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REVIEW ARTICLE

Lipidated COVID-19 Localizes into Mitochondria and Causes Oxidative Damage to Mitochondrial DNA–Pathophysiology of long COVID

Moragot Chatatikun [1,2†], Hiroko P. Indo [3,4†], Motoki Imai [5–7], Fumitaka Kawakami [5–9], Makoto Kubo [7,10], Takafumi Ichikawa [7,8], Hiroshi Abe [3], Jun Urano [11], Fuyuhiko Tamanoi [12], Warinda Prommachote [1,13], Suriyan Sukati [1,13], Jirapat Namkeaw [14,15], Thiranut Jaroonwitchawan [14,15], Hiroshi Ichikawa [16], Voravuth Somsak [1,2], Jitbanjong Tangpong [1,2], Sachiyo Nomura [17-19] and Hideyuki J. Majima [1,2]

1. School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat, 80161, Thailand
2. Research Excellence Center for Innovation and Health Products (RECIHP), School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat 80160, Thailand
3. Department of Oncology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima City, Kagoshima 890-8544, Japan
4. Amanogawa Galactic Astronomy Research Center (AGARC), Kagoshima University Graduate School of Sciences and Engineering, 1-21-40 Korimoto, Kagoshima 890-0065, Japan
5. Department of Molecular Diagnostics, School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara 252-0373, Japan
6. Department of Applied Tumor Pathology, Graduate School of Medical Sciences, Kitasato University, , 1-15-1 Kitasato, Minami-ku, Sagamihara 252-0374, Japan
7. Regenerative Medicine and Cell Design Research Facility, School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, 252-0373, Japan
8. Department of Regulation Biochemistry, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, 252-0373, Japan
9. Department of Health Administration, School of Allied Health Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, 252-0373, Japan
10. Department of Environmental Microbiology, Graduate School of Medical Sciences, Kitasato University, 1-15-1 Kitasato, Minami-ku, Sagamihara, Kanagawa 252-0373, Japan
11. Cellbre, San Diego, CA 92121, USA
12. Institute for Integrated Cell-Material Sciences, Institute for Advanced Study, Kyoto University Kyoto 606-8501 Japan
13. Hematology and Transfusion Science Research Center (HTSRC), School of Allied Health Sciences, Walailak University, Nakhon Si Thammarat 80160, Thailand
14. Futuristic Science Research Center, School of Science, Walailak University, Thasala, Nakhon Si Thammarat 80160, Thailand
15. Research Center for Theoretical Simulation and Applied Research in Bioscience and Sensing, Walailak University, Thasala, Nakhon Si Thammarat 80160, Thailand
16. Department of Medical Life Systems, Graduate School of Life and Medical Sciences, Doshishia University, Kyoto 610- 0394, Japan
17. Department of Clinical Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo, 142-8501 Japan
18. Isotope Science Center, The University of Tokyo, 2-22-16 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan
19. Department of Gastrointestinal Surgery, The University of Tokyo Hospital, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

† Those authors contributed equally on this paper.
* Corresponding author (HJM)

ABSTRACT

Protein lipidation modifies proteins in eukaryotic cells, including cysteine prenylation, and N-terminal glycine myristoylation, and regulates many biological pathways. We summarize the history of lipidation and the roles of prenylation, myristoylation, and palmitoylation. Lipidation modifies other molecules and takes them to the cellular membrane. Lipidized proteins also go to mitochondria. Prenylation links protein C end motif, and myristoylation links protein N end motif. Palmitoylation links protein C or N motif. It is possible to take both prenylation and myristoylation. Previously, we showed that HSP47 interacts with prenylated and myristoylated proteins, goes to mitochondria, and generates reactive oxygen species (ROS) from mitochondria. It is known that mitochondrial ROS (mtROS) further causes apoptosis and intra-mitochondrial damage to lipids, proteins, and mtDNA. Viral proteins are lipid-modified by infected cells and go to mitochondria, and the electron transport chain (ETC) generates further reactive oxygen species (ROS). The excess ROS may cause lipid peroxidation and damage to mitochondrial DNA (mtDNA). COVID-19, also prenylated and myristoylated, goes to mitochondria, generates mtROS, and damages mtDNA. It is the pathophysiology of long COVID.

Keywords: mitochondria; lipidation; prenylation; myristoylation, palmitoylation; virus; HSP47

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Introduction

Life appeared on Earth 3.8 billion years ago, when the atmosphere had almost no oxygen [1,2]. A membrane surrounds cells, the components of which include lipids, and then cells can maintain their circumstances. Life has evolved to have organelles to share their functions inside cells. Cells must import many substances inside them. Stein described the detailed systems of cell membrane transport and diffusion [3]. Most large molecules can't cross cell membranes without their carriers. Reactive oxygen species (ROS) without electric charge across the membrane [4–7].

