

NOVEL CERAMIDE COMPOUND, CANDIDATE FOR HEAD AND NECK CANCER  
TREATMENT, INDUCES MITOPHAGY THROUGH CERAMIDE SIGNALING



by

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## **ABSTRACT**

### **NOVEL CERAMIDE COMPOUND, CANDIDATE FOR HEAD AND NECK CANCER TREATMENT, INDUCES MITOPHAGY THROUGH CERAMIDE SIGNALING**

Ceramide is a bioactive sphingolipid that is involved in signaling pathways regulating cancer cell death and tumor growth. In several types of cells, the ceramide synthesis or accumulation upon cellular stress is known to bring about one of the ceramide-induced cancer cell death mechanism; lethal mitophagy. Mitochondria-targeted ceramide analogs constitute one of the anticancer strategies that are applied to induce lethal mitophagy in cancer cells. In this study, we revealed that LCL768, a new pyridinium ceramide analog conjugated with selenium, induced lethal mitophagy in Head and Neck Squamous Cell Carcinoma. Mechanistically, LCL768 was implicated for Drp1 activation through nitrosylation at C644. Also, the activation of Drp1 regulates CerS1 trafficking to mitochondria for CerS1/C18-ceramide mediated signaling. Besides, our data demonstrated that LCL768 along with endogenous C18-ceramide accumulated mainly in mitochondria. This accumulation of LCL768 significantly impaired the mitochondrial functions in HNSCC. Moreover, LCL768 resulted in ceramide-mediated growth inhibition in xenograft-derived tumors and also mitophagy for both xenograft-derived tumors and 3D patient organoids. Overall, this study reveals a novel mechanism for LCL768 in the regulation of ceramide-mediated mitophagy in HNSCC.

## ÖZET

### **BAŞ VE BOYUN KANSERİ TEDAVİSİNDE YENİLİKÇİ SERAMİD BİLEŞİĞİ ADAYI, SERAMİD SİNYAL YOLAĞI İLE MİTOFAJİ TETİKLENMESİ**

Seramid, kanser hücresi ölümünü ve tümör büyümesini düzenleyen sinyal yollarında yer alan biyoaktif bir sfingolipiddir. Çeşitli hücre tiplerinde, hücrel strese yanıt olarak seramid sentezi veya birikiminin, seramidin neden olduğu kanser hücresi ölüm mekanizmalarından birini indüklediği bilinmektedir; ölümcül mitofaji. Mitokondri hedefli seramid analogları, kanser hücrelerinde indükletal mitofajiye uygulanan antikanser stratejilerinden birini oluşturur. Bu çalışmada, selenyum LCL768 ile konjuge edilmiş yeni bir piridinyum seramid analogu olan LCL768'in HNSCC'de ölümcül mitofajiye neden olduğunu ortaya koyduk. Mekanik olarak, verilerimiz LCL768'in C644'te nitrosilasyon yoluyla Drp1 aktivasyonunu indüklediğini gösterdi. Ayrıca, Drp1 aktivasyonu, CerS1/C18-seramid aracılı seramid sinyalleme için CerS1 trafiğini mitokondriye düzenledi. Ayrıca, verilerimiz LCL768'in esas olarak mitokondride biriktiğini ve endojen C18-seramidin mitokondriyal birikimini indüklediğini göstermiştir. Önemli olarak, LCL768, HNSCC'de mitokondriyal fonksiyonu azaltır. Ayrıca, LCL768, ksenograft türevli tümörlerde seramid aracılı büyüme inhibisyonunu ve hem ksenograft türevli tümörlerde hem de 3D hasta organoidlerinde seramid aracılı mitofajiyi indükledi. Bu nedenle, bu çalışma, HNSCC'de seramid aracılı mitofajiyi düzenleyen yeni bir LCL768 mekanizmasını ortaya koymaktadır.

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**LIST OF SYMBOLS / ABBREVIATIONS**

|            |                                               |
|------------|-----------------------------------------------|
| HNSCC      | Head and neck squamous cell carcinoma         |
| SPT        | Serine palmitoyl transferase                  |
| CerS1 to 6 | Ceramide synthase 1 to 6                      |
| DES        | Dihydroceramide desaturase                    |
| TNM        | Tumor-node-metastasis                         |
| SM         | Sphingomyelin                                 |
| GlcCer     | Glucosylceramide                              |
| GalCer     | Galactosylceramide                            |
| S1P        | Sphingosine-1-phosphate                       |
| SMase      | Sphingomyelinase                              |
| DOI        | Depth of invasion                             |
| GlcCDase   | Glucosylceramidase                            |
| GCDase     | Galactosylceramidase                          |
| CDases     | Ceramidases                                   |
| SPHK1      | Sphingosine kinase 1                          |
| AC         | Acid ceramidase                               |
| NC         | Neutral ceramidase                            |
| ACER       | Alkaline ceramidase                           |
| AML        | Acute myeloid leukemia                        |
| MOMP       | Mitochondrial outer membrane permeabilization |
| CERK       | Ceramide kinase                               |
| LAPTM4B    | Lysosome-associated protein transmembrane-4b  |
| Drp1       | Dynamin-related protein 1                     |
| HNC        | Head and neck cancer                          |
| GVHD       | Graft versus host disease                     |
| SGPP1      | Sphingosine-1-phosphate phosphatase 1         |

|          |                                                           |
|----------|-----------------------------------------------------------|
| AJCC     | American Joint Committee on Cancer                        |
| FDA      | Food and Drug Administration                              |
| RRM2     | Ribonucleoside-diphosphate reductase subunit M2           |
| Pyr-cer  | Pyridinium ceramide                                       |
| ATG      | Autophagy-related genes                                   |
| ESCRT    | Endosomal sorting complex required for transport          |
| LC3      | Light chain 3                                             |
| AIM      | Atg8-interacting motif                                    |
| OMM      | Outer mitochondrial membrane                              |
| OSCC     | Oral squamous cell carcinoma                              |
| LIR      | LC3-interacting region                                    |
| mTOR     | Mammalian target of rapamycin                             |
| VPS34    | Vacuolar protein sorting 34                               |
| PI3P     | Phosphatidylinositol 3-phosphate                          |
| ROS      | Reactive oxygen species                                   |
| Hsp60    | Heat shock proteins 60                                    |
| PI3K     | Phosphatidylinositol 3-kinase                             |
| NEMP     | Nuclear-encoded mitochondrial proteins                    |
| MDV      | Mitochondria-derived vesicles                             |
| PTEN     | Phosphatase and tensin homolog                            |
| TOM      | Translocase of outer membrane                             |
| DMEM     | Dulbecco's modified eagle's medium                        |
| ULK1     | Unc-51-like kinase 1                                      |
| L-NAME   | N $\omega$ -Nitro-L-arginine methyl ester hydrochloride   |
| DMSO     | Dimethyl sulfoxide                                        |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| ACO2     | Aconitase 2                                               |
| OCR      | Oxygen consumption rate                                   |

|        |                                  |
|--------|----------------------------------|
| IP     | Immunoprecipitation              |
| DHF    | Dichlorofluorescein              |
| PLA    | Proximity ligation assay         |
| PINK1  | PTEN-induced putative kinase 1   |
| PBS    | Phosphate buffered saline        |
| shRNA  | Short hairpin RNA                |
| PDI    | Protein disulfide isomerase      |
| WT     | Wildtype                         |
| EV     | Empty vector                     |
| SoSe   | Sodium selenite                  |
| iNOS   | inducible nitric oxide synthase  |
| Mfn1/2 | mitofusins                       |
| VDAC   | voltage-dependent anion channel  |
| NO     | Nitric oxide                     |
| RNS    | Reactive nitrogen species        |
| HPV    | Human papillomavirus             |
| S1PR   | Sphingosine-1-phosphate receptor |
| TIM    | Translocase of inner membrane    |
| FBS    | Fetal bovine serum               |

## **1. INTRODUCTION**

### **1.1 HEAD AND NECK SQUAMOUS CELL CARCINOMA**

Cancer that arises from head and neck areas is called head and neck cancers (HNC) and head and neck squamous cell carcinoma (HNSCC) is the most common type of HNC. HNSCCs develop from the epithelium lining of the upper aerodigestive region which have functions in talking, breathing, and eating (1).

#### **1.1.1. Head and Neck Anatomy**

HNCs include malignancies, originate from the epithelium lining the oral cavity and pharynx (1). Although head and neck cancers can be developed from salivary glands, sinuses, or muscles and nerves in the same region, these cancers are rare species than head and neck cancer squamous cell carcinoma (HNSCC) (2,3).

