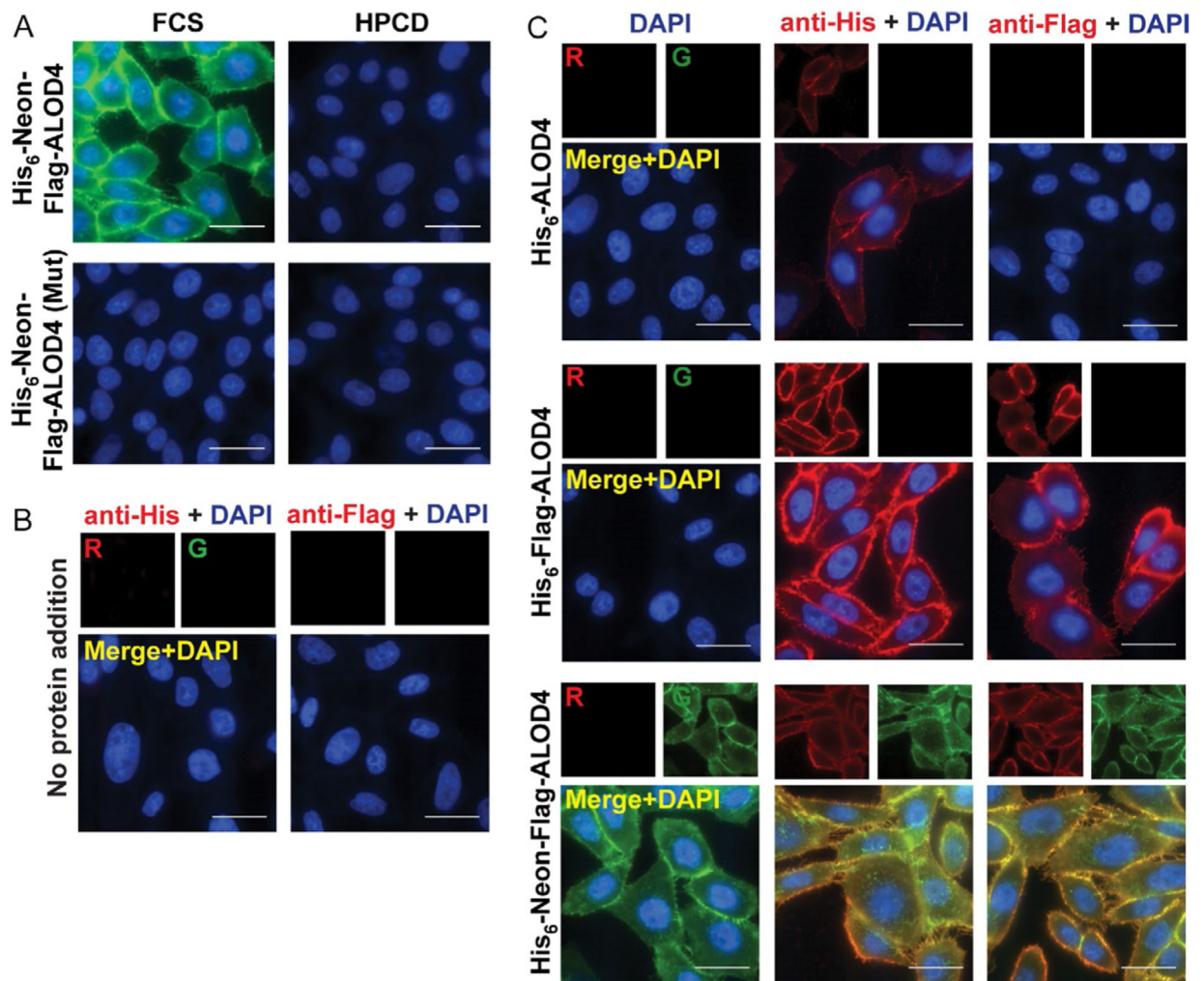
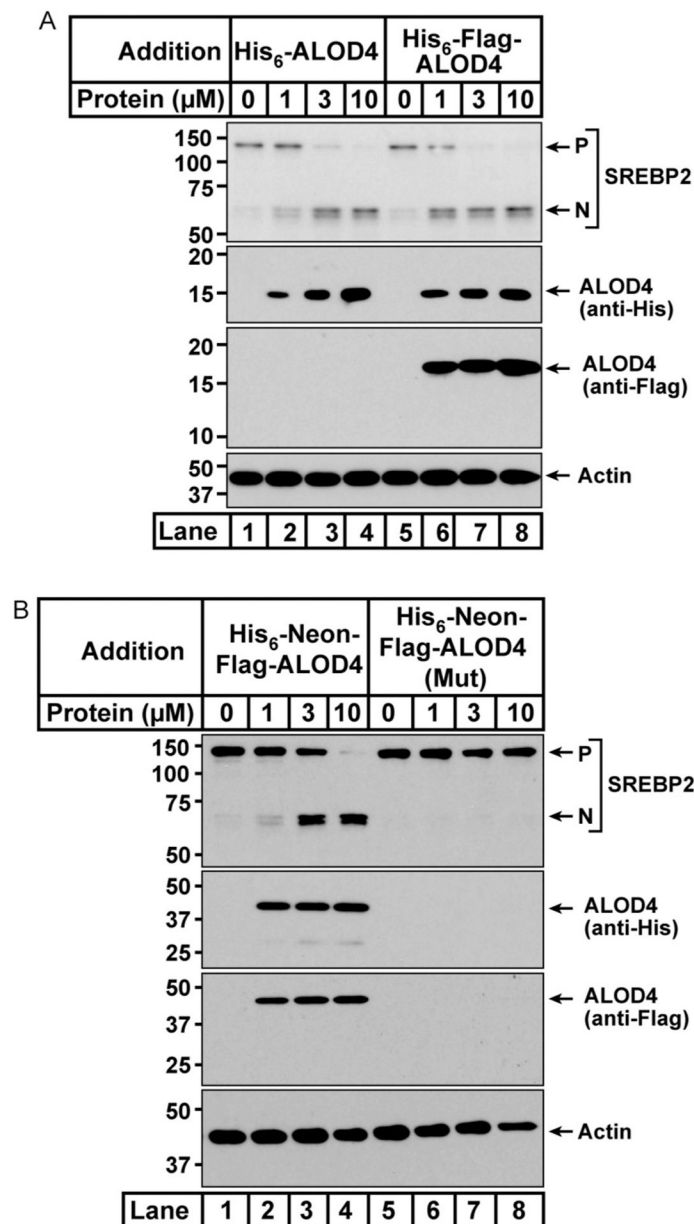