Oxidative phosphorylation produces ATPs via the electron transport chain (ETC), which is localized in the inner membrane of mitochondria. However, this biological machine is imperfect, and 2 – 3% of electrons leak during electron transportation. Oxygen molecules react with those electrons, producing superoxide anion ($O_2^{\bullet-}$) in mitochondria, and $O_2^{\bullet-}$ produces several different ROS. The authors demonstrated that mitochondrial ROS (mtROS) caused apoptosis for the first time [8] and showed that mtROS initiates intracellular signals [5–7,9].

Heat shock protein-47 (HSP47) is present in the endoplasmic reticulum of cells and performs several functions regarding cartilage and bone formation, autophagy, and fibrosis [10,11]. Post-translational modifications (PTMs) have their roles in protein and cellular signaling [12]. Previously, we reported electron and X-ray irradiation can induce different changes in the protein via PTMs that lead to significant physiological and pathophysiological effects in the cells and tissues [13]. We found that lipidated, farnesylated, and myristoylated HSP47 went to mitochondria and generated reactive oxygen species from mitochondria [13]. We have shown mitochondria as the target of lipidation.

In this review, we describe the history of lipidation, the roles of prenylation, myristoylation, and palmitoylation, mitochondria as the target of protein lipidation, and viruses. We focus a new role on mitochondria as the target of viral lipidation. Then, the excess ROS generated from mitochondria may cause lipid peroxidation and further damage mitochondrial DNA (mtDNA). These mtDNA damages will cause 'long COVID'.

Unique properties of protein lipidation

HISTORY OF LIPIDATION

A lipid membrane surrounds the cell, shielding it from its environment [3]. The membrane also functions to communicate with the exterior environment. The lipid components involved in this communication must be close to the hydrophobic cell membrane. However, most proteins are water-soluble. Protein lipidation, such as prenylation, myristylation, and palmitoylation, overcomes this obstacle.

Myristate and palmitate represent the most common fatty acid modifying groups. Each fatty acid manages distinct biochemical properties that regulate intracellular trafficking, subcellular localization, protein-protein, and protein-lipid interactions [14]. Over the past two decades, protein S-acylation (S-palmitoylation) has emerged as an essential regulator of crucial signaling pathways. S-acylation is a reversible post-translational modification involving a fatty acid attachment to a cysteine residue of proteins through a thioester bond. The balance of protein S-acylation and deacylation has profoundly affected various cellular processes, including innate immunity, inflammation, glucose metabolism, and fat metabolism [15]. Defects of prenylation promote the activation of inflammation and induce a dysfunction of mitochondrial activity and oxidative stress [16]. Genetic and pharmacological manipulation of protein prenylation has provided insights into several cellular processes and the etiology of diseases involved in prenylation, such as interference of RAS protein prenylation and cancer[17].

Protein lipidation, referring to lipid attachment to proteins, is prominent and occurs in many proteins in eukaryotic cells and regulates numerous biological pathways [18]. Protein lipidation is a significant co- or post-translational modification that markedly increases the hydrophobicity of proteins, resulting in changes to their conformation, stability, membrane association, localization, trafficking, and binding affinity to their co-factors [19]. These modifications increase proteins' complexity and functional diversity in response to complex external stimuli and internal changes. Protein lipidations primarily encompass five types: S-prenylation, N-myristoylation, S-palmitoylation, glycosylphosphatidylinositol (GPI) anchor, and cholesterylation. Yuan et al. described the timeline of protein lipidation research, regulatory enzymes, important protein substrates, clinical trials, and the development of inhibitors. They described the importance of these modifications for protein regulation, cell signaling, and diseases [20]. They illustrated the timeline of protein lipidation research, including regulatory enzymes, important protein substrates, clinical trials, and the development of inhibitors. Proteins can be modified by at least seven types of lipids, including fatty acids, lipoic acids, isoprenoids, sterols, phospholipids, glycosylphosphatidylinositol (GPI) anchors, and lipid-derived electrophiles (LDEs) [19,21]. Numerous genetic, structural, and biomedical studies have consistently shown that protein lipidation is pivotal in regulating diverse physiological functions and is inextricably linked to various diseases [21]. Furthermore, cellular lipid metabolism affects the availability of fatty acyl-CoA and other lipid derivatives used as substrates for protein lipidation. The 16-carbon fatty acid palmitate, is a critical intermediate for the biosynthesis of other cell lipids [19].