The first part of the digestive system is oral cavity, including mucosal lips, the mouth epithelium, hard palate, anterior tongue, and gums. The oral pharynx is a tube that lies between the nose to the esophagus. It includes the nasopharynx, oropharynx, and hypopharynx. The larynx comes after pharynx and have functions in air passage, sphincter, and vocalization (Figure 1.1). HNC can also arise from salivary glands, paranasal sinuses, and the nasal cavity (4).

#### **1.1.2. Head and Neck Cancers and Head and Neck Squamous Cell Carcinoma**

Cancer that arises from head and neck areas is called HNC (5). Most of these cancers are HNSCCs develop from the epithelium tissue which have functions in talking, breathing, and eating. These cancers are related to expose of tobacco-derived carcinogens, excessive

alcohol consumption, or preceding human papillomavirus (HPV) infection (6,7). Also, they need complex approaches to treatment and diagnosis (8).

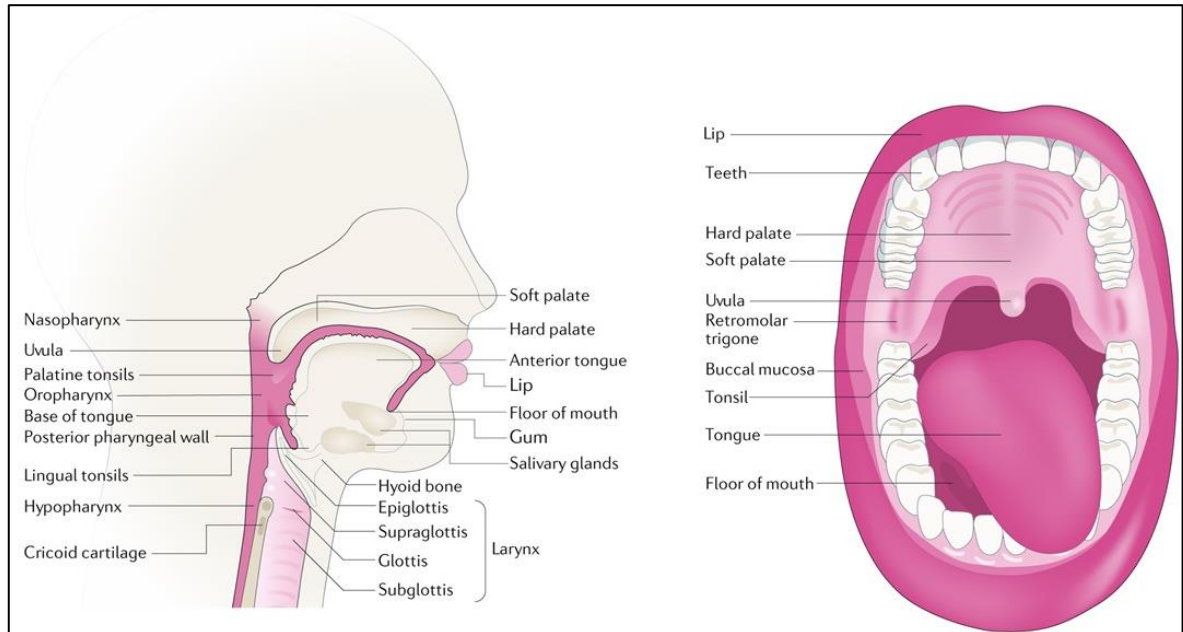


Figure 1.1. Anatomical sites of head and neck region (9).

### 1.1.3. Epidemiology

The annual incidence of HNC is more than 890000, accounting for 4.6 % of all cancers and 4.5 % of cancer-related deaths (10). 90 % of HNC is HNSCC in various anatomic parts in Figure 1.1, and it is the sixth most commonly diagnosed type of cancer for incidence with a 66 % 5-survival rate worldwide (9,11). Around 75 % of HNSCCs are associated with tobacco, carcinogens, and alcohol consumption (12–14). However, the case incidence related to those is slowly declining because of less usage (15,16). Conversely, the cases related to HPV infections are increasing, which is a small portion of the cases and occurs mainly in young people (17). Besides, the prognosis in HPV-positivity is amended because of a better response to treatment than the HPV-negative counterparts (18–20).

#### **1.1.4. Diagnosis and Staging**

Diagnosis starts with radiologic imaging of the mass after a physical examination. It then continues with fine-needle aspiration, an exceedingly sensitive and specific technique to collect specimens from hard anatomical accessibility (21). The most common staging method used in HNSCC is tumor-node-metastasis (TNM) staging by the American Joint Committee on Cancer (AJCC) (22). The staging differs in each region of TNM and the patient's HPV status, represented as an extranodal extension (ENE) since the prior infection with HPV has improved survival rates compared to non-infected patients (Table 1.1) (23,24). The early stages of the classification (I and II) involve smaller tumors without any lymph node invasion. However, later stages (III and IV) include invasive tumors to surrounding regions and metastasis in stage IV (24,25).

#### **1.1.5. Treatment**

The treatment strategy for HNSCC depends on the stage and the tumor's location. Early-stage patients, which comprise 40 % of all cases, can be cured by surgery or radiotherapy with better survival rates (26). Advanced-stage patients with III or IV, multimodal therapy is required for surgery, chemotherapy, or chemoradiation therapy. Finally, systemic therapies, chemotherapy, or immunotherapy with radiation therapy are needed for recurrent or metastatic cancers. The standard chemotherapy includes cisplatin, a platinum-based alkylating agent (27–29). In addition, cetuximab, an anti-epidermal growth factor receptor monoclonal antibody, was approved by the FDA for late-stage HNSCC. Immune checkpoint inhibitors (ICI) are new systemic treatment options that are recently getting attention for recurrence and/or metastatic (R/M) HNSCC. The FDA approved Pembrolizumab and then nivolumab as anti-PD-1 agents to treat those R/M patients (30,31).

## 1.2. SPHINGOLIPIDS

Sphingolipids are membrane-associated lipids responsible for the fluidity and barrier function (32). Besides the structural function, sphingolipids are required for signaling pathways such as cell death, cell growth, proliferation, and migration (33). For the concept of cancer, sphingolipids have regulative roles as bioactive molecules in diverse biological processes: invasion, metastasis, apoptosis, and lethal mitophagy (34). The bioactive sphingolipid such as ceramide, which induces cell death and tumor suppression. Also sphingosine-1-phosphate is another bioactive sphingolipids that causes cell survival and drug resistance (35). The alteration in these sphingolipids have an influential role in cancer progression and therapy.

### 1.2.1. Sphingolipid Structure and Metabolism

Ceramide is the pivotal molecule in this lipid metabolism, and it consists of a long-chain sphingosine base and amide-linked fatty acyl chain, which varies in length between C14 and C26 (35–37). Endogenous ceramide is generated by the *de novo* pathway, which start with condensation of serine with palmitoyl-CoA to produce 3-keto-sphinganine via serine palmitoyl transferase (SPT) (38–40). Then, 3-ketosphinganine reductase prompts reduction of 3-ketosphinganine to sphinganine. This sphinganine is further metabolized by (dihydro)ceramide synthases (CerS1–CerS6) to produce dihydroceramide via the addition of the variable fatty acyl chain (41,42). Lastly, dihydroceramide is desaturated by dihydroceramide desaturase (DES) to produce endogenous ceramide (43–45). CerS1-6 enzymes are responsible for ceramide generation and they provide different ceramide with different fatty acyl lengths and different cellular functions (45–48). The salvage pathway is one of the pathways connected in ceramide generation where sphingosine and exogenous short-chain ceramides (C2-C6) are recycled to produce ceramide by CerS1-6 enzymes (49). In addition, hydrolysis of complex sphingolipids is another alternative pathway to produce

ceramide. These complex sphingolipids are sphingomyelin (SM), C1P, glucosylceramide (GlcCer), and galactosylceramide (GalCer). Sphingomyelinases (Smases) cleaves sphingomyelin to ceramide; glucosylceramidase (GlcCDase) and galactosylceramidase (GCDase) catalyze the generation of ceramide from complex sphingolipids glucosylceramide and/or galactosylceramide (35,44,50). Ceramide can be metabolized by ceramidases (CDases) to generate sphingosine, which is phosphorylated by sphingosine kinase 1 (SPHK1) or sphingosine kinase 2 (SPHK2) to produce sphingosine-1-phosphate (S1P) (37). S1P is metabolized via S1P lyase to make ethanolamine-1-phosphate and hexadecanol or hydrolyzed to generate sphingosine via S1P phosphatases (51,52). Moreover, ceramide is used as a substrate to form complex sphingolipids. Glucosylceramide synthase (GCS) converts ceramide to glucosylceramide (GlcCer). This GlcCer is used for synthesis of lactosylceramide and complex glycosphingolipids involving in plasma membrane (53). Otherwise, ceramide is utilized as a substrate for sphingomyelin by sphingomyelin synthase (SMS) and ceramide-1-phosphate by ceramide kinase (CERK) and 1-O-acylceramide by diacylglycerol acyltransferase (DGAT1/2) as shown in Figure 1.2 (54–56).

