

Introducing inducible fluorescent split cholesterol oxidase to mammalian cells

Received for publication, October 3, 2016, and in revised form, April 5, 2017. Published, Papers in Press, April 7, 2017, DOI 10.1074/jbc.M116.761718

Konstantin G. Chernov[‡], Maarit Neuvonen^{§¶}, Ivonne Brock^{§¶},  Elina Ikonen^{§¶1,2}, and  Vladislav V. Verkhusha^{¶||1,3}

From the [‡]Department of Biochemistry and Developmental Biology and [§]Department of Anatomy, Faculty of Medicine, University of Helsinki, Helsinki 00290, Finland, [¶]Minerva Foundation Institute for Medical Research, Helsinki 00290, Finland, and ^{||}Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Edited by George M. Carman

Cholesterol oxidase (COase) is a bacterial enzyme catalyzing the first step in the biodegradation of cholesterol. COase is an important biotechnological tool for clinical diagnostics and production of steroid drugs and insecticides. It is also used for tracking intracellular cholesterol; however, its utility is limited by the lack of an efficient temporal control of its activity. To overcome this we have developed a regulatable fragment complementation system for COase cloned from *Chromobacterium* sp. The enzyme was split into two moieties that were fused to FKBP (FK506-binding protein) and FRB (rapamycin-binding domain) pair and split GFP fragments. The addition of rapamycin reconstituted a fluorescent enzyme, termed split GFP-COase, the fluorescence level of which correlated with its oxidation activity. A rapid decrease of cellular cholesterol induced by intracellular expression of the split GFP-COase promoted the dissociation of a cholesterol biosensor D4H from the plasma membrane. The process was reversible as upon rapamycin removal, the split GFP-COase fluorescence was lost, and cellular cholesterol levels returned to normal. These data demonstrate that the split GFP-COase provides a novel tool to manipulate cholesterol in mammalian cells.

Cholesterol is an essential component of the eukaryotic cell plasma membrane and is critical for its structural integrity (1). It is derived either exogenously or synthesized endogenously in the endoplasmic reticulum (ER).⁴ A continuous flow of cholesterol

from the ER to the plasma membrane (PM) results in an intracellular gradient of cholesterol with the majority of cholesterol (60–70%) localized in the PM (2). In the PM, cholesterol is able to associate with saturated phospholipids and sphingolipids, forming membrane nanodomains (3). These structures, called lipid rafts, are enriched e.g. in signal transduction molecules (4).

Several compounds such as statins, filipin, and cyclodextrins can be used to manipulate cellular cholesterol. Statins inhibit the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis (5). Filipin is a cholesterol-binding fluorescent antibiotic that permeabilizes membranes (6). Cyclodextrins form soluble complexes with cholesterol and can be used to extract cholesterol from or deliver cholesterol to membranes (7). Another commonly used method to manipulate cholesterol levels is treatment of cells with cholesterol oxidase (COase) (8). This enzyme also has multiple biotechnological applications, including clinical diagnostics of cholesterol content and production of sterol drugs and insecticides (9).

Bacteria produce COases to utilize cholesterol for their energy metabolism, which is initiated by a multistep oxidation reaction. First, the FAD cofactor accepts hydride from cholesterol. Then the reduced flavin reacts with dioxygen to generate peroxide and re-oxidizes the enzyme. Finally, oxidized cholesterol isomerizes to a final product, cholest-4en-3-one (10). Cholesterol oxidases bind to FAD either non-covalently (class 1) or covalently (class 2). Classes differ in the kinetics of the oxidation reaction, class 2 being faster than class 1 (11). Both classes are described as being composed of two functional domains: the FAD-binding domain and the cholesterol-binding domain. Topologically they can also be considered as single domain proteins as the polypeptide chain meanders back and forth. The class 1 enzymes possess the typical Rossmann fold found in nucleotide-binding proteins (12). The class 2 enzymes belong to the vanillyl-alcohol oxidase family (13, 14). In both classes FAD and cholesterol are buried from the solvent inside the enzyme, although the nature of the binding pockets is different (10).

The effect of COase on the cell membrane is profound, as it rapidly converts membrane cholesterol to cholestenone. In cholestenone, the initial hydroxyl group is converted to a keto group that has limited capacity for hydrogen bonding with other plasma membrane components. This increases the flip-flop rate of cholestenone that is in the order of magnitude higher than that for cholesterol (15). COase treatment also

This work was supported by Academy of Finland Grants 263371 and 266992 (to V. V. V.) and 272130 and 284667 (to E. I.) and by an EU FP7 program ERC-2013-ADG-340233 grant (to V. V. V.). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supplemental Table 1 and Figs. S1–S3.

¹ Both authors contributed equally to this work.

² To whom correspondence may be addressed: Dept. of Anatomy, Faculty of Medicine, University of Helsinki, Helsinki 00290, Finland. Tel.: 358-91-912-5277; Fax: 358-91-912-5261; E-mail: elina.ikonen@helsinki.fi.

³ To whom correspondence may be addressed: Dept of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY 10461. Tel.: 718-430-8591; Fax: 718-430-8996; E-mail: vladislav.verkhusha@einstein.yu.edu.

⁴ The abbreviations used are: ER, endoplasmic reticulum; PM, plasma membrane; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; COase, cholesterol oxidase; PPI, protein-protein interaction; FRB, rapamycin-binding domain of kinase mammalian target of rapamycin; FKBP, FK506-binding protein; FP, fluorescent protein; EGFP, enhanced GFP; sfGFP, super-folder GFP; LPDS, lipoprotein-deficient serum; SREBP-2, sterol regulatory element-binding protein 2; CT, threshold cycle; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

Split cholesterol oxidase

decreases membrane order because of cholesterol lowering and the disordering effect of cholestenone. Raft disruption leads to a spontaneous clustering of death factor Fas, formation of Fas-FADD complexes, activation of caspase-8, and apoptosis (16). An additional cytotoxic effect is caused by peroxide that increases the intracellular reactive oxygen species level (17). Despite that, a lysosome-targeted COase has been reported to attenuate the cytotoxicity of 7-ketocholesterol in human fibroblasts (18). Thus, COase activity influences multiple cellular processes.

Reconstitution of enzymatic activity through the noncovalent association of complementing fragments has been widely used to monitor dynamic protein-protein interactions (PPI) and to discover drugs modifying them (19). Development of split GFP and other fluorescent proteins enabled the visualization of PPI in a wide variety of organisms (20). Recently, a split horseradish peroxidase was developed to detect PPI in the extracellular space for studying communication between different cell types (21). Beyond that, complementing fragments are used in chemically induced dimerizers to restore enzymatic activity and trigger biological responses. In this case one fragment of an enzyme is fused with FRB (rapamycin-binding domain of kinase mammalian target of rapamycin) and the other with FKBP (FK506-binding protein), which form a pair upon the addition of rapamycin, reconstituting the functional enzyme (22, 23). For example, split Cre and Cas9 nucleases are versatile tools for genome editing and transcriptional regulation (24–26). Split ubiquitin and tobacco etch virus protease are broadly used to regulate protein degradation and apoptosis (27, 28). Additionally, splitting may increase spatiotemporal precision of enzymatic reactions and make them tissue-specific (24, 29).

In this study we engineered a rapamycin-inducible fluorescent cholesterol oxidase from *Chromobacterium* sp. DS-1 (split GFP-COase) that belongs to class 2 of COase enzymes (13, 30). Induction by rapamycin allows temporal control over COase activity and rapidly leads to an efficient reduction of intracellular cholesterol. After rapamycin removal, the cells lose the holoenzyme expression and recover their cholesterol levels. We propose this drug-inducible split GFP-COase as a new tool to manipulate the cellular cholesterol content and gain insights into cholesterol-dependent proteins and cholesterol-related cell pathologies.

Results

Engineering of split GFP-COase enzyme

COase from *Chromobacterium* sp. strain DS-1 is composed of 21 α -helices and 20 β -strands that form four structural domains (13). We used two approaches to find successful sites for the split of the COase. Assuming that protein domains fold independently, we decided on three split sites that would separate entire domains and may complement in restoring enzymatic activity (Fig. 1A). Our second approach was to split COase in the loops with maximum flexibility. We chose several loop regions with maximal temperature B-factor that reflects their maximal mobility (Fig. 1A) (13). Presumably, splitting at these sites should preserve α -helices and β -sheets as well as bind to FAD and cholesterol.