Roles of Prenylation, myristoylation, and palmitoylation

PRENYLATION

Roles of prenylation

Prenylation, the modification of eukaryotic proteins by isoprenoid lipids, controls the localization and activity of a range of proteins with critical functions. Protein prenylation is a ubiquitous covalent post-translational modification found in all eukaryotic cells, and the roles of prenylated proteins are well conserved across species. Wang and Casey highlighted this lipid modification pathway's biological and evolutionary importance [17]. Protein prenylation is a ubiquitous covalent post-

translational modification found in all eukaryotic cells. It comprises the attachment of either a farnesyl or a geranylgeranyl isoprenoid. Prenylation is essential for the proper cellular activity of numerous proteins, including Ras family GTPases and heterotrimeric G-proteins. Inhibition of prenylation has been extensively investigated to suppress the activity of oncogenic Ras proteins to achieve antitumor activity [22].

Wang and Casey described the roles of prenylation on aging [17]. There is considerable interest in targeting protein prenylation in premature aging disorders and understanding its role in normal aging. The final maturation step involves removing the C-terminal 15-amino-acid peptide by the endoprotease ZMPSTE24, which is dependent on prenylation. The most well-studied form of the premature aging disorder, Hutchinson–Gilford progeria syndrome (HGPS), is caused by a genetic mutation of prelamin A that leads to alternative splicing and the consequent loss of the ZMPSTE24-recognition site [23]. A more severe form of progeria is caused by a mutation resulting in the loss of ZMPSTE24 protease activity. In all forms of progeria, the resulting incompletely processed protein, termed progerin, accumulates on the nuclear envelope, triggering a range of molecular disturbances that lead to premature aging. Interestingly, the treatment of farnesyltransferase inhibitors (FTIs) reverses the phenotypical abnormalities in progeroid cells [24,25], and the knock-in of a form of prelamin A that cannot be farnesylated in mice with the genetic background of progeria yielded relatively average survival [26]. These studies led to a clinical trial using FTIs to treat progeroid syndromes. Although FTIs had only a modest effect on disease progression [27], the results prompted an ongoing trial using a combination of FTI with a statin and bisphosphonate compounds that affect the farnesyl biosynthesis pathway, and early data have shown that the triple-drug combination can reduce the multi-organ phenotype of aging and significantly prolong survival. Progress has also been made in investigating the effects of targeting isoprenylcysteine carboxymethyltransferase (ICMT), the enzyme that carries out the terminal carboxyl methylation after prenylation; introduction of a hypomorphic allele of ICMT into the Zmpste24 −/− mouse model of progeria largely rescued both the premature aging phenotype and premature death [17].

Discovery of protein prenyl groups and structural varieties

The posttranslational modification of proteins with lipid moieties, known as protein lipidation, was first recognized in the late 1970s and the beginning of 1980s [28]. The discovery of protein S-prenylation was in 1978, S-palmitoylation was in 1979, and protein N-myristylation was in 1982 [29]. The first reports of prenylated proteins and peptides described the secreted pheromone peptides from yeasts [30,31]. Sakagami et al. isolated a novel sex hormone tremerogen A-10 that controlled conjugation tube formation in *Tremella mesenterica* [32,33]. Protein prenylation was also described by Kamiya et al. [34] and Ishibashi et al. [35].

Protein prenylation: C-terminal motifs

Protein prenylation involves the transfer of either a farnesyl or a geranylgeranyl moiety to C-terminal cysteine(s) of the target protein [36]. The CaaX and C-seven motifs were found as C-terminal motifs for the prenylation [37]. The CaaX motif consists of a cysteine (C), two aliphatic amino acids ("aa"), and if X position is serine, alanine, or methionine, the protein is farnesylated, or if X is a leucine, the protein is geranylgeranylated [38]. The second motif for prenylation is CXC, which, in the Ras-related protein Rab3A, leads to geranylgeranylation on both cysteine residues and methyl esterification [38]. The third motif, CC, is also found in Rab proteins, where it appears to direct only geranylgeranylation but not carboxyl methylation [38]. Carboxyl methylation only occurs on prenylated proteins [38]. Prenylation includes dimethylallylation (C5), geranylation (C10), farnesylation (C15), and geranylgeranylation (C20), depending on the number of isoprenyl (C) motifs [39] (Table 1).

Myristoylation

Myristoylation is the covalent attachment of a 14-carbon myristoyl group to the alpha-amino group of an N-terminal glycine residue via an amide bond [40]. Myristoylation was first described in 1967 by Ochoa-Solano, Romero, and Gitler, who showed histidine lipidation by myristoylation [41].

N-myristoylation can occur both post-translationally and co-translationally. Co-translational N-myristoylation can influence the transport and localization of the protein, which likely affects the protein's function [29]. Yuan et al. highlight N-myristoylation's numerous roles in normal cell physiology and human diseases like cancer and infection. Furthermore, inhibitors of N-myristoylation are being investigated to treat these diseases [29].