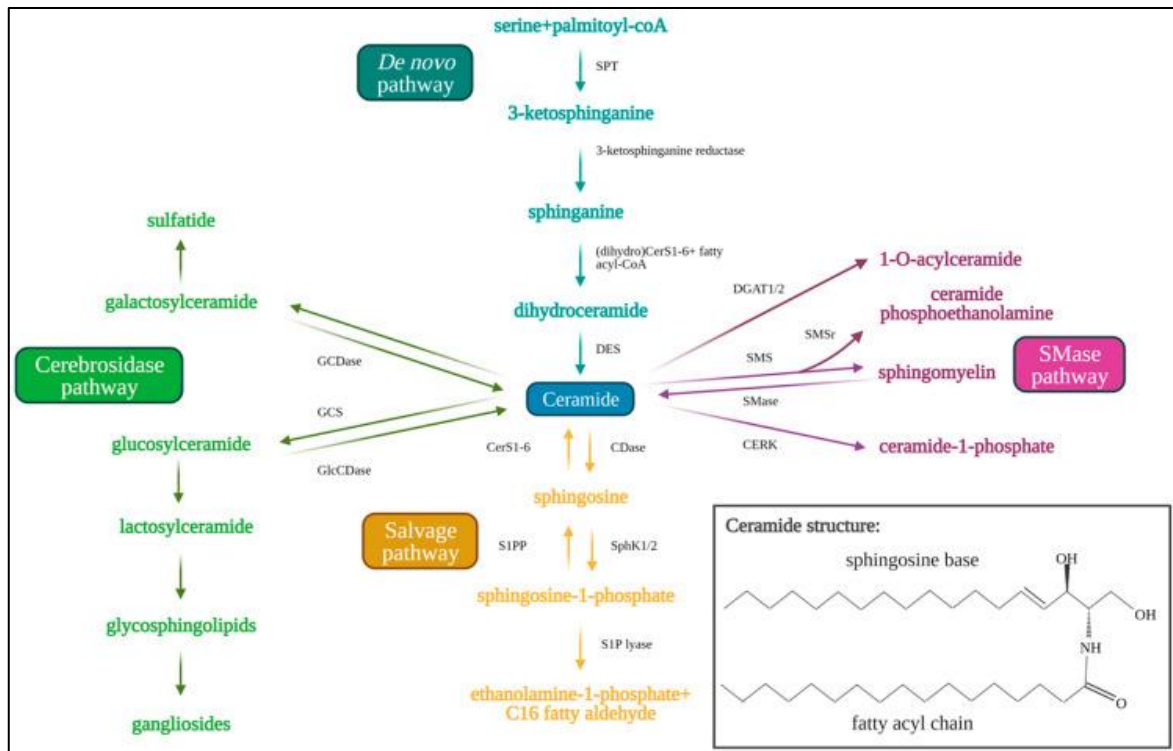


Figure 1.2. The pathway of sphingolipid metabolism (57).

### 1.2.2. Sphingolipid Metabolism and Cancer

Bioactive sphingolipids are involved in many processes that regulate signaling pathways for cell growth, death, proliferation, and migration. Cellular stress induces sphingolipid generation, which participates in many significant cellular actions such as apoptosis, necroptosis, senescence, and lethal mitophagy (50). In cancer cell reprogramming, these protective mechanisms can be changed and impaired that lead to support their survivability by dysregulating the sphingolipid metabolism by upregulating pro-survival sphingolipids such as sphingosine-1-phosphate and downregulating pro-death sphingolipids such as ceramide (58). In addition, metabolic enzymes regulate the abundance of sphingolipids in cellular content by altering the expression or activity (59).

### 1.2.2.1. Ceramide Synthases 1-6

Ceramide synthesis with different fatty acyl chain lengths is provided by CerS1-6 enzymes, which has distinct effects on cancer cell progression (45,60). CerS1 is responsible for C18-ceramide generation, a pro-death ceramide that was found to be repressed in HNSCC (61–64). A decrease in C18-ceramide has affiliated with poor breast cancer and neuroblastoma prognosis (65–68). Similarly, CerS4, which is also responsible for C18-C20 ceramide generation, has been shown to be at low levels in high-stage HNSCC patients compared to early-stage patients (69).

Moreover, CerS2 has a role in producing long-chain ceramides (C22-C24), whose overexpression was found to decrease migration and invasion in breast cancer cells (70). Conversely, CerS5 level is increased in colorectal cancer which leads to bad patient outcomes (71–73). Finally, CerS6 has shown to increase in gastric cancer, which has a role in C16-ceramide generation, was demonstrated as a biomarker in gastric cancer (74). However, radiation therapy in HeLa cells induces CerS6-generated C16-ceramide-mediated apoptosis (75). Ceramides with differences in fatty acid chain lengths have definite roles in cancer progression, standing on the context or cell type (59).

### 1.2.2.2. Sphingosine Kinase 1 and 2

SPHK1 and SPHK2 produce sphingosine-1-phosphate, a pro-survival sphingolipid that is elevated in secretion or production in cancer. Although both kinases produce S1P from sphingosine, their downstream targets are changed based on their localization in the cell. SPHK1 is localized in the cytosol, but SPHK2 remains in the nucleus, cytosol, and mitochondria (76,77). SPHKs are important for cancer progression since they maintain the dynamic between pro-survival and pro-death signaling through S1P and ceramide metabolism. SPHK1 is elevated in many cancers, such as breast, and glioblastoma (78–81). Likewise, SPHK2 was shown to be increased in colorectal and non-small-cell lung cancers

(82,83). Therefore, inhibition of S1P production provides a potential target for developing a treatment. FTY20 (Fingolimod) is a sphingosine analog that SPHK2 phosphorylates to produce S1P analog, an antagonist for S1PR1, resulting in tumor suppression in lung cancer and colon cancer (84,85). Sphingosine kinase inhibitor I (SKI) is another sphingosine analog that was shown to inhibit proliferation in glioblastoma cell lines (86). Similarly, ABC294640 is an SPHK2 inhibitor that has been used in clinical trials against sorafenib-resistant hepatocellular carcinoma, multiple myeloma, and prostate cancer (87,88). Thus, SPHK1 and SPHK2 are key players in tumor growth, and antagonizing S1P signaling could provide an anticancer therapeutic strategy for many cancer types (50).

#### **1.2.2.3. Ceramide Kinase**

Ceramide kinase (CERK) generates ceramide-1-phosphate by phosphorylating ceramide, whose overexpression was found to induce growth and migration in triple-negative breast cancer (89–91). Also, elevated CERK expression has been demonstrated to involve in the recurrence of the breast cancer (92). Conversely, NVP-231, an inhibitor of CERK, was found to decrease migration and invasion potential of metastatic breast cancer cells (93).

#### **1.2.2.4. Ceramidases**

Ceramidases (CDases) produce sphingosine by hydrolyzing ceramide. There are three different CDases classified in sphingolipids (94).

AC are lysosomal ceramidases that are shown to be increased in many cancers, such as prostate, and also play a role in developing resistance after radiation therapy (95,96). In addition, AC expression levels were shown to be decreased in patients with more radiosensitive organoids compared to patients with upregulated AC expression (97).

NC generates sphingosine from ceramide, further utilized for pro-survival S1P production (98). Inhibition of NC via pharmacological or molecular inhibition was found to activate ceramide-mediated cell death in colon cancers (99).

ACERs are classified into three different groups (ACER1, ACER2, and ACER3), which have roles in sphingosine production from ceramide. Although the function of ACER1 in cancer progression and survival is currently unknown, it has been shown to have a part in skin homeostasis (100). However, in the database of The Cancer Genome Atlas, ACER1 was reported to be decreased in human skin cutaneous melanoma, HNSCC, and esophageal carcinoma (101). ACER2 was found to be increased in expression in hepatocellular carcinoma (HCC) and DNA-damaged cancer cells (102). ACER3 selectively hydrolyzes unsaturated long-chain ceramides (C18:1, C20:1, and C20:4) to make sphingosine (103). Molecular inhibition of ACER3 was found to induce apoptosis in HCC, and deficiency in ACER3 was reported to decrease tumor growth in acute myeloid leukemia (AML) (104,105).