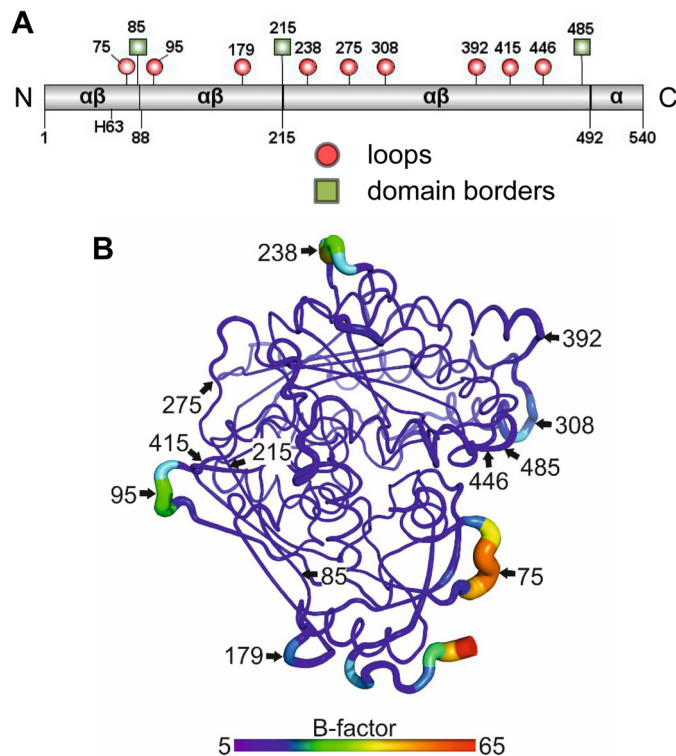


Figure 1. Structure of COase and mapping of putative split sites. A, schematic diagram of cholesterol oxidase domain structure and positions of split sites. Split sites that separate domains are shown as green squares, and sites located in the loops are shown as red circles. B, cholesterol oxidase structure (PDB ID: 3JS8) generated with PyMol software. The range of B-factor values is shown with a color scale from blue to red, where red corresponds to the most disordered regions, and blue corresponds to most ordered regions. The black arrows depict the split sites; the corresponding amino acid number is indicated.

We first tested the activity of split COase in bacteria, co-expressing both fragments from a single plasmid. To induce complementation of the separated N- and C-terminal fragments, they were fused to coils with oppositely charged amino acid residues (named E-coil and K-coil). These coils electrostatically interact with each other with high affinity (31) and bring COase fragments together, restoring their enzymatic activity. COase activity in bacterial extracts was detected for the constructs split at positions 75, 95, 415, and 485 (Fig. 2A). Notably, all these splits located in close proximity to N-terminal or C-terminal ends of the protein. The most active split at the position 485 had a 20-fold lower enzymatic activity than the full-length enzyme. The remaining active constructs (splits at positions 75, 95, and 415) were even less active. This result was expected because splitting may decrease solubility and destabilize protein structure. Importantly, no enzymatic activity was detected when only one COase part was expressed in the cells (data not shown).

We further tested four splits with maximum activity (75, 95, 415, 485) in mammalian cells. For this purpose we fused the N-terminal COase part with FRB and the respective C-terminal part with membrane-targeted FKBP constructs (Fig. 2B). COase activity was only detected in splits 75 and 95, whereas splits 415 and 485 were inactive in HeLa cell lysates (Fig. 2C). COase activity in splits 75 and 95, however, was detected even in the absence of rapamycin (Fig. 2C).

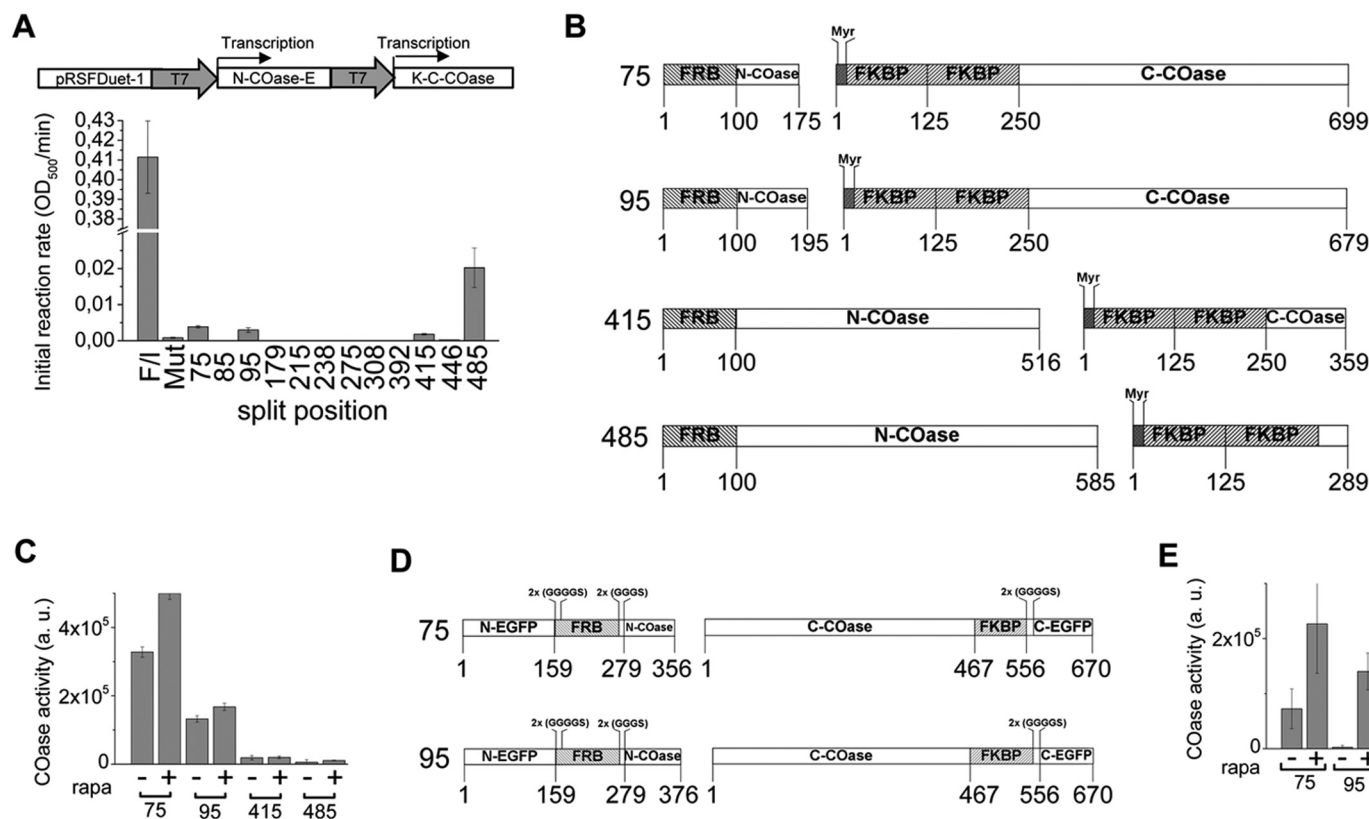


Figure 2. Reconstitution and evaluation of the split COase constructs. A, reconstitution of split COase from E-coil and K-coil fusion constructs expressed in bacteria. Split constructs 75, 95, 415, and 485 had the highest initial reaction rates and were chosen for further evaluation in HeLa cells. F/I, full-length COase; Mut, COase H63A mutant. Error bars, S.D. ($n = 3$). B, schematic diagram of the FRB and FKBP fusion constructs for the splits 75, 95, 415, and 485. C, COase activity of FRB and FKBP fusion constructs expressed in HeLa cells. Only splits 75 and 95 possessed activity. Error bars, S.D. ($n = 3$). a.u., arbitrary units. Schematic diagram (D) and COase activity (E) of rapamycin-inducible split EGFP-COase expressed in HeLa cells. Error bars, S.D. ($n = 3$).

Detection of COase activity in living mammalian cells requires incubation of cells with dichlorofluorescein diacetate, which does not react directly with peroxide but with hydroxyl radicals generated by the Fenton reaction (32). Therefore, dichlorofluorescein diacetate-dependent fluorescence reflects the release of redox-active metals from lysosomes or cytochrome *c* from mitochondria rather than accumulation of peroxide. A better detection method would involve the co-transfection of cells with HyPer peroxidase biosensor (33), but this biosensor is very pH-sensitive, posing another drawback.

To avoid these obstacles, we decided to monitor COase activity by generating chimera of the COase fragments fused to split-halves of a fluorescent protein (FP). Consequently, fusion of the COase fragments would induce fluorescence and allow the detection of cells expressing the active COase by microscopy and cell sorting. Thus, we fused the COase parts with rapamycin-inducible split EGFP constructs (Fig. 2D). The addition of EGFP parts only slightly decreased the enzymatic activity of both 75 and 95 splits. More importantly, the fusion of the EGFP parts to split 95 abolished background activity in the absence of rapamycin (Fig. 2E). This effect might be explained by the lower amount of expressed protein and increased solubility of the chimeric constructs. In conclusion, the split 95 COase fragments (split-EGFP COase) generated the most promising combination to allow efficient control of COase enzymatic activity by rapamycin induction.

To investigate the kinetics of split-EGFP COase reconstitution in living HeLa cells, we induced cells continuously with rapamycin and analyzed them by flow cytometry. Unexpectedly, the EGFP signal was barely detected by flow cytometry and was too dim to be detected by fluorescence microscopy (Fig. 3, A and B). This is explained by the very low amount of reconstituted split-EGFP COase as shown in Fig. 3C.

To increase construct brightness, we replaced parts of EGFP with the corresponding fragments of super-folder GFP (sfGFP) (Fig. 3A), which has been shown to fold very efficiently with a high chromophore formation rate (34). However, a split sfGFP construct gave a strong fluorescent signal even in the absence of rapamycin due to spontaneous self-association of the complementing fragments (Fig. 3B). This self-association was eliminated by using a pair that contained the N-terminal fragment of sfGFP and C-terminal fragment of EGFP (Fig. 3, A and B). This construct, termed split GFP-COase, possessed the same brightness as sfGFP after 4 h of continuous induction but was practically undetectable in the absence of rapamycin (0 h) (Fig. 3, B and C). Both the protein amount of the complemented split GFP-COase chimera and its COase activity increased linearly after induction, reflecting a constant rate of protein synthesis (Fig. 4, A–C). Finally, we compared the enzymatic activity of split GFP-COase with full-length EGFP-COase. The specific activity (ratio of resorufin to GFP fluorescence) of split GFP-COase was about half that of the full-length enzyme (Fig. 4D).