Palmitoylation

Palmitoylation, which modifies a protein with a 16-carbon palmitate, was discovered in the 1960s. Three types of palmitoylation have been described: S-palmitoylation, N-palmitoylation, and O-palmitoylation [21] (Table 1).

S-palmitoylation

S-palmitoylation is the reversible attachment of palmitic acid via a thioester bond on cysteine residues. Palmitoylation is carried out by palmitoyl acyltransferases (PATs, also known as ZDHHC-PATs) and acyl protein thioesterases (APTs) removes the palmitoyl group. PATs have a conserved cysteine-rich zinc-finger Asp-His-His-Cys (ZDHHC) motif in the catalytic domain and is essential for activity [21]. Examples of proteins that are S-palmitoylated include the proto-oncogene GTPase NRas, cell surface death receptor Fas, apoptosis regulator BCL2-associated X (BAX), and the proto-oncogene non-receptor tyrosine kinase Src [21].

N-palmitoylation

The attachment of palmitic acid via an amide bond to the amino group of the N-terminal residue of a protein is N-palmitoylation. Attachment of palmitic acid can also occur

on the ε-amino group of a lysine residue at the N-terminus, referred to as N-palmitoylation. An example of N-palmitoylation is human Sonic Hedgehog (SHh), which is palmitoylated on the amino group of the N-terminal cysteine residue by Hedgehog acyltransferase (HHAT) [21].

O-palmitoylation

In O-palmitoylation, palmitic acid is irreversibly linked to the side chain of serine residues through an ester bond. Only a handful of proteins have been reported to be O-palmitoylated. One example is histone H4, which is O-palmitoylated at Ser-45 by lysophosphatidylcholine acyltransferase 1 (LPCAT1) [42]. In addition, the blocking of presynaptic voltage-gated Ca^{2+} channels by the spider venom PLTX-II is thought to be mediated by O-palmitoylation at a threonine residue in the C-terminal region of the neurotoxin [21].

Mitochondria as the target of protein lipidation

Mitochondria comprise 1,000 to 1,500 proteins [43]. The protein import machinery of the mitochondrial membranes and aqueous compartments reveals a remarkable variability of protein recognition, translocation, and sorting mechanisms [44]. The electron transport chain (ETC) is in the inner membrane, comprises nearly 100 proteins, and should be appropriately brought to ETC [45]. Further, many proteins go outside and inside mitochondria to maintain homeostasis against physiological and pathological interference [46]. Mitochondrial lipids modulate protein import on various levels involving precursor targeting, membrane potential generation, stability, and activity of protein translocases [47]. To reach mitochondria, proteins should be modified with lipidation [48].

Boveris and Navarro described reduced mitochondrial biogenesis as correlated with aging [42]. Archaea use prenylated hemes as cofactors of cytochrome oxidases [21,49]. In mitochondria, many components are prenylated, myristoylated, and palmitoylated; many are related to aging and diseases. For example, S-palmitoylation of Bax [50], and myristoylation of AMP-activated protein kinase (AMPK) [51]. Kostiuk et al. identified 21 putative palmitoylated proteins in the rat liver mitochondrial matrix [52]. Simvastatin treatment inhibits the biosynthesis of mevalonate, causing cytochrome c to be released from the mitochondria to the cytosol. This effect is associated with activation of caspase 9 and 3 during apoptosis. This effect was reversed by adding mevalonate, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), suggesting protein prenylation's involvement in the apoptosis mechanisms [53].

It is known that some lipid-modified proteins go to mitochondria. Zha et al. demonstrated that Bid is N-myristoylated, goes to mitochondria and cause apoptosis [54]. Utsumi et al. (2003) demonstrated that the C-terminal 15 kDa fragment of cytoskeletal actin is targeted to mitochondria via posttranslational N-myristoylation during apoptosis [55]. Bivona et al. demonstrated that K-Ras, when phosphorylated by protein kinase C (PKC), rapidly dissociates from the plasma membrane and, through association with Bcl-XL on the outer mitochondria membrane, induces apoptosis [56]. Beauchamp et al. demonstrated that N-myristoylation of dihydroceramide Delta4-desaturase 1 (DES1) results in targeting DES1 to the mitochondria, leading to an increase in ceramide levels, which contributes to the apoptosis effect of myristic acid, in COS-7 cells [57]. Lynes et al. demonstrated that palmitoylation of calnexin serves to enrich calnexin on the mitochondria-associated membrane (MAM) [58]. Palmitoylated calnexin interacts with sarcoendoplasmic reticulum and Ca^{2+} transport ATPase (SERCA) 2b, and this interaction determines ER Ca^{2+} content and the regulation of ER-mitochondria Ca^{2+} crosstalk. AMP-activated protein kinase (AMPK) plays a central role in cellular energy sensing and bioenergetics. Liang et al. demonstrated that N-myristoylation of AMPKβ by the type-I N-myristoyl transferase 1 (NMT1) confers the recruitment of AMPK to the mitochondria [51]. Bhat et al. showed that FBXO10, the interchangeable component of the cullin-RING-ligase 1 complex, is geranylgeranylated and localizes to the outer mitochondrial membrane [59]. These examples show that many lipid-modified proteins localize to the mitochondria and perform various critical functions.