#### **1.2.2.5. Sphingomyelinases**

Sphingomyelinases (SMases) generate ceramide by catalyzing the sphingomyelin. There are three different classes which are grouped relying on their pH-dependent activity (106). Alkaline SMase deficiency was found to decrease ceramide levels and elevate pro-survival S1P levels, which enhanced tumor progression in a mouse model of colon cancer. Neutral SMase 2 (NSMase2) downregulation has been demonstrated to induce tumor progression in oral squamous cell carcinoma (OSCC) and melanoma (107). Although SMases play key roles in pro-death ceramide generation, induction of acid SMases (ASMases) in B16F10 melanoma induces metastasis, indicating the prominence of cell/tissue-specific expression of sphingolipid metabolism-associated enzymes in the signaling mechanism (108).

#### **1.2.2.6. Sphingomyelin Synthase**

The ceramide is catalyzed to produce sphingomyelin by sphingomyelin synthase (SMS), which includes isoforms of SMS1 and SMS2 (109). Although the function of SMS1 in cancer progression and survival is currently unknown, SMS2 was found to be upregulated in ovarian cancer (110).

#### **1.2.3. Sphingolipids in Cancer Cell Death**

The synthesis of pro-cell death ceramide upon cellular stress feedback such as heat and chemotherapeutic agents mediates cell death via different mechanisms (44). These mechanisms include apoptosis, necroptosis, ER stress, and autophagy (111). The regulation of the cell death mechanism relies on the tissue type and the subcellular localization of ceramides (112).

##### **1.2.3.1. Apoptosis**

Ceramide was demonstrated to induce apoptosis in leukemic cells. Then, ceramide was shown to be generated in response to radiation-mediated apoptosis and this apoptosis is provided by the involvement of ceramide-rich macrodomains for Bax integration on the mitochondrial membrane (113,114). Ceramide channels were shown to be involved in mitochondrial outer membrane permeabilization (MOMP) to induce mitochondrial apoptosis(115). Lysosome-associated protein transmembrane-4b (LAPTM4B) facilitates ceramide removal from late endosomal organelles. Silencing of LAPTM4B was shown to induce sphingolipid accumulation in late endosomal organelles and destabilize the lysosomal membrane, resulting in resistance to apoptosis through developing resistance against caspase 3 activation upon chemotherapy (116).

### **1.2.3.2. Necroptosis**

Necroptosis is induced by ceramide signaling when the ceramide levels are elevated through CERS1 overexpression, C18 ceramide generation, and FTY720 treatment (37,117). In addition, the interaction between phosphatase 2A inhibitor I2PP2A and ceramide or FTY720 was shown to activate the tumor suppressor serine/threonine-protein phosphatase 2A and induce necroptosis in lung xenograft-derived tumors (37,118).

### **1.2.3.3. ER Stress and Cell Cycle Arrest**

The association between ceramide and ER was shown to have a role as an upstream manager of autophagy (119). Methotrexate-induced folate stress was shown to increase ER stress-related protein aggregates augmented with CERS6, which involved in p53 activation (120). Sphingosine kinase 1 deletion in Tp53-null mice was shown to induce the formation of thymic lymphomas, which lead to elevated levels of ceramide, cell cycle inhibitors, and cancer cell senescence (121).

### **1.2.3.4. Autophagy**

Dihydroceramide and ceramide accumulation induces autophagy, followed by context-dependent cancer cell death or survival (122–124). Mitophagy, selective autophagy of mitochondria, plays an influential role in tumor suppression via lethal mitophagy, where the degradation is extended to the cell and results in cell death (125). Lethal mitophagy is induced upon ceramide exposure through chemotherapy or exogenous ceramides that increase CerS1-generated C18-ceramide accumulation at the OMM, which then recruits autophagosomes to mitochondria to induce mitophagy in AML and head and neck cancers (126). In addition, it was discovered that Drp1 activation and mitochondrial fission were the essential steps of ceramide-dependent lethal mitophagy (126,127)

#### **1.2.4. Sphingolipid and Cancer Therapy**

##### **1.2.4.1. Chemotherapy and Radiotherapy**

In many cancers, pro-cell death ceramide level was shown to be reduced while pro-survival S1P generation is elevated. Chemotherapy and radiotherapy induce sensitivity and apoptosis by restoring ceramide levels in cancer cells. Daunorubicin has been found to induce ceramide-related apoptosis in leukemia and histiocytic lymphoma cancers (128). Combination drug therapy of gemcitabine and doxorubicin was demonstrated to induce cell death by CerS1-dependent C18 ceramide generation *in vitro* and *in vivo* xenograft mouse model of metastatic HNCs (129,130). Gemcitabine and doxorubicin were reported to have improved responses with increased serum levels of C18 ceramide for recurrent HNC patients in Phase II clinical trials. Additionally, high ceramide levels were shown to be related to patients with positive responses upon treatment with hypofractionated stereotactic body radiation therapy (131).

##### **1.2.4.2. Immunotherapy**

In addition to chemotherapy and radiotherapy, sphingolipids have essential effects on immune cells, which have antitumor functions toward transformed cells. S1P/S1PR signaling has been found to involve lymphocyte egress, which is vital for immunity (132–134). Also, S1PR1 signaling in CD<sup>+</sup> T cells induces melanoma and breast cancer in mice and activates STAT3, which causes tumor growth and metastasis (135,136). In Gaucher disease, deficiency in glucocerebrosidase results in elevated levels of lyso-glucosylceramide. Patients with Gaucher disease have reactive clonal immunoglobulin against these lyso-glucosylceramides and have a high risk of developing melanoma (137). Moreover, CerS6-derived C16 ceramide generation was shown to enhance T cell activation as a response to alloantigens throughout allogeneic hematopoietic stem cell transplantation against hematologic malignancies, which causes graft versus host disease (GVHD) (138).

#### **1.2.4.3. Drug Resistance**

The generation of pro-survival S1P by ceramidases and glucosylceramide by glucosylceramide synthase from pivotal molecule ceramide has been found to be involved in drug resistance. (139,140). Moreover, SPHK1 overexpression was shown to have resistance to cetuximab in colorectal cancer (141). Also, overexpression of SPHK1 was found to promote the resistance against imatinib-mediated apoptosis in chronic myeloid leukemia (142). Furthermore, sphingosine-1-phosphate phosphatase 1 (SGPP1) silencing via miR-95 was found to mediate radiation resistance in mouse models of prostate and breast cancers (143).

#### **1.2.4.4. Therapeutic Targeting of Sphingolipids**

Fingolimod (FTY20) was approved by the US Food and Drug Administration (FDA) for treating relapsing multiple sclerosis, an analog of sphingosine and a practical antagonist of S1PR1 after SPHK2 phosphorylation to P-FTY20 (144,145). FTY20 was shown to induce tumor suppression via S1PR-dependent and receptor-independent mechanisms (146,147). The S1PR-dependent mechanism involves the internalization of P-FTY20 and degradation of S1PRs (148). In receptor-independent mechanism, FTY20 was found to induce necroptosis and tumor suppression by forming ceramidosomes (ceramide enriched membrane) by inhibiting oncoprotein SET and activating tumor suppressor PP2A signaling (149).

ABC294640 is an inhibitor of SPHK2 that has been shown to suppress tumor growth via inhibiting telomerase stability, suppressing c-Myc and ribonucleoside-diphosphate reductase subunit M2 (RRM2) expression, and inhibiting dihydroceramide desaturase in cancer cells (150,151). Pyridinium ceramide was demonstrated to cumulate in mitochondria selectively and mediate lethal mitophagy in the head and neck, acute myeloid leukemia, and prostate cancers (126,152–154).

### **1.3. AUTOPHAGY**

Autophagy is an evolutionary conserved intracellular catabolism that removes damaged organelles, aggregated proteins, or pathogens by delivering the cargo to the lysosomes, which hydrolases them into amino acids, nucleotides, fatty acids and recycles into cytoplasm under stressful conditions such as nutrient deficiency, hypoxia, growth factor depletion, and infection (155–159). Autophagy also has roles in recycling redundant or damaged proteins with a long half-life to regulate cell homeostasis and protein-organelle quality by way of its selective and non-selective mechanisms (160–163). Thus, autophagy plays a vital role in many pathologies such as neurodegeneration, infections, and tumorigenesis (164–168).