Fig. 4.

Visualizing PM accessible cholesterol with fluorescent ALOD4. On day 0, CHOK1 cells were set up in medium A at a density of 2×10^4 cells per well of an 8-well glass bottom MatTek imaging plate. (A) On day 1, cells were washed twice with 500 μ L PBS then treated with 200 μ L of either lipoprotein-rich medium A or cholesterol-depleting medium B. After 1h at 37°C, cells were washed twice with 500 μ L PBS, then treated with 200 μ L medium A supplemented with either 3 μ M His₆-Neon-Flag-ALOD4 or His₆-Neon-Flag-ALOD4 (Mut). After 15 min at RT, cells were washed twice with 500 μ L PBS, then fixed with 200 μ L buffer D. After incubation for 15 min at RT, cells were washed five times with PBS and then treated with 500 μ L blocking buffer E for 30 min. Cells were then treated with 500 μ L buffer E containing 1 μ g/mL DAPI. After incubation for 1h at RT, cells were washed five times with PBS, post-fixed with buffer D, then imaged with a Nikon Eclipse microscope. Scale bars are 20 μ m. (B and C) On day 1, cells were washed twice with 500 μ L PBS then treated with 200 μ L of either medium A (B) or medium A supplemented with 3 μ M of either His₆-ALOD4, His₆-Flag-ALOD4, or His₆-Neon-Flag-ALOD4 (C). After incubation for 15 min at RT, cells were washed twice with 500 μ L PBS, then fixed with 200 μ L buffer D. After incubation for 15 min at RT, cells were washed five times with PBS, then

treated with 500 μ L buffer E. After incubation for 30 min at RT, buffer E was removed and replaced with 200 μ L buffer E (C, *left panel*) or buffer E containing 1:1000 of either anti-His (B, *left panel*; C, *center panel*) or anti-Flag (B and C, *right panel*) antibodies. After incubation for 1h at RT, cells were washed six times with 500 μ L PBS (5 min per wash), then treated with 200 μ L buffer E containing 1:400 goat anti-mouse-594 secondary antibody and 1 μ g/mL DAPI. After incubation for 1h at RT, cells were washed six times with 500 μ L PBS (5 min per wash), then post-fixed with 200 μ L buffer D for 10 min at RT, then washed five times with PBS. Cells were imaged with a Nikon Eclipse microscope. Scale bars are 20 μ m.

**Fig. 5.**

Depletion of ER cholesterol by ALOD4 binding to accessible cholesterol in PM. On day 0, CHOK1 were set up in medium A at a density of 6×10^4 cells per well of a 48 well plate. On day 1, cells were washed twice with 500 μL PBS, then treated with 200 μL medium A supplemented with the indicated concentration of either His₆-ALOD4 or His₆-Flag-ALOD4 (A) or with either His₆-Neon-Flag-ALOD4 or His₆-Neon-Flag-ALOD4 (Mut) (B). After incubation for 1h at 37°C, cells were washed twice with 500 μL PBS, then harvested with 200 μL buffer C. 10% of each sample was subjected to immunoblot analysis as described in Section 5. *P*, precursor form of SREBP2; *N*, cleaved nuclear form of SREBP2.