Coenzyme Q (CoQ) is not protein, but CoQ or ubiquinone is a crucial lipid component of the electron transport chain and is required for ATP generation in mitochondria. CoQ is the generic name of a class of lipid-soluble electron carriers formed of a redox-active benzoquinone ring attached to a prenyl side chain [60]. Mutations in CoQ biosynthetic genes are associated with rare but ROS infantile multisystemic diseases [61]. CoQ10 supplementation rescues cerebellar ataxia with CoQ10 deficiency [62]. CoQ10 supplementation rescues renal disease in Pdss2kd/kd mice with mutations in prenyl diphosphate synthase subunit 2 [63].

Protein lipidation: Where do they go, the cellular membrane or mitochondria?

Jiang et al. published a review of protein lipidation [18]. They described that protein lipidation, cysteine prenylation, N-terminal glycine myristoylation, cysteine palmitoylation, and serine and lysine fatty acylation occur on many proteins in eukaryotic cells. The lipid-modified proteins go to cellular membranes and regulate many biological pathways, such as membrane trafficking, protein secretion, signal transduction, and apoptosis [18].

Jiang et al. posed several interesting questions: how can the same modification target different proteins to different organelles, e.g., N-terminal glycine myristoylation targets specific proteins to the mitochondria and others to the plasma membrane[18]. Do lipid modification and its local environment have an intrinsic affinity for different membranes, or are different trafficking machinery engaged by the modified proteins. The difficulty might be the situation's complexity, e.g., the existence of different trafficking machinery [18]. Table 1 shows classifications of fatty acylation and their protein-modification sites. The modified residue of prenylation is Cys (C) in CaaX, CXC, and CC motif of C-terminal protein (Table 1) [39,40]. At the same time, those of myristoylation is Gly (G), and Lys (K) of N-terminal protein, those of S-palmitoylation are Cys (C) of C-terminal protein, those of N-palmitoylation, O-palmitoylation are Lys (K) of C-terminal protein and Ser (S), Thr (T) of C-terminal protein, respectively. The modified residue of N-terminal

palmitoylation is Cys (C) of N-terminal protein (Table 1) [12,19]. C- and N-terminal modifications may quickly occur in cells and go to mitochondria (Figure 1).