#### **1.3.1. Types of Autophagy**

Four different groups of autophagy are distinguished based on the delivery process to lysosomes upon cellular stresses or transduction of signaling pathways (169).

##### ***1.3.1.1. Macroautophagy***

Macroautophagy is the most common type of autophagy directed by autophagy-related genes (ATG). They are responsible for regulating autophagosome production, a double-membrane vesicle that engulfs cytoplasmic fragments by forming a C-shaped membrane and extending around cargo (170–172). Then autophagosomes merge with the lysosome to form autolysosomes, where hydrolytic enzymes are used to degrade autophagic cargo (Figure 1.3) (173,174). Macroautophagy is an important process during tumorigenesis since it can be an inhibitor or stimulator of cell growth. It prevents cancer by eliminating organelles and proteins with long half-lives or damage. However, it can induce carcinogenesis via cytoprotective response to chemotherapy and/or metabolite recycling to fulfill tumor growth and proliferation needs (175).

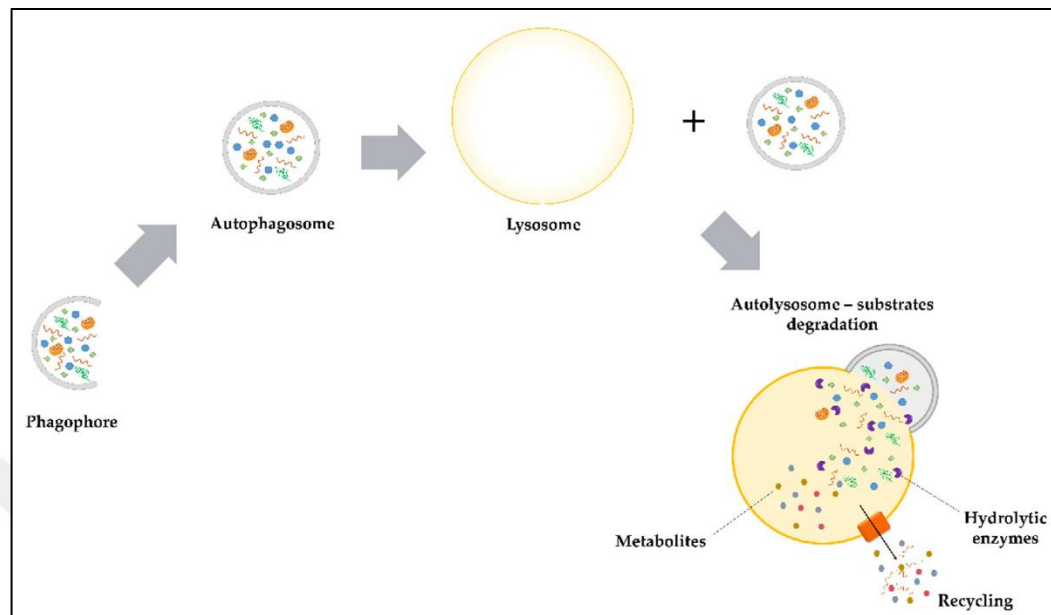


Figure 1.3. The overview of the macroautophagy (176).

#### 1.3.1.2. Microautophagy

Microautophagy is a process of degradation of substrates directly in lysosomes (177). First, redundant or damaged proteins, organelles, or fragments of cytoplasmic substrates are collected and transported into endosomes in a vesicle by heat shock cognate protein 70 (Hsc70) (178). Then, membrane invagination happens by Endosomal Sorting Complex Required for Transport (ESCRT), and the cargo is degraded inside the endosome (179,180).

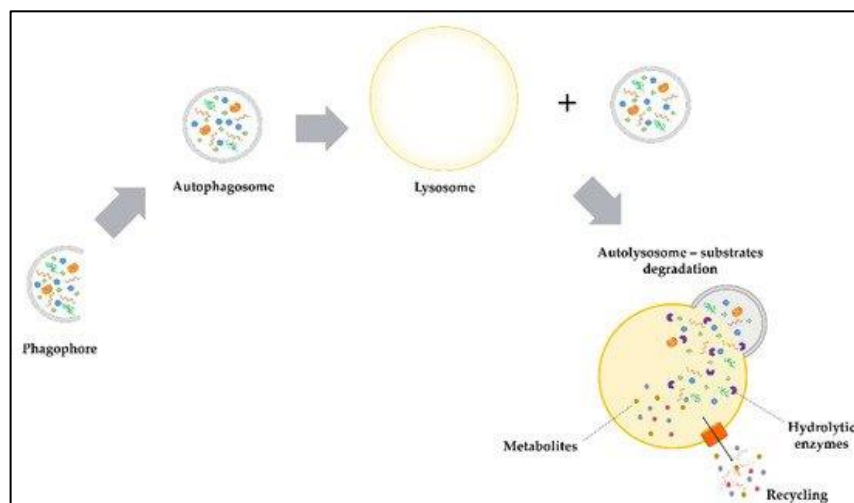


Figure 1.4. The diagram of the microautophagy (176).

### 1.3.1.3. Chaperone-Mediated Autophagy

Substrate fragments in the cytoplasm with the KFERQ motif are found in all proteins, but in some cases, the motif is found to be incomplete due to post-translational modifications (181). The recognized proteins are transported by Hsc70 with its co-chaperones to the lysosome surface, where Lysosome-Associated Membrane Protein type 2A (LAMP2A) binds and transports them into the lysosome for hydrolytic degradation as shown in Figure 1.5 (182–184). CMA selectively controls the levels of many proteins, including proto-oncogenic protein MYC (185,186).

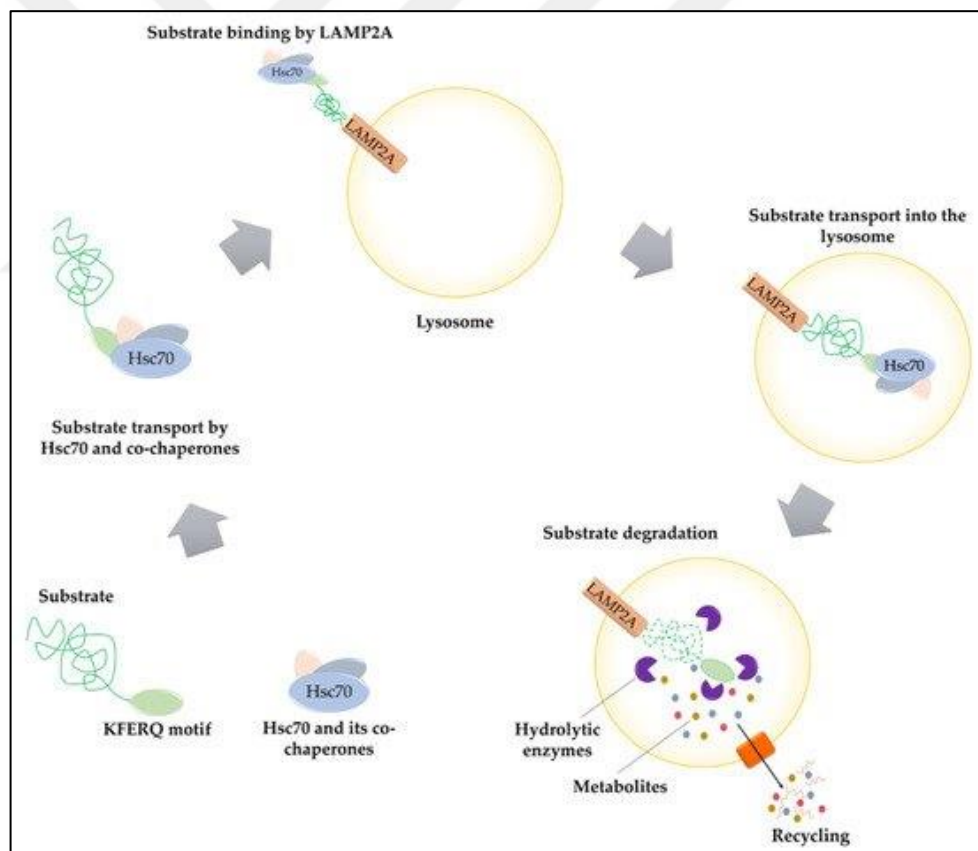


Figure 1.5. The diagram of the chaperone-mediated autophagy(176).