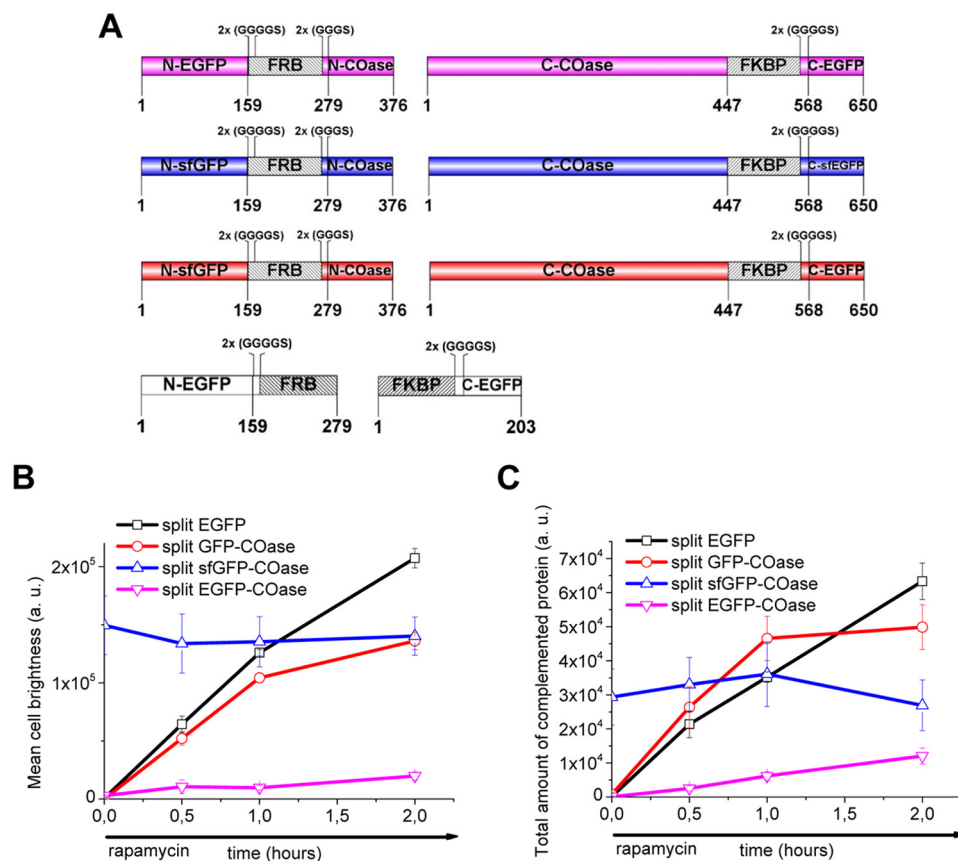


Figure 3. Fluorescent properties of split cholesterol oxidase in cultured cells. A, schematic diagram of constructs. B and C, flow cytometry analysis of transfected HeLa cells incubated continuously with rapamycin. Shown are mean cell brightness (B) and total amount of complemented protein (C). Error bars, S.D. a.u., arbitrary units. ($n = 3$).

Intracellular distribution and activity of split GFP-COase

We next studied the intracellular distribution of split COase in transfected HeLa cells after 2 and 4 h of rapamycin induction. Split GFP-COase was rather uniformly distributed over the cytoplasm and was not detected in the nucleus, in contrast to split EGFP control that was found in both cytoplasm and nucleus (Fig. 5, A and B). The absence of cytoplasmic aggregation suggested correct folding of the protein. Exclusion of split GFP-COase from the nucleus indicated that the chimeric protein exceeds the diffusion limit of nuclear pores.

To determine the amount of oxidized cholesterol, we stained rapamycin-induced cells with filipin. This fluorescent antibiotic detects both plasma membrane and endomembrane pools of cholesterol and allows their quantification in fixed cells (35). Upon the induction of split GFP-COase, filipin largely disappeared from the plasma membrane region within 2 h, whereas intracellular accumulation still remained intense (Fig. 5A). This remaining signal corresponded to ~40% of filipin intensity compared with control cells expressing split EGFP (Fig. 5C). Within 4 h of induction, filipin almost totally disappeared from cells with the highest level of split GFP-COase expression (Fig. 5B), with the average remaining signal being ~20% (Fig. 5C). Because the binding of filipin to residual cholesterol can be blocked by some membrane components (36), we confirmed this finding by biochemical cholesterol determination. We sorted cells expressing split GFP-COase using FACS and extracted lipids and analyzed them by thin layer chromatogra-

phy (TLC). This verified that the cholesterol amount of the COase-expressing cells was about half that in split-EGFP transfected or non-transfected cells after 2 h of rapamycin induction (Fig. 5D; supplemental Fig. S1). To assess if intra-endosomal/lysosomal cholesterol is protected from oxidation, we incubated split GFP-COase-expressing cells with U18666A, an inhibitor of the late endosomal cholesterol transporter NPC1 (37). This resulted in a filipin-staining pattern typical of storage lysosomes (Fig. 5E) and elevated filipin intensity compared with control split GFP-COase-expressing cells (Fig. 5F). This suggests that the enzyme did not access intra-endosomal/lysosomal cholesterol.

Split GFP-COase promotes apoptosis

The accumulation of cholestenone is highly toxic to cells and organisms (38). In most cells lipids are distributed asymmetrically in the plasma membrane, with phosphatidylserine found in the inner leaflet of the plasma membrane (39). Upon induction of apoptosis, phosphatidylserine becomes translocated to the outer surface of the membrane and exposed to annexin V (40). To assess the effect of cholesterol oxidation on phosphatidylserine exposure, we stained split GFP-COase-expressing CHO cells with annexin V and propidium iodide (41). Cells expressing split GFP-COase showed a time-dependent increase in the fraction of annexin V- and propidium iodide-positive cells compared to control split EGFP cells (Fig. 6). These results suggest that COase expression changes membrane asymmetry

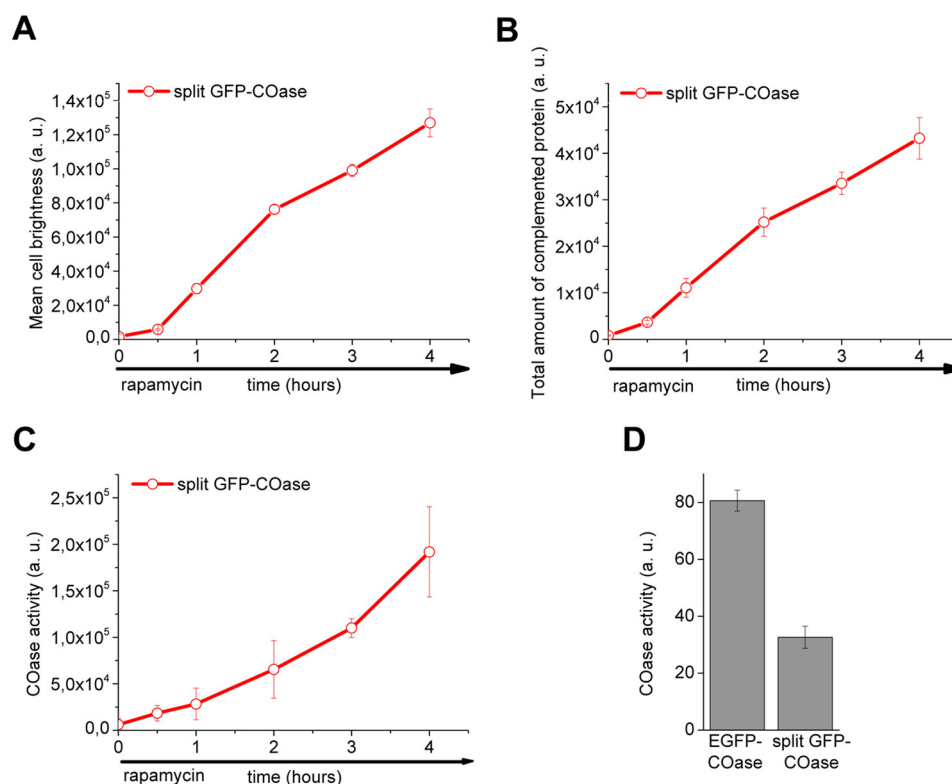


Figure 4. Correlation of green fluorescence with COase activity for split constructs. HeLa cells were transfected for 24 h and induced continuously with rapamycin. *A* and *B*, flow cytometry analysis of HeLa cells. Shown are mean cell brightness (*A*) and total amount of complemented protein (*B*). *Error bars*, S.D. ($n = 3$). *C*, measurement of COase activity in cell extracts. *Error bars*, S.D. ($n = 3$). *D*, comparison of activity of non-split EGFP-COase and split GFP-COase. HeLa cells were transfected for 4 h (EGFP-COase) or induced continuously with rapamycin for 4 h after 24 h transfection (split GFP-COase). Samples were analyzed by flow cytometry, and activity was measured in cell extracts. *Bars* represent COase activity divided by total amount of complemented protein. *Error bars*, S.D. *a.u.*, arbitrary units. ($n = 3$).