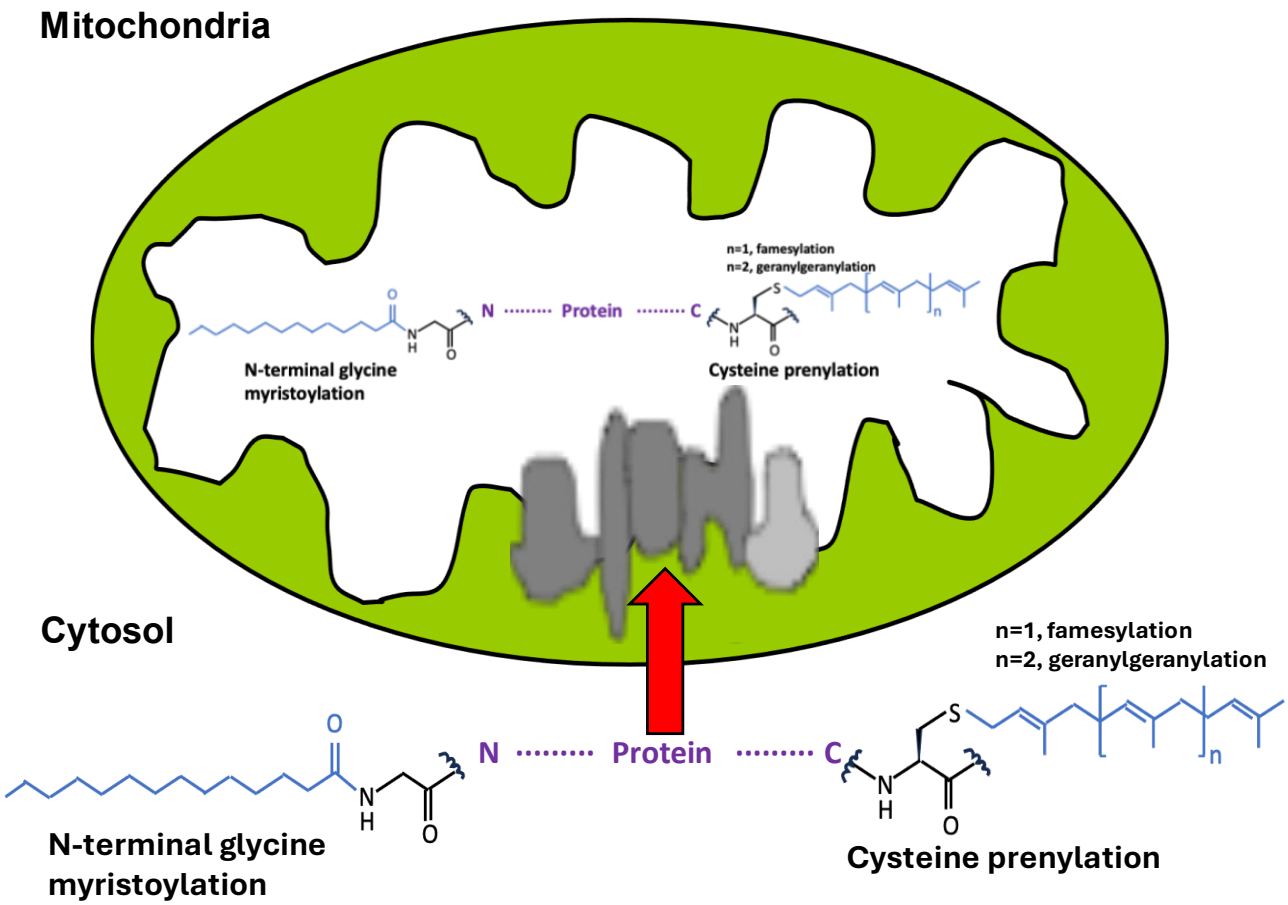
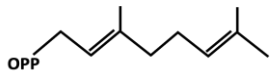
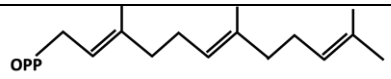
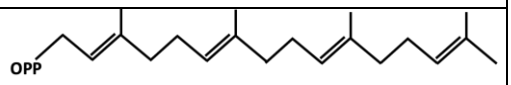
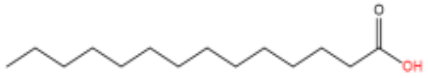
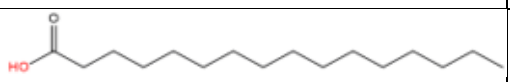


Figure 1: Prenylation binds to the C-terminal end, while myristoylation binds to the N-terminal end of proteins. They go to mitochondria, bind to ETC (Gray color), produce ROS, and further cause apoptosis or damage to mtDNA.

Table 1: Fatty Acylation of Proteins-modification sites. Classifications of fatty acylation, their structure formula, proteins-modification sites, and fatty acid transferase.

Types of lipidation		**Structure formula**	**Modified Residue**	**Fatty acyl Transferase**	**References**
Prenylation	geranylation (C10)		Cys (C) in CaaX, CXC, and CC motif of **C-terminal** protein	Prenyltransferases	[39, 40]
	farnesylation (C15)				
	geranylgeranylation (C20)				
Myristoylation			Gly (G), Lys (K) of **N-terminal** protein	N-myristoyl transferase (NMT)	[12]
Palmitoylation	S-palmitoylation		Cys (C) of **C-terminal** protein	Palmitoyl acyltransferases (PATs, also known as ZDHHC-PATs) acyl protein thioesterases (APTs), and the conserved zinc-finger Asp-His-His-Cys (ZDHHC) motif	[19]
	N-palmitoylation		Lys (K) of **C-terminal** protein		
	O-palmitoylation		Ser (S), Thr (T) of **C-terminal** protein		

Virus, lipidation and mitochondria

Viral proteins are also substrates for prenylation; viral proteins can be post-translationally modified by protein lipidation mechanisms of their host cells [64]. Some inhibitors of prenylation exhibit potent antiviral activities [65].

Viral proteins containing the C-terminal CaaX motif can be prenylated by host prenyltransferases. A clinically relevant example is the large antigen of the hepatitis delta virus. Glenn et al. first demonstrated the evidence for the role of prenylation in viral replication [66]. During replication, the hepatitis delta virus (HDV) switches from producing small to large delta antigens. Both antigen isoforms have an HDV genome binding domain and are packaged into hepatitis B virus (HBV)-derived envelopes but differ at their carboxy termini. The large antigen was shown to contain a terminal CXXX box and undergo prenylation [66]. Bordier et al. found that prenylation inhibitors prevent infectious hepatitis delta virus particles [67] and found in vivo antiviral activity against hepatitis delta virus [67]. The phase 2A clinical trial results showed that the prenylation inhibitor lonafarnib significantly reduces hepatitis delta virus levels [65].