#### **1.3.1.4. Selective Autophagy**

Selective autophagy is the process of degradation that selectively degrades specific organelles and targets such as mitochondria, endoplasmic reticulum, ribosomes, lipid droplets, and nuclei by binding to autophagy receptors of the cargo (187–189). These receptors have fragments of Atg8-Interacting Motif (AIM) and LC3-Interacting Region (LIR) on their surface, enabling the binding of receptors and protein from the Atg8/LC3/GABARAP family selectively, and finally, autophagosome formation (190,191). Based on their targets for degradation, selective autophagy is divided into different categories such as proteaphagy (proteasomes), nucleophagy (nuclei), ER-phagy (endoplasmic reticulum), ribophagy (ribosomes), and mitophagy (mitochondria) (176). Selective autophagy removes the damaged or redundant organelles, proteins, or fragments. Thus, it is an essential mechanism for maintaining cellular homeostasis, which protects from disorders such as neurodegenerative or inflammatory diseases, metabolic abnormalities, or cancer (192–195).

#### **1.3.2. Mechanism of Autophagy**

Autophagy is an evolutionarily conserved intracellular catabolic process that removes damaged organelles, aggregated proteins, pathogens upon intracellular or extracellular stimulus, which includes several steps (196,197).

##### **1.3.2.1. Induction of Autophagy and Phagophore Nucleation**

Under the nutrient-rich condition, ATG13 and Unc-51-like kinase 1 (ULK1) are phosphorylated by the mTORC1 mammalian target of rapamycin (mTOR) and bound directly to each other in an inactive form (198). When intracellular or extracellular stimuli induce cells, the ULK1 complex becomes the most common autophagy target, including ULK1, ATG13, FIP200, and ATG101. The mTORC1 binding sites of ULK1 are

dephosphorylated, resulting in ULK1 dissociation and autophosphorylation. Then ULK1 phosphorylated ATG13 and FIP200 (RBI-inducible coiled-coil protein 1) (199,200). ATG13 anchors ULK1 and then the other Atg proteins to pre-autophagosomal structure (PAS), which is responsible for the phagophore nucleation (201,202). The recruitment of the ULK1 complex acts as an autophagy initiation complex. It initiates the engagement and localization of other necessary units to the PAS and involvement in autophagosome formation by phosphorylating then class III of phosphatidylinositol 3-kinase (PI3K) (PI3KC3) complex I. This complex then activates the phosphatidylinositol 3-phosphate (PI3P) production at the ER, which recruits the proteins involved in phagophore expansion (203–208).

#### ***1.3.2.2. Phagophore Expansion***

Ub-like ATG8 family proteins are the most influential members of ATGs involved in phagophore expansion which include the light chain 3 (LC3) subfamily (microtubule-associated protein 1 light chain 3, LC3): LC3A, LC3B, LC3C, and  $\gamma$ -aminobutyric acid receptor-associated protein (GABARAP) subfamily: GABARAP, GABARAPL1, GATE-16/GABARAPL2) (209,210). They start to work after the process at their C-terminus for phosphatidylethanolamine (PE) conjugation in which phagophore formation is induced (211,212). This conjugation produces the membrane-bound lipidated form observed in LC3-I conversion to LC3-II. Moreover, ATG8s have role selective autophagy where they enable the recruitment of cargo via the LC3 interacting regions (LIRs) on the cargo receptors as shown in Figure 1.6 (213).

#### ***1.3.2.3. Autophagosome Maturation***

After phagophore expansion and sealing, maturation is needed before lysosomal fusion. In this step, the ATGs are cleared from the outer membrane, and the lysosomal system is recruited, which is mainly driven by ATG8s (214,215).

### **1.3.3. Autophagy in Cancer**

Autophagy is fundamental process for maintaining cellular homeostasis by controlling organelle and protein quality and sustaining energy (216–218). In diseases such as infection and neurodegenerative, dysfunctional autophagy results in the accumulation of damaged or redundant proteins and organelles which causes severe disturbance in the function of the cells (219–221). In cancer progression, autophagy functions mostly in a context-dependent. It can either promote tumor suppression or tumor growth in cancer development (222–225). Its cryoprotective responsibilities, such as maintaining genome stability, reducing DNA damage, and preventing oncogenic protein accumulation, are found to be tumor-suppressive. However, in tumors with higher stage, autophagy can behave as cellular protector by escaping from cellular stress such as chemotherapy, nutrient deprivation, and DNA damage, providing cells with metabolic plasticity, which promotes tumor growth and resistance to therapeutic agents (226,227). The effects of autophagy can be both suppressive or tumor-promoting on the cells depending on various factors such as cancer type, stage, or genetic background (225,228–230).

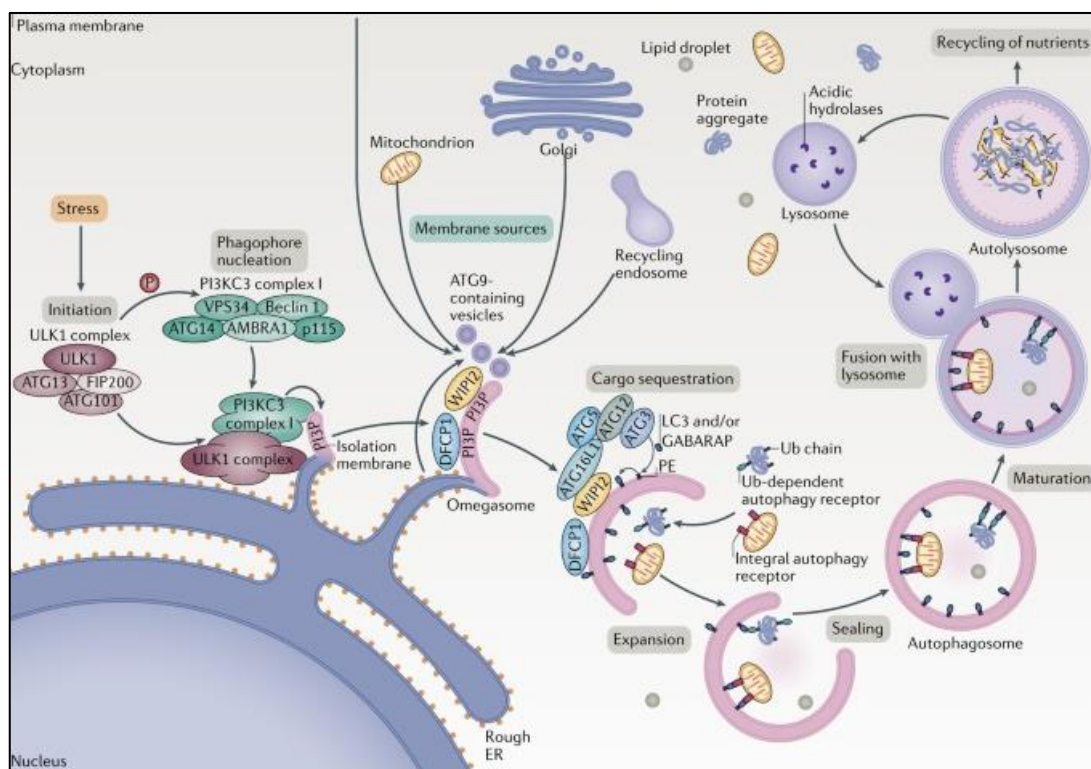


Figure 1.6. The overview of the autophagy mechanism (213).

#### 1.4. MITOPHAGY

Mitochondria are highly dynamic and double-membrane organelles that are the leading site for energy production (231). In addition to their role in energy production, mitochondria have roles in many other crucial functions such as biomolecule synthesis, stress regulation, calcium buffering, and pro-survival or pro-apoptotic signaling (232–234). Since mitochondria play an important role in ATP production via ETC, they are the center of reactive oxygen species (ROS) production, whose higher levels can damage mitochondria at the protein or organelle level by disturbing protein folding and structure and causing mutations in mitochondrial DNA (235–237). Mitochondria have quality control systems that are consistently active and protect the organelle from stress and damage by regulating two opposing processes. First, the system removes the damaged components or the organelle and then sustains the needs of pre-existing mitochondria via mitochondrial biogenesis in a

dynamic manner (238). Damages beyond the protein levels induce mitophagy, including mitochondrial biogenesis, fusion, fission, and the removal of mitochondria (239).