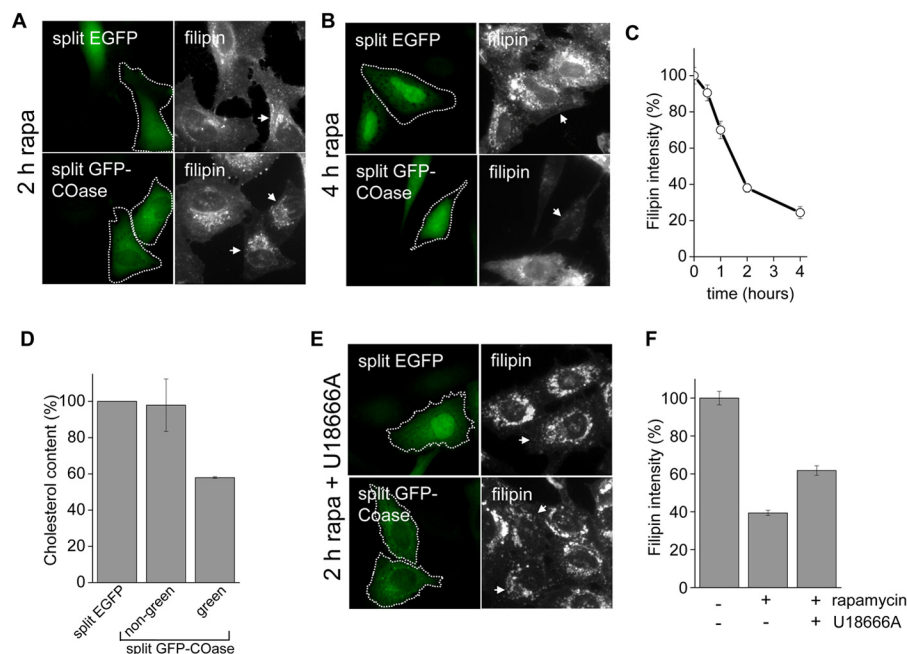


Figure 5. Effect of cholesterol oxidase on cholesterol levels in HeLa cells. *A* and *B*, images of split EGFP or split GFP-COase expressing HeLa cells induced with rapamycin for 2 h (*A*) or 4 h (*B*) and stained with filipin are shown. *C*, quantification of filipin staining for split GFP-COase expressing HeLa cells. A total of 70–136 cells for each time point were analyzed. Data show the mean $\pm$ S.E. for three independent experiments. *D*, measurement of cholesterol content in FACS-sorted split GFP-COase expressing HeLa cells by TLC. Data show the mean $\pm$ S.D. from two independent experiments. *E*, HeLa cells were transfected with split EGFP and split GFP-COase constructs for 16 h in the presence of 2 μ g/ml U18666A and induced for 2 h with rapamycin. *F*, quantification of filipin staining for split GFP-COase expressing HeLa cells with and without U18666A treatment. Intensities were normalized to the value of the non-induced sample (defined as 100%).

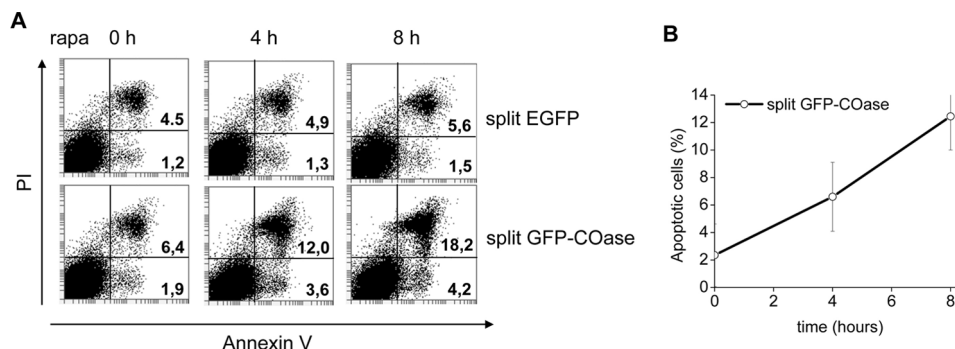


Figure 6. Analysis of apoptosis induced by split GFP-COase. A, CHO cells expressing split EGFP or split GFP-COase were induced with rapamycin (*rapa*), stained with annexin V and propidium iodide, and subjected to flow cytometry analysis. *PI*, propidium iodide. B, quantification of data from three independent experiments. Error bars, S.D. ($n = 3$).

and enhances apoptosis over time. The precise mechanism(s) involved remains to be elucidated, but the rapid flip-flop and membrane-disordering effects of cholestenone (15) may contribute to phosphatidylserine translocation.

Recovery of cellular cholesterol after short induction of split GFP-COase

Analysis of split GFP-COase showed that it rapidly and efficiently reduces cellular cholesterol in a few hours after induction. The longer the COase is active, the more cells become apoptotic. To evaluate the potential recovery of cholesterol levels after a short oxidation by split GFP-COase, we pulsed transfected HeLa cells with rapamycin for 30 min, washed it out, and then continued cultivation in the absence of the drug. The intensity of the green signal of split GFP-COase increased rapidly and linearly reached its maximum at 2 h, followed by an exponential decrease. The calculated half-life time of split GFP-COase complex was 6 h (Fig. 7A). For control cells expressing split EGFP the mean cell brightness was ~5-fold higher than for split GFP-COase-expressing cells (Fig. 7B). In addition, the intensity of the green signal of split EGFP increased and decreased more slowly than of the split GFP-COase. The half-life time of split EGFP was 12 h, *i.e.* double that of split GFP-COase (Fig. 7B).

These findings suggested that the GFP-COase complex is less stable than the EGFP complex, and the formation of the GFP-COase complex might destabilize the enzyme. Consequently, this property might allow cells to restore their cholesterol level once split GFP-COase dissociates and/or is degraded in the cells. To investigate this hypothesis, we followed the cholesterol levels of HeLa cells after short rapamycin induction of the split GFP-COase by filipin staining. We observed a rapid decrease of filipin staining already at 2 h after the rapamycin pulse that correlated with the formation of the GFP-COase complex and the increase in green fluorescence (Fig. 7C). Filipin intensity was minimal between 2 and 6 h. After 6 h, the filipin signal started to gradually recover, reaching half of the initial level at 24 h and a full recovery at 48 h of chase. This corresponded to almost total disappearance of green fluorescence (Fig. 7, A–D). These data indicate that a short pulse of rapamycin causes rapid cholesterol oxidation within few hours, whereas replenishment of cholesterol levels is a slower process. Notably, the transfected cells remained viable until the end of the experiment and restored their cholesterol content (Fig. 7D).

To study the mechanisms by which the cells regain cholesterol, we performed the chase after short split GFP-COase induction in DMEM with 5% lipoprotein-deficient serum (LPDS). Under these conditions, cells rely on endogenous synthesis for their cholesterol supply. We found that in LPDS medium, cells replenished ~75% of the cholesterol based on filipin intensity (Fig. 7C). This implies that cells rely both on *de novo* cholesterol synthesis and lipoprotein cholesterol uptake in regaining cholesterol. We also noted that upon adding statin during the recovery from split GFP-COase expression, most of the cells actually died, implicating the necessity of cholesterol synthesis during recovery. Accordingly, the active cleaved form of the major transcriptional regulator sterol regulatory element-binding protein 2 (SREBP-2) was increased upon split GFP-COase expression (supplemental Figs. S2, A and B). The kinetics of SREBP-2 cleavage indicated that the maximal amount of the active form of SREBP-2 accumulated between 2 and 4 h of split GFP-COase induction (supplemental Fig. S2C). To study whether the cleaved SREBP-2 activates transcription of genes involved in cholesterol metabolism, we analyzed the kinetics of mRNA expression of two representative SREBP-2 gene targets, *i.e.* low density lipoprotein receptor and HMG CoA reductase. We found that the transcripts were up-regulated upon induction of split GFP-COase and SREBP-2 activation (supplemental Fig. S2D), with low density lipoprotein receptor mRNA showing a more substantial effect. The modest up-regulation of HMG CoA reductase mRNA may be accompanied by post transcriptional regulation of the enzyme, contributing to increasing cholesterol synthesis.