After infection with SARS-CoV-2, virus-derived proteins are produced inside the host cell [68]. Zhou et al. described protein-protein interactions that comprise the SARS-CoV-2 and human protein interactome using high-throughput two-hybrid experiments and mass spectrometry [69]. Viral proteins were found to be lipid-modified, and some viral proteins will be localized to the mitochondria. ROS are generated once the virus localizes in mitochondria, damaging mitochondrial DNA (mtDNA). Then, mtDNA will be more damaged and generate more ROS for an extended period. This will be a pathophysiology of "long COVID" (Figure 2).

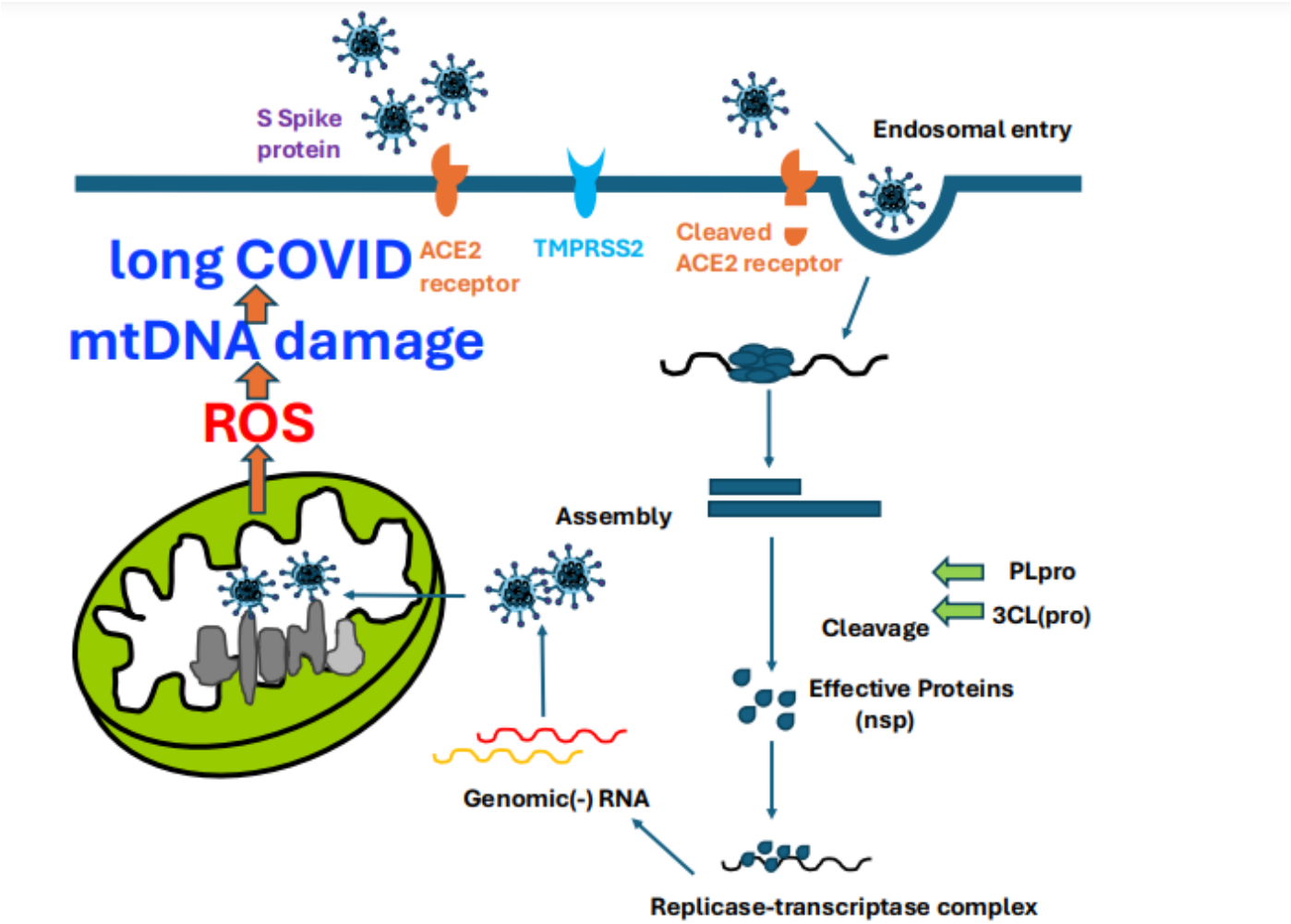
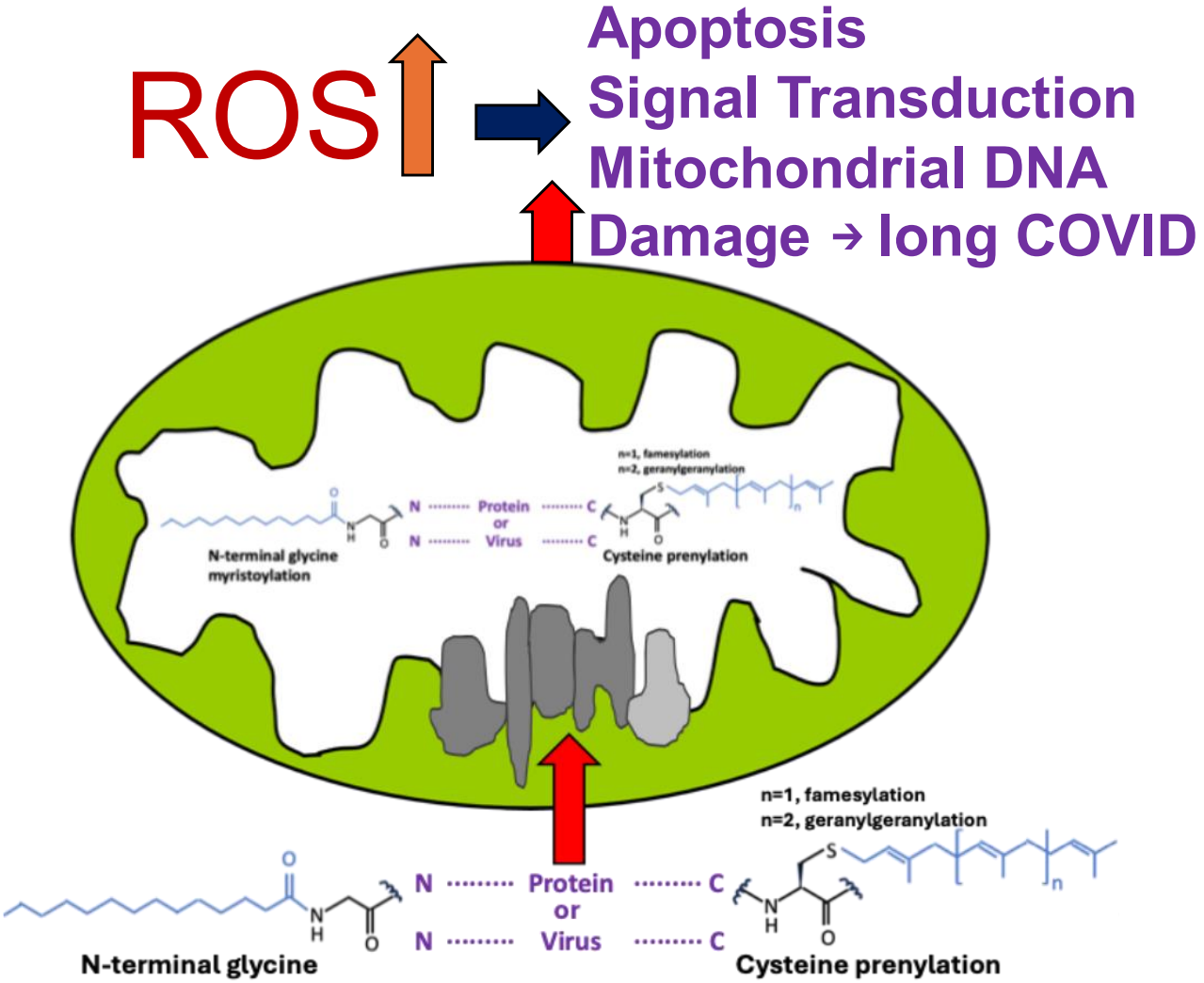


Figure 2: Prenylation binds to the C-terminal end, while myristoylation binds to the N-terminal end of viral protein. They go to mitochondria, bind to ETC, produce ROS, and further cause apoptosis or damage to mtDNA.



Graphic Figure Legends: Prenylation binds to the C-terminal end, while myristoylation binds to the N-terminal end of proteins and virus-translated proteins. They go to mitochondria, bind to ETC, produce ROS, and further cause apoptosis or damage to mtDNA.

8. Conclusion

Protein lipidation, including prenylation, myristoylation, and palmitoylation, targets proteins to various cellular membranes. Mitochondria, which have two membranes, are also target of protein lipidation. Previously, we showed that HSP47 is associated with prenylation and myristoylation, goes to mitochondria, and generates mtROS. It is known that mtROS further causes apoptosis and intra-mitochondrial damage to lipids, proteins, and mtDNA. Viral proteins are also modified by protein lipidation by the prenylation and myristoylation in host cells and localize to mitochondria, generating excess ROS and mitochondrial damage, including mtDNA. This is a story of 'long COVID'.

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