#### **1.4.1. Mitochondrial Quality Control at Protein Level**

Mitochondria exposed to damage first respond through antioxidants (such as superoxide dismutase, catalase, vitamin E, and C), DNA repair, protein folding, and degradation (240). Mitochondrial DNA damage is repaired by mitochondrial DNA polymerase gamma (POLG), whose mutation causes neurodegenerative diseases (241). At the protein level, mitochondrial chaperones such as heat shock proteins 60 and 70 (Hsp60 and Hsp70) import nuclear-encoded mitochondrial proteins (NEMP) to the matrix to repair the damage (242). In the case of the accumulation of damaged proteins, LON, caseinolytic peptidase P (ClpP), and ATPases associated with diverse cellular activities (AAA), proteases degrade them in the mitochondria matrix and the inner membrane (243). For the outer membrane proteins, E3 ligase MARCH5 ubiquitinates and targets them to the 26S proteasome (244). Also, the mitochondrial damage is removed via micromitophagy, in which only the damaged part of the mitochondria is delivered to endosomes or lysosomes for degradation without autophagosome formation (245–247). The partial delivery of mitochondria is provided by mitochondria-derived vesicles (MDV), which are generated independently from mitochondrial depolarization, ATG, LC3, Beclin-1, or Drp-1 mediated mitochondrial fission as shown in Figure 1.7 (248).

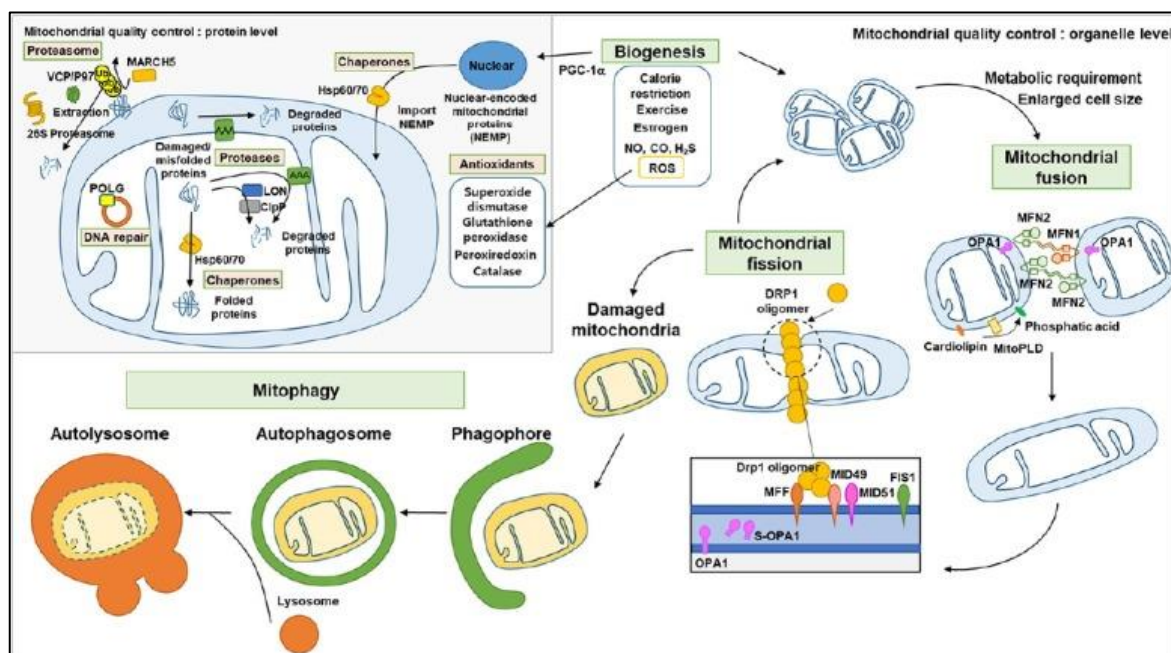


Figure 1.7. The diagram of the mitochondrial quality control mechanism (249).

#### 1.4.2. Mitochondrial Quality Control at Organel Level and Mitophagy

The quality of mitochondria is provided by synthesizing new mitochondria, fusion and fission dynamics. In addition, the aged or damaged organelle is removed, called mitophagy, when the mitochondrial damages beyond the protein level (250). This process is regulated by ubiquitin-dependent or ubiquitin-independent, or lipid-based pathways (251).

##### 1.4.2.1. Ubiquitin-Dependent Mitophagy

After ubiquitin-dependent pathway uses ubiquitin as a signal molecules on the surface of mitochondria to recruit autophagic machinery, which is maintained in different ways (251).

##### 1.4.2.1.1. Parkin-PINK1-mediated mitophagy

When mitochondrial health and function are at the sufficient levels, the phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) is imported into the inner mitochondrial membrane via mitochondrial translocases of outer and inner membrane (TIM

and TOM) and cleaved by matrix processing peptidase (MMP) and Preisilin-Associated Rhomboid-Like Protein (PARL) (252–254). Proteasomes degrade truncated PINK1 in the cytoplasm (255). Under membrane stress conditions, mitochondrial import of PINK is inhibited, and PINK is stabilized on the OMM (256–258). Then, PINK1 is activated by autophosphorylation, which induces the transfer of Parkin from the cytoplasm to the mitochondrial surface (259). PINK1-dependent phosphorylation of Parkin induces mitochondrial localization of Parkin and also E3 ligase activity, which generates the formation of the ubiquitin chain on the OMM (260–262). PINK1 phosphorylates ubiquitin chains on the damaged mitochondria. The Parkin translocation induces the ubiquitination and degradation of OMM proteins such as mitofusins, VDAC, and TOMs (263–266). The autophagy receptors bind (267) first to ubiquitinated cargo and then to LC3 on the autophagosomal membrane to bring ubiquitinated cargo and autophagosome for degradation (268,269). In mammalian cells, different receptors are effective in ubiquitin-dependent mitophagy: p62, optineurin (OPTN), nuclear domain 10 protein 52 (NDP52), which promotes phagophore generation by gathering ULK1 and WIPI1 upstream of LC3 (270,271). Finally, targeted mitochondria are surrounded by autophagosomes and degraded by fusion with the lysosomes as shown in Figure 1.8 (272).

#### 1.4.2.1.2. Parkin- Independent Ubiquitin-dependent Mitophagy

Removal of mitochondria via Parkin-independent however, ubiquitin-dependent mitophagy promotes the removal of mitochondria via the ubiquitination of mitochondrial proteins, which then recruits autophagosomes by using autophagy receptors (251). These mitochondrial proteins are glycoprotein 78, SYNPHILIN1-SIAH1, mitochondrial E3 ubiquitin ligase 1 (MUL1), and p62/SQSTM (273–276).

#### 1.4.2.2. Ubiquitin-Independent Mitophagy

Damaged mitochondria can be removed by direct association between LC3/ GABARAP and mitochondrial proteins with LC3-interacting region (LIR) that acts as a receptor (251). These

mitochondrial proteins are NIX (NIP3-like protein X), BNIP3 (BCL2/Adenovirus E1B 19 kDa interacting protein 3), FUNDC1 (FUN14 domain-containing protein 1), BCL2L13 which locate on the OMM and regulated by post-translational modifications in a stress-dependent manner (272,277–279).

#### ***1.4.2.3. Lipid-Based Mitophagy***

Lipids including cardiolipin and ceramide have been found to initiate mitophagy by localizing on the OMM (251) and interacting with LC3 upon mitochondrial stress conditions. (280–282). Also, mitochondrial ceramide or ceramide analog was found to induce lethal mitophagy in HN and AML cells which are regulated by CerS1-mediated C18-ceramide accumulation at the OMM (283–285).