Split GFP-COase induces translocation of a cholesterol biosensor

Because filipin staining cannot distinguish between cholesterol residing in the cytosolic *versus* exofacial/luminal leaflets of the plasma membrane, we analyzed the localization of mCherry-D4H cholesterol biosensor. This probe has been reported to recognize cholesterol in the cytosolic leaflet of the plasma membrane and to dissociate upon cholesterol extraction and perturbation of its trafficking (42). Moreover, D4H did not have affinity for cholestenone (supplemental Fig. S3). Consistent with Maekawa *et al.* (42), mCherry-D4H mainly localized at the plasma membrane of CHO cells, whereas in HeLa cells there were also some cytoplasmic mCherry-D4H aggregates (Fig. 8A). When split GFP-COase was induced, we

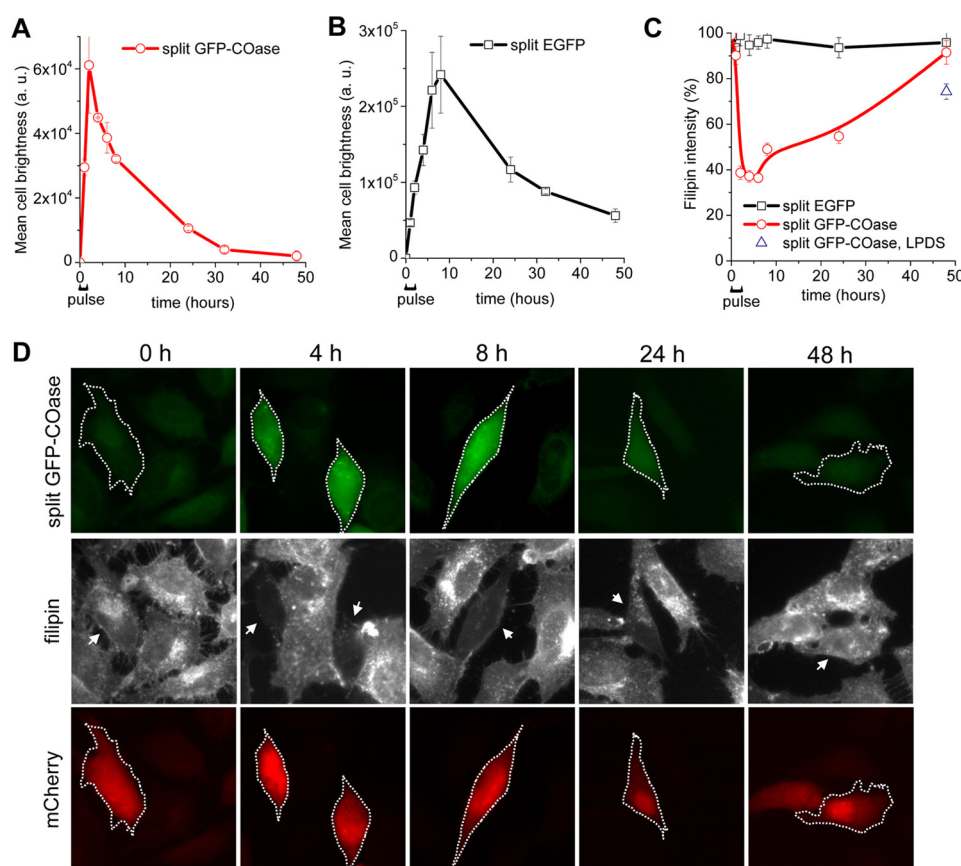


Figure 7. Reduction of split GFP-COase green fluorescence and recovery of cholesterol levels after rapamycin removal. HeLa cells expressing split GFP-COase were induced with rapamycin for 30 min, washed, and incubated in cultured medium for the indicated times. *A* and *B*, flow cytometry analysis of split GFP-COase (*A*)- and split EGFP (*B*)-expressing cells. Error bars, S.D. ($n = 3$). *C*, measurement of filipin intensity in split GFP-COase- and control (split EGFP)-expressing cells. Filipin intensity in split GFP-COase-expressing cells after 48 h of recovery in 5% LPDS medium is shown for comparison. A total of 50–60 cells were analyzed for each time point. Data show the mean $\pm$ S.E. for three independent experiments. Intensities were normalized to the value of the non-induced sample (defined as 100%). *D*, images of split GFP-COase-expressing HeLa cells induced with rapamycin. For imaging, the split GFP encoding plasmids were cotransfected with mCherry to identify transfected cells when GFP expression decreased. a.u., arbitrary units.

observed translocation of mCherry-D4H from the plasma membrane to intracellular aggregates within 8 h in most cells (Fig. 8, *A–C*). After COase induction, the cells gradually changed their morphology and became more rounded. Dissociation of mCherry-D4H suggested that prolonged COase treatment effectively oxidizes cholesterol in the cytoplasmic leaflet of the plasma membrane. This in turn may result in the perturbation of actin cytoskeleton and altered cell morphology.

Discussion

Engineering of split GFP-COase included selection of active split enzyme variants in bacteria followed by their testing and further optimization in mammalian cells to make them inducible and easily detectable. To date, only *Chromobacterium* sp. DS-1 COase demonstrated ultimate stability in organic solvents, detergents, and at high temperatures (30). It has an isoelectric point of 8.6 and possesses high positive electrostatic surface potential that increases its affinity toward negatively charged membranes. Additionally, its activity in mammalian cells is reported (18), providing an additional justification for choosing this enzyme. The best splitting strategy generates separate fragments containing isolated domains that are correctly folded and functional by themselves, as in the classical two-

hybrid system. COase consists of four structural domains (Fig. 1A); however, splitting between them failed to produce active fragments, probably because they were misfolded. Splitting in loops with maximal mobility resulted in several fragments that were active in bacterial extracts (Fig. 2A). This strategy was also successful in generating split ubiquitin, GFP, and DHFR (43–45).

COase contains a large central water filled cavity and a channel that connects it with the surface (13). This channel serves as an entrance for substrate and molecular oxygen to the active site (14). The cavity consists of two oppositely charged areas bound to a cofactor and a substrate. Negatively charged phosphate groups of FAD bind to a positively charged part of the cavity, whereas a cholesterol molecule interacts with a polar part near the channel entry (Fig. 9). The FAD is deeply buried inside the protein structure, and movement in positions 75 and 95 allows cofactor entry into the cavity. Splitting at position 75 (or 95) removes the N-terminal part that covers the cavity together with His-63, which makes a covalent bond with the cofactor. In the remaining C-terminal fragment, FAD is totally exposed to water and cannot reside in COase, whereas a cholesterol-binding pocket remains intact (Fig. 9). Splitting at position 485 also opens a substrate-binding cavity, leaving the FAD

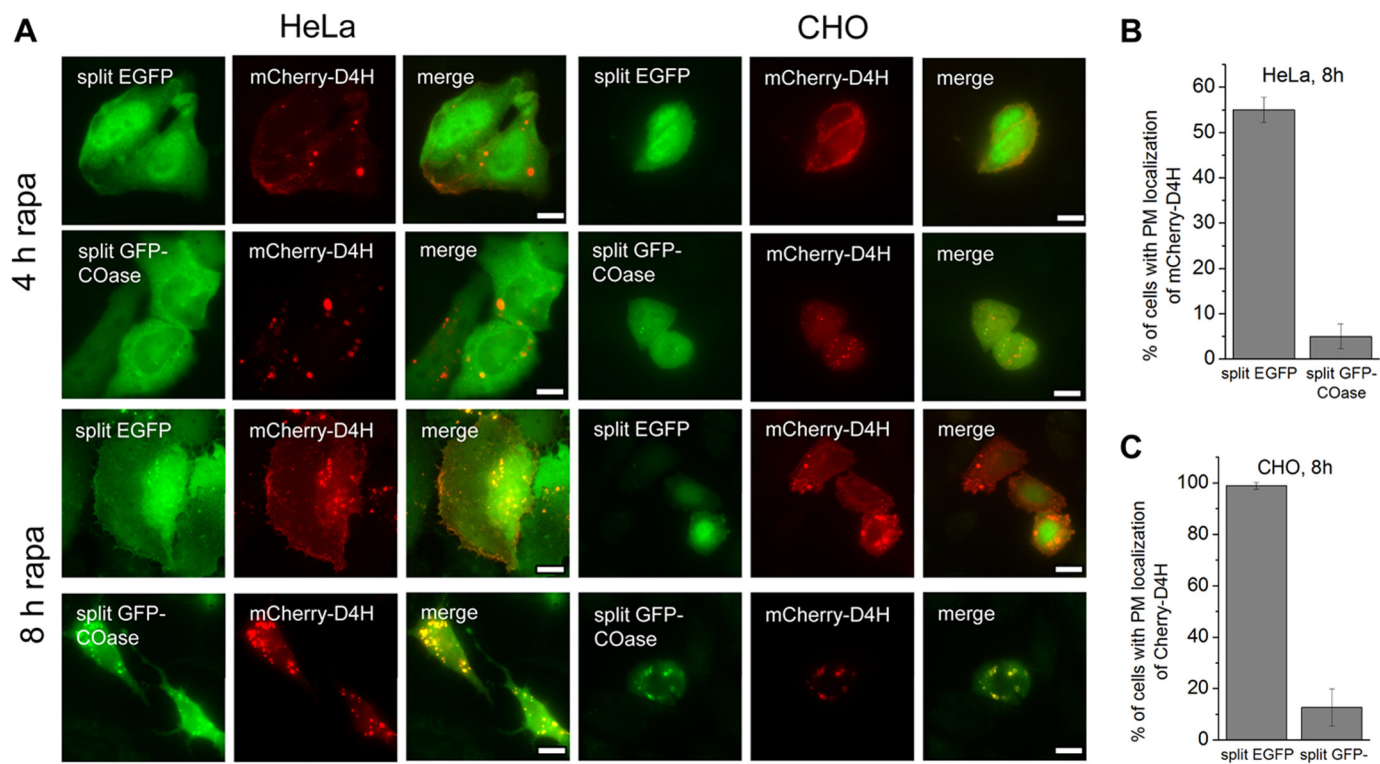


Figure 8. Split GFP-COase oxidizes cholesterol in the cytosolic leaflet of the plasma membrane. A, images of HeLa and CHO cells co-transfected with Cherry-D4H and split EGFP or split GFP-COase and induced continuously with rapamycin (*rapa*). B and C, evaluation of Cherry-D4H localization at the plasma membrane. A total of 100 cells from three independent experiments were analyzed. Data show the mean $\pm$ S.E.

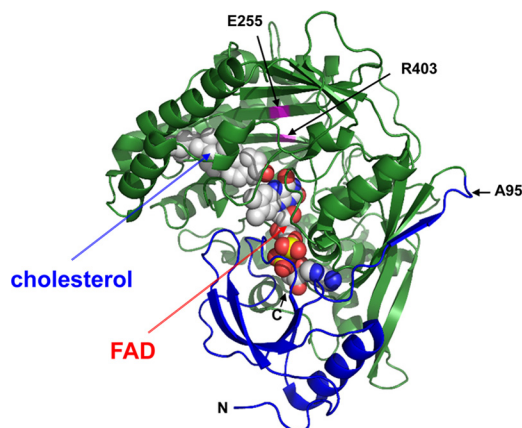


Figure 9. Schematic presentation of split COase structure. Split position 95 is indicated with an arrow, the N-terminal fragment of split COase is shown in blue, and the C-terminal fragment is in green. An FAD molecule was present in the original structure (PDB ID: 3JS8), and a cholesterol molecule was inserted using PyMol software. FAD is located in the center, and cholesterol is located in the upper left corner of the cavity. These two molecules are shown as spheres with carbon in gray, oxygen in red, nitrogen in blue, and phosphorus in yellow. Reacting hydroxyl groups of cholesterol and N5 atom of FAD are indicated in close proximity. Residues Glu-255 and Arg-403, which are located at adjacent β -stands and form the channel, are in magenta.

binding site intact; however, this construct was inactive in mammalian cells, likely due to its low stability.

The addition of rapamycin brings the COase fragments close together, resulting in the formation of the roof of the FAD-binding cavity. In the reconstituted split COase, the FAD must be covalently bound because full-length H63A COase mutant is inactive (Fig. 2A). Typically, the activity of split enzymes is on the order of magnitude lower than full-length proteins. The

activity of split GFP-COase is half of the full-length enzyme, which also indicates formation of stabilizing covalent bond with cofactor (Fig. 4D). The maximum of fluorescence signal for split GFP-COase was obtained at 2 h, that corresponded to the drop of filipin intensity. After that, the fluorescence of split GFP-COase exponentially decreased with a half-time of 6 h, whereas the signal for split EGFP exhibited prolonged growth and twice slower decay (Fig. 7, A and B). This indicates lower stability of split GFP-COase compared with split EGFP. Similar induction and degradation kinetics were observed for split luciferase in live mice after single administration of rapamycin (46).

Perhaps even faster degradation of split GFP-COase could be achieved by fusing them with a destabilizing tag, named FRB* (47). In the FRB*-FKBP system, a half-life of the fusion protein was 2 h, which is 3-fold shorter than for split GFP-COase. The FRB*-tagged protein was continuously degraded, which resulted in a loss-of-function, whereas stabilization that occurred only upon the addition of C20-methylrapamycin (C20-MaRap), an improved analogue of rapamycin. However, the complete stabilization of tagged protein was obtained after 24 h, the time much longer than observed for split GFP-COase activity.

Our system employs two separate vectors encoding the complementing parts. The enzymatic activity of a single fusion protein containing the FRB and FKBP pairs can be significantly higher than that of two separately expressed fragments (48). However, single fusion-based enzymes often demonstrate a high level of background activity in the absence of rapamycin. In our system the fragments of the final split GFP-COase

Table 1
A list of the plasmids designed in this study

Plasmid	Vector backbone	Promoter	Insert
pRSFDuet-N-COase-C-COase	pRSFDuet-1	T7	BamHI-N-COase-Sall-XhoI-C-COase-AvrII
FRB-N-COase	pC4-RHE	CMV	FRB-XbaI-N-COase-SpeI
FKBP-C-COase	pC4M-F2E	CMV	XbaI-2x FKBP-C-COase-SpeI
N-EGFP-FRB-COase	pcDNA3.1+	CMV	NheI-N-EGFP-BamHI-linker-FRB-COase (1–96)-XhoI
C-COase-FKBP-C-EGFP	pcDNA3.1+	CMV	NheI-COase (97–538)-FKBP-BamHI-C-EGFP-XhoI
N-sfGFP-FRB-COase(1–96)	pcDNA3.1+	CMV	N-sfGFP-BamHI-linker-FRB-COase (1–96)-XhoI
C-COase (97–538)-FKBP-C-sfGFP	pcDNA3.1+	CMV	NheI-COase (97–538)-FKBP-BamHI-C-EGFP-XhoI

construct expressed separately show no enzymatic activity, indicating that the affinity of the fragments in the absence of rapamycin is negligible. Therefore, the complemented split GFP-COase provides an easily measurable fluorescent readout that correlates with COase activity.

Recombinant COase has been used to probe the cellular distribution of cholesterol (8). When added to cultured cells extracellularly, purified COase oxidizes only about half of the cholesterol in intact cells because it does not penetrate the cell membrane (8, 17). We found a similar fraction of cholesterol oxidized in HeLa cells expressing split GFP-COase, suggesting that only cholesterol in the inner leaflet of the plasma membrane was attacked by intracellular COase. The split GFP-COase changes membrane organization and produces multiple effects on cell metabolism and architecture. Previous findings have revealed that both cholesterol depletion and peroxide generation are implicated in COase action (17). Cholesterol depletion results in down-regulation of the Akt/PIK3 kinase pathway, whereas elevation of reactive oxygen species up-regulates BAX and increases the amount of released cytochrome *c* (16, 17, 49). Notably, the oxidation product, cholestenone, produces long-lasting defects in actin dynamics and cell mobility (15). Moreover, cholesterol oxidation may indirectly affect cell shape, *e.g.* via proteolysis of cytoskeletal elements induced by apoptotic signaling (50).

Interestingly, expression of the lysosome-targeted COase alleviated oxysterol cytotoxicity (18). Oxysterols accumulate in lysosomes and cause membrane permeabilization and provoke cell death in a concentration-dependent manner. Overexpression of lysosome-targeted COase was found to decrease oxysterol-induced apoptosis, although the exact mechanism of protection is unknown (18). Our observations show that cytoplasmically expressed COase results in cellular cholesterol reduction and plasma membrane cholesterol biosensor translocation.

In summary, we have engineered the cytoplasmic rapamycin-inducible split GFP-COase and characterized it biochemically *in vitro* and functionally in mammalian cells. Short expression of split GFP-COase is reversible and well tolerated by mammalian cells. This new tool allows easy visualization of cells expressing the active chimeric COase using microscopy and flow cytometry and estimation of its enzymatic activity by quantification of the fluorescence signal. In comparison to other experimental methods of cholesterol depletion, split GFP-COase acts more acutely than statins. Moreover, the split GFP-COase acts exclusively from inside the cell, targeting the cytoplasmic leaflet, as compared with cyclodextrins that also act rapidly but target the exoplasmic leaflet. This is also the major difference between split GFP-COase and extracellularly

applied COase. Overall, our findings suggest that intracellular COase is a potent cellular regulator and that the inducible split GFP-COase with improved temporal control of enzyme activity may provide new insights into cholesterol-dependent functions.

Experimental procedures

Design of plasmids

pC4-RHE and pC4M-F2E were obtained from Ariad Pharmaceuticals. Split EGFP plasmids were obtained from S. Gambhir (48). sfGFP fragments were amplified from sfGFP plasmids provided by E. Snapp and by G. Waldo (54). Desired DNA sequences were amplified with primers (Biomers), digested with endonucleases (New England BioLabs) and ligated. Constructs were verified by sequencing. The designed plasmids are summarized in Table 1.

Cholesterol oxidase assay in bacterial cells

Both N- and C-terminal parts of cholesterol oxidase were co-expressed in *Escherichia coli* host BL21(DE3) upon overnight induction with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside. Cells from 20 ml of culture were collected and disrupted in 1 ml of 1× PBS buffer, and 50 μ l of clarified supernatant was added to 1 ml of reaction buffer (50 mM sodium potassium phosphate, 64 mM sodium cholate, 0.34% Triton X-100, 1.4 mM 4-aminoantipyrine, 21 mM phenol, 0.89 mM cholesterol, and 5 units/ml horseradish peroxidase). Development of red color in the assay mixture was tracked by monitoring the absorbance at 500 nm at 24 °C for 30 min.

Cholesterol oxidase assay in HeLa cell lysate

HeLa cells grown in DMEM complete medium supplemented with 1 units/ml of penicillin, 100 μ g/ml streptomycin, and 10% FBS were transfected with split constructs by the calcium phosphate method (51) for 24 h and induced with 5 nM rapamycin for the indicated times. Cells were washed 3 times with PBS, cholesterol oxidase activity was assayed by the addition of reaction buffer (1× PBS, 0.5% Triton X-100, 0.1 mM cholesterol, 20 μ M Amplex Red (fresh batch), 20 units/ml catalase, 1 units/ml horseradish peroxidase), and cells were incubated for 30 min at 37 °C. The addition of catalase to the reaction buffer significantly reduced background signal. Fluorescence of oxidation product resorufin (excitation/emission maxima are at 570/585 nm, respectively) was measured on Victor X3 multilabel plate reader (PerkinElmer Life Sciences). To compare the activity of full-length EGFP-COase with split GFP-COase, resorufin fluorescence was divided by the total amount of green signal.

Split cholesterol oxidase

Filipin staining

HeLa cells grown in complete medium were seeded on glass coverslips and transfected with split GFP-COase or split EGFP constructs by calcium phosphate for 24 h (51) and induced with 5 nM rapamycin for the indicated times. The cells were fixed for 15 min with 4% paraformaldehyde in PBS, quenched with 50 mM NH₄Cl in PBS for 10 min at room temperature, and stained with filipin (500 µg/ml, Sigma) in PBS supplemented with 10% FBS for 30 min at 37 °C. After washing with PBS, the cells were mounted with Mowiol 4–88/Dabco (100 mg/ml), both from Sigma.

Flow cytometry

Flow cytometry analysis of rapamycin-induced split protein complementation was performed using Accuri C6 flow cytometer (BD Biosciences) equipped with a 488-nm laser and a 533/30-nm emission filter. Green cells were gated using a negative control (0.9% of live cells in the gate). Mean fluorescence intensity multiplied by a number of green cells gave an integrated signal in the gate. Each time point was made in triplicate, and minimum of 10⁴ cells were analyzed per sample. The data were analyzed using a CFlow v. 1.0.264.15 and OriginPro v. 8.6 software.

Fluorescence microscopy and quantification of fluorescence intensity

Filipin and GFP were imaged using a Nikon Eclipse Ti-E inverted microscope equipped with a Nikon Intensilight metal arc light source and a 40 × 0.75 NA air objective lens (Plan-Fluor, Nikon). mCherry-D4H imaging was performed using an Olympus IX83 inverted epifluorescence microscope equipped with a 200-watt metal halide arc lamp (Lumen 220PRO, Prior) and a 100 × 1.35 NA oil immersion objective lens (UPlanSApo, Olympus). Filipin and GFP intensities were quantified using Fiji ImageJ software. Regions of interest were drawn manually, and mean fluorescence intensities were measured. Average background mean intensities from two regions outside the cells were subtracted from the values. Samples from at least two independent experiments with 60–120 transfected cells per time point were quantified. All the filipin intensities were normalized to the value of the non-induced sample (defined as 100%).

Purification of GST-D4H

E. coli BL21 (DE3) cells harboring the GST-D4H plasmid were grown in autoinduction medium (6 g/liter Na₂HPO₄, 3 g/liter KH₂PO₄, 10 g/liter NaCl, 20 g/liter Tryptone, 5 g/liter yeast extract, 0.5 g/liter glucose, 2 g/liter lactose, 0.6% glycerol, 100 µg/ml carbenicillin) at 20 °C for 24 h (final A₆₀₀ = 5) (52). Cells were collected by centrifugation and lysed using B-PER (Pierce). GST-D4H was bound to glutathione-agarose (Macherey-Nagel) and eluted with buffer (50 mM Tris-HCl, 5 mM EDTA, 25 mM glutathione). Protein fractions were dialyzed against buffer (25 mM Hepes-KOH, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, pH 7.3).

Liposome preparation

Liposomes with various lipid compositions were prepared by mixing the required amount of lipid stocks and drying them under nitrogen gas and hydrating in the assay buffer (25 mM Hepes-KOH, 100 mM NaCl, 0.5 mM EDTA, pH 7.3) followed by ultrasound sonication.

Quantitative RT-PCR

Total RNA from HEK293 cells was extracted with the Nucleospin RNA extraction kit (Macherey-Nagel), and cDNA was synthesized from total RNA using ProtoScript II first strand cDNA synthesis kit (New England BioLabs). PCR reactions were performed using cDNA, LightCycler 480 SYBR Green I master mix (Roche Applied Science), and the corresponding primers (supplemental Table 1) in a LightCycler 480 system (Roche Applied Science). Samples were heated for 5 min at 95 °C and amplified in 45 cycles of 10 s at 95 °C, 15 s at 64 °C, and 20 s at 72 °C. Analyses were conducted using LightCycler 480 software, and the threshold cycle (CT) was extracted from the PCR amplification plot. The Δ CT values were calculated to evaluate the difference between the CT of a target gene and the CT of the housekeeping gene SP-1, as follows: Δ CT = Δ CT (target gene) – Δ CT (SP-1). mRNA expression level for each data point was normalized to the corresponding level in split EGFP expressing cells. All samples were analyzed in quadruplicate.

Liposome sedimentation assay

GST-D4H protein (0.2 mg/ml) was incubated with 1 mM multilamellar vesicles for 20 min at 24 °C, and the mixture was centrifuged at 100,000 × *g* for 30 min at 20 °C. Supernatants and pellets were analyzed by 12% SDS-PAGE. The lipid compositions (mol %) were as follows: POPE:DOPC, 60:40; POPE:DOPC:cholesterol, 60:20:20; POPE:DOPC:cholestenone, 60:20:20.

mCherry-D4H distribution

HeLa and CHO cells were seeded on glass coverslips and incubated with calcium phosphate-DNA complexes. The mass ratio of split EGFP or split GFP-COase constructs per mCherry-D4H construct with was 10:1. After 4 h of incubation, CHO cells were briefly treated with 1 × PBS supplemented with 20% glycerol, media from both HeLa and CHO were replaced, and cells were allowed to grow for 16 h. Cells were induced with 5 nM rapamycin continuously, fixed with 4% paraformaldehyde, and mounted with UltraCruz mounting medium (Santa Cruz Biotechnology).

Biochemical lipid analysis

Lipids were extracted according to Bligh and Dyer (53) and separated by high performance thin layer chromatography using hexane/diethyl ether/acetic acid (80:20:10) as the running solvent. Lipids were visualized by dipping the TLC plate in 3% CuSO₄, 8% H₂PO₄ and charring. Lipids were identified based on standards, and the signal intensity was quantified using ImageJ software.

Annexin V analysis

CHO cells were transfected with Turbofect (Thermo Scientific) for 24 h on 12-well plates. Expression of chimeric con-

struct was induced with 5 nM rapamycin. Cells were collected, resuspended in 200 μ l of binding buffer (10 mM Hepes-KOH, 150 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 μ g/ml propidium iodide, pH 7.3), and incubated with 1 μ l of annexin V-Cy5 (Abcam) for 5 min. A minimum of 10⁴ cells were analyzed by flow cytometry in red (excitation 488 nm, emission 585/40 nm) and far red (excitation 640 nm, emission 675/25 nm) channels. All experiments were performed in triplicate.

Author contributions—K. G. C., M. N., and I. B. acquired the data. K. G. C., E. I., and V. V. V. analyzed and interpreted the data. K. G. C., E. I., and V. V. V. wrote the manuscript. All authors reviewed the manuscript.

Acknowledgments—This work used the services of the Biomedicum Imaging Unit and Flow Cytometry core facilities of the University of Helsinki. We thank S. Gambhir for the split EGFP plasmids, E. Snapp and G. Waldo for the sfGFP plasmids, G. Fairn for the mCherry-D4H plasmid, N. Doukyu for the COase plasmid, S. Virtanen for the design of initial split COase constructs, S. Pfisterer for help with the experimental design, and Y. Sidorova for the assistance with manuscript preparation.

References

- Ikonen, E. (2008) Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* **9**, 125–138
- Wüstner, D., and Solanko, K. (2015) How cholesterol interacts with proteins and lipids during its intracellular transport. *Biochim. Biophys. Acta* **1848**, 1908–1926
- Lingwood, D., and Simons, K. (2010) Lipid rafts as a membrane-organizing principle. *Science* **327**, 46–50
- Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–39
- Demierre, M. F., Higgins, P. D., Gruber, S. B., Hawk, E., and Lippman, S. M. (2005) Statins and cancer prevention. *Nat. Rev. Cancer* **5**, 930–942
- Börnig, H., and Geyer, G. (1974) Staining of cholesterol with the fluorescent antibiotic “filipin.” *Acta Histochem.* **50**, 110–115
- Zidovetzki, R., and Levitan, I. (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions, and control strategies. *Biochim. Biophys. Acta* **1768**, 1311–1324
- Lange, Y. (1992) Tracking cell cholesterol with cholesterol oxidase. *J. Lipid Res.* **33**, 315–321
- Pollegioni, L., Piubelli, L., and Molla, G. (2009) Cholesterol oxidase: biotechnological applications. *FEBS J.* **276**, 6857–6870
- Vrielink, A., and Ghisla, S. (2009) Cholesterol oxidase: biochemistry and structural features. *FEBS J.* **276**, 6826–6843
- Gadda, G., Wels, G., Pollegioni, L., Zucchelli, S., Ambrosius, D., Pilone, M. S., and Ghisla, S. (1997) Characterization of cholesterol oxidase from *Streptomyces hygroscopicus* and *Brevibacterium sterolicum*. *Eur. J. Biochem.* **250**, 369–376
- Yue, Q. K., Kass, I. J., Sampson, N. S., and Vrielink, A. (1999) Crystal structure determination of cholesterol oxidase from *Streptomyces* and structural characterization of key active site mutants. *Biochemistry* **38**, 4277–4286
- Sagermann, M., Ohtaki, A., Newton, K., and Doukyu, N. (2010) Structural characterization of the organic solvent-stable cholesterol oxidase from *Chromobacterium* sp. DS-1. *J. Struct. Biol.* **170**, 32–40
- Coulombe, R., Yue, K. Q., Ghisla, S., and Vrielink, A. (2001) Oxygen access to the active site of cholesterol oxidase through a narrow channel is gated by an Arg-Glu pair. *J. Biol. Chem.* **276**, 30435–30441
- Neuvonen, M., Manna, M., Morkkila, S., Javanainen, M., Rog, T., Liu, Z., Bittman, R., Vattulainen, I., and Ikonen, E. (2014) Enzymatic oxidation of cholesterol: properties and functional effects of cholestenone in cell membranes. *PLoS ONE* **9**, e103743
- Gniadecki, R. (2004) Depletion of membrane cholesterol causes ligand-independent activation of Fas and apoptosis. *Biochem. Biophys. Res. Commun.* **320**, 165–169
- Liu, J., Xian, G., Li, M., Zhang, Y., Yang, M., Yu, Y., Lv, H., Xuan, S., Lin, Y., and Gao, L. (2014) Cholesterol oxidase from *Bordetella* species promotes irreversible cell apoptosis in lung adenocarcinoma by cholesterol oxidation. *Cell Death Dis.* **5**, e1372
- Mathieu, J. M., Wang, F., Segatori, L., and Alvarez, P. J. (2012) Increased resistance to oxysterol cytotoxicity in fibroblasts transfected with a lysosomally targeted *Chromobacterium* oxidase. *Biotechnol. Bioeng.* **109**, 2409–2415
- Wehr, M. C., and Rossner, M. J. (2016) Split protein biosensor assays in molecular pharmacological studies. *Drug Discov. Today* **21**, 415–429
- Kodama, Y., and Hu, C. D. (2012) Bimolecular fluorescence complementation (BiFC): a 5-year update and future perspectives. *Biotechniques* **53**, 285–298
- Martell, J. D., Yamagata, M., Deerinck, T. J., Phan, S., Kwa, C. G., Ellisman, M. H., Sanes, J. R., and Ting, A. Y. (2016) A split horseradish peroxidase for the detection of intercellular protein-protein interactions and sensitive visualization of synapses. *Nat. Biotechnol.* **34**, 774–780
- Fegan, A., White, B., Carlson, J. C., and Wagner, C. R. (2010) Chemically controlled protein assembly: techniques and applications. *Chem. Rev.* **110**, 3315–3336
- Shekhawat, S. S., and Ghosh, I. (2011) Split-protein systems: beyond binary protein-protein interactions. *Curr. Opin. Chem. Biol.* **15**, 789–797
- Zetsche, B., Volz, S. E., and Zhang, F. (2015) A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nat. Biotechnol.* **33**, 139–142
- Jullien, N., Sampieri, F., Enjalbert, A., and Herman, J. P. (2003) Regulation of Cre recombinase by ligand-induced complementation of inactive fragments. *Nucleic Acids Res.* **31**, e131
- Wehr, M. C., Laage, R., Bolz, U., Fischer, T. M., Grünewald, S., Scheek, S., Bach, A., Nave, K. A., and Rossner, M. J. (2006) Monitoring regulated protein-protein interactions using split TEV. *Nat. Methods* **3**, 985–993
- Pratt, M. R., Schwartz, E. C., and Muir, T. W. (2007) Small-molecule-mediated rescue of protein function by an inducible proteolytic shunt. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 11209–11214
- Gray, D. C., Mahrus, S., and Wells, J. A. (2010) Activation of specific apoptotic caspases with an engineered small-molecule-activated protease. *Cell* **142**, 637–646
- Davis, K. M., Pattanayak, V., Thompson, D. B., Zuris, J. A., and Liu, D. R. (2015) Small molecule-triggered Cas9 protein with improved genome-editing specificity. *Nat. Chem. Biol.* **11**, 316–318
- Doukyu, N., Shibata, K., Ogino, H., and Sagermann, M. (2009) Cloning, sequence analysis, and expression of a gene encoding *Chromobacterium* sp. DS-1 cholesterol oxidase. *Appl. Microbiol. Biotechnol.* **82**, 479–490
- De Crescenzo, G., Litowski, J. R., Hodges, R. S., and O'Connor-McCourt, M. D. (2003) Real-time monitoring of the interactions of two-stranded *de novo* designed coiled-coils: effect of chain length on the kinetic and thermodynamic constants of binding. *Biochemistry* **42**, 1754–1763
- Karlsson, M., Kurz, T., Brunk, U. T., Nilsson, S. E., and Frennsson, C. I. (2010) What does the commonly used DCF test for oxidative stress really show? *The Biochemical journal* **428**, 183–190
- Belousov, V. V., Fradkov, A. F., Lukyanov, K. A., Staroverov, D. B., Shakhbazov, K. S., Terskikh, A. V., and Lukyanov, S. (2006) Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nature methods* **3**, 281–286
- Pédélecq, J. D., Cabantous, S., Tran, T., Terwilliger, T. C., and Waldo, G. S. (2006) Engineering and characterization of a superfolder green fluorescent protein. *Nat. Biotechnol.* **24**, 79–88
- Pörn, M. I., and Slotte, J. P. (1995) Localization of cholesterol in sphingomyelinase-treated fibroblasts. *Biochem. J.* **308**, 269–274
- McGookey, D. J., Fagerberg, K., and Anderson, R. G. (1983) Filipin-cholesterol complexes form in uncoated vesicle membrane derived from

- coated vesicles during receptor-mediated endocytosis of low density lipoprotein. *J. Cell Biol.* **96**, 1273–1278
37. Lu, F., Liang, Q., Abi-Mosleh, L., Das, A., De Brabander, J. K., Goldstein, J. L., and Brown, M. S. (2015) Identification of NPC1 as the target of U18666A, an inhibitor of lysosomal cholesterol export and Ebola infection. *eLife* **4**, e12177
38. Bernheimer, A. W., Robinson, W. G., Linder, R., Mullins, D., Yip, Y. K., Cooper, N. S., Seidman, I., and Uwajima, T. (1987) Toxicity of enzymically-oxidized low-density lipoprotein. *Biochem. Biophys. Res. Commun.* **148**, 260–266
39. Op den Kamp, J. A. (1979) Lipid asymmetry in membranes. *Annu. Rev. Biochem.* **48**, 47–71
40. Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., and van Oers, M. H. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**, 1415–1420
41. Kuypers, F. A., Lewis, R. A., Hua, M., Schott, M. A., Discher, D., Ernst, J. D., and Lubin, B. H. (1996) Detection of altered membrane phospholipid asymmetry in subpopulations of human red blood cells using fluorescently labeled annexin V. *Blood* **87**, 1179–1187
42. Maekawa, M., and Fairn, G. D. (2015) Complementary probes reveal that phosphatidylserine is required for the proper transbilayer distribution of cholesterol. *J. Cell Sci.* **128**, 1422–1433
43. Pelletier, J. N., Campbell-Valois, F. X., and Michnick, S. W. (1998) Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 12141–12146
44. Johnsson, N., and Varshavsky, A. (1994) Split ubiquitin as a sensor of protein interactions *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10340–10344
45. Ghosh, I. H., Hamilton, A. D., and Regan, L. (2000) Antiparallel leucine zipper-directed protein reassembly: application to the green fluorescent protein. *J. Am. Chem. Soc.* **122**, 5658–5659
46. Luker, K. E., Smith, M. C., Luker, G. D., Gammon, S. T., Piwnicka-Worms, H., and Piwnicka-Worms, D. (2004) Kinetics of regulated protein-protein interactions revealed with firefly luciferase complementation imaging in cells and living animals. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 12288–12293
47. Stankunas, K., Bayle, J. H., Gestwicki, J. E., Lin, Y. M., Wandless, T. J., and Crabtree, G. R. (2003) Conditional protein alleles using knockin mice and a chemical inducer of dimerization. *Mol. Cell* **12**, 1615–1624
48. Paulmurugan, R., and Gambhir, S. S. (2005) Novel fusion protein approach for efficient high-throughput screening of small molecule-mediating protein-protein interactions in cells and living animals. *Cancer Res.* **65**, 7413–7420
49. Zhuang, L., Lin, J., Lu, M. L., Solomon, K. R., and Freeman, M. R. (2002) Cholesterol-rich lipid rafts mediate akt-regulated survival in prostate cancer cells. *Cancer Res.* **62**, 2227–2231
50. Desouza, M., Gunning, P. W., and Stehn, J. R. (2012) The actin cytoskeleton as a sensor and mediator of apoptosis. *Bioarchitecture* **2**, 75–87
51. Jordan, M., and Wurm, F. (2004) Transfection of adherent and suspended cells by calcium phosphate. *Methods* **33**, 136–143
52. Studier, F. W. (2014) Stable expression clones and auto-induction for protein production in *E. coli*. *Methods Mol. Biol.* **1091**, 17–32
53. Bligh, E. G., and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917
54. Lajoie, P., and Snapp, E. L. (2013) Detecting soluble polyQ oligomers and investigating their impact on living cells using split-GFP. *Methods Mol. Biol.* **1017**, 229–239

Introducing inducible fluorescent split cholesterol oxidase to mammalian cells
Konstantin G. Chernov, Maarit Neuvonen, Ivonne Brock, Elina Ikonen and Vladislav V. Verkhusha

J. Biol. Chem. 2017, 292:8811-8822.

doi: 10.1074/jbc.M116.761718 originally published online April 7, 2017

Access the most updated version of this article at doi: [10.1074/jbc.M116.761718](https://doi.org/10.1074/jbc.M116.761718)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

Supplemental material:

<http://www.jbc.org/content/suppl/2017/04/07/M116.761718.DC1>

This article cites 54 references, 15 of which can be accessed free at
<http://www.jbc.org/content/292/21/8811.full.html#ref-list-1>