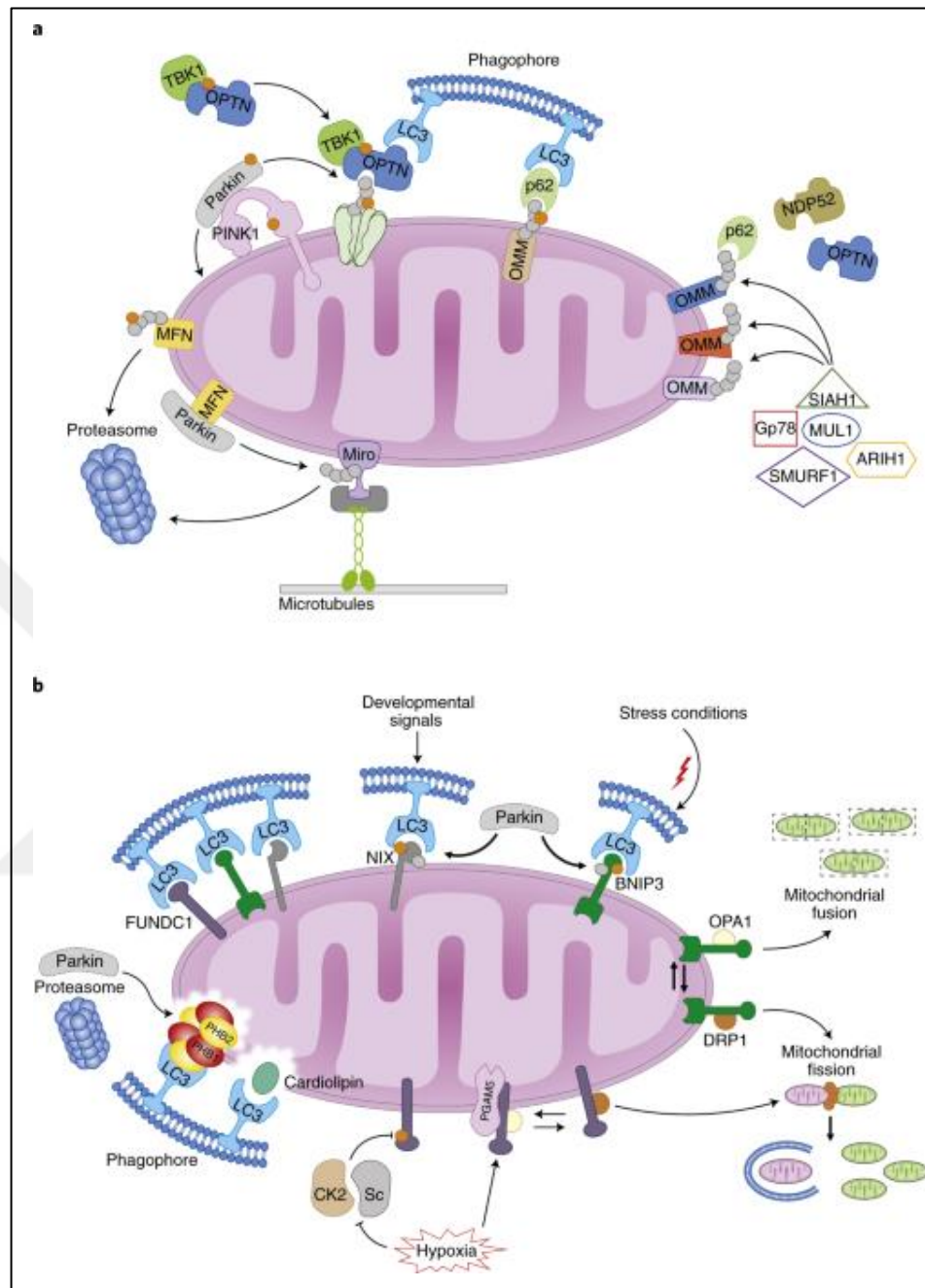


Figure 1.8. The overview of the mitophagy process. a. The PINK1-Parkin regulated mitophagy pathway, b. Receptor-regulated mitophagy pathway(272).

### 1.5. AIM OF THE STUDY

Ceramide is an emerging tumor suppressor lipid that mediates anti-proliferative signaling against various stress stimuli (36). Endogenous C18-ceramide is selectively elevated in response to many anti-cancer therapeutics causing growth arrest, apoptosis, and/or mitophagy-mediated cell death (63,286,287). On the other hand, the amount of C18-ceramide is significantly reduced in HNSCC patients. The reconstitution of C18-ceramide level with the endogenous generation or exogenous ceramide analogs leads to cancer death/tumor suppression through mitochondrial dysfunction (125,127,287). However, conventional ceramide analogs present challenges including low solubility, poor cellular uptake, and high toxicity due to non-specific accumulation in tissues.

In this study, it is aimed to develop novel mitochondria targeting pyridinium-ceramide analog, LCL768 that induces mitophagy and tumor suppression in HNSCC, which will provide a way to overcome challenges in efficacy. So, this study is proposed to determine the mechanism of LCL768 in tumor suppression by inducing mitophagy in HNSCC *in vivo*, xenograft-derived tumors, and 3D organoids.

## 2. MATERIALS

### 2.1. INSTRUMENTS

CO<sub>2</sub> incubator (VWR, 51014995, USA), Laminar flow cabinet (Nuaire, Class II Type A2, USA), Centrifuge (Eppendorf, 5702, Germany), Centrifuge (Beckman Coulter, Microcentrifuge 20, USA), Inverted microscope (Motic, AE20, USA), -80°C freezer (PHCbi, USA), pH meter (Thermo Scientific, Orion Star A111, USA), Mini vortexer (VWR, 58816-121, USA), Heater (Thermolyne, 16500 Dri-Bath, USA), Microplate reader (Biotek, Synergy HT, USA), Waterbath (VWR, 89032, USA), Mini-PROTEAN Tetra Cell Electrophoresis System, Trans-Blot Turbo Transfer System (BIORAD, USA), ChemiDOC XRS+Gel Imaging System, Confocal Microscopy (Olympus, FluoView FV10i LIV, Japan), Seahorse Analyzer (Agilent, XF96 USA), Automated Cell Counter (BIO-RAD, VM-300, USA).

### 2.2. EQUIPMENTS

Falcon tubes (15 mL, 50 mL) (Cellstar, Greiner Bio-One, Germany), Microcentrifuge tubes (VWR, 20170-038, USA), Electronic pipette (Heathrow Scientific, RF300, USA), Micropipettes (10 µL, 20 µL, 200 µL, 1000 µL) (Gilson, USA), Serological pipettes (5 mL, 10 mL, 25 mL) (Cellstar, Greiner Bio-One, Germany), Cell culture flasks (Cellstar, Greiner Bio-One, Germany), Cell culture plates (6-well, 12-well, 24-well, 96-well) (Cellstar, Greiner Bio-One, Germany), Glass Bottom 35 mm Petri Dish (MatTek, USA), Cryovials (Greiner Bio-One, Germany), Filter (0.22 µm - 0.45 µm) (Pall Corporation, USA), Counting Slides (BIO-RAD, USA), Cell culture dishes (Greiner Bio-One, Germany), Counting Chamber (Hausser Scientific, USA), Whatman Paper (BIO-RAD, 1703967, USA), PVDF 0.2 µm (GE, Germany), Syringe (BD, USA), Insulin syringe (28G) (BD, USA), 4–20% Mini-PROTEAN

Precast Protein Gel (BIO-RAD, USA), Magnetic Rack (BIO-RAD, USA), Parafilm (Parafilm, USA), Standard Analog Rocker (VWR, USA).

## **2.3. CHEMICALS**

### **2.3.1. Cell Line**

HNSCC cell line UM-SCC-1A was obtained from Dr. Ogretmen (Department of Biochemistry, Medical University of South Carolina).

### **2.3.2. Cell Culture Media and Growth Supplements**

Dulbecco's Modified Eagle's Medium (Corning, 10-013-CV, USA), Opti-MEM Reduced Serum Media (gibco, 31-985-062), Fetal Bovine Serum (VWR, 97068-085, USA), Penicillin Streptomycin Solution 100X (Corning, 30-002-CI, USA), Plasmocin (Invivogen, ant-mpp, USA), PBS pH 7.4 (1X) (gibco, 10010-023, US), Puromycin (Invivogen, ant-pr1, USA), 0.25 % Trypsin-EDTA (1X) (gibco, 25200-050).

### **2.3.3. Other Reagents**

Ethanol 200 Proof (Decon, 2716, US), Hypure WFI Quality Water (GE, SH30221.17, US), Dimethyl sulfoxide (Sigma, 34869, Germany), Bovine Serum Albumin (Sigma, A7906, Germany), Protease Inhibitor Cocktail (Sigma, p8340, Germany), Phosphate Inhibitor Cocktail (Thermo Scientific, 78428, USA), PR-619 (Cayman Chemical, 16276, USA), Bortezomib (Cayman Chemical, 10008822, USA), SIN-1 (Cayman Chemical, 82220, USA), N $\omega$ -Nitro-L-arginine methyl ester hydrochloride (Sigma, N5751, Germany), Protein Assay Dye Reagent Concentrate (BIO-RAD, 5000006, USA), Protein Standards (BIO-RAD, 161-037, USA), Coomassie G-250 Stain (BIO-RAD, 161-0786, USA), Paraformaldehyde, 4 %

in PBS (Alfa Aesar, J61899, USA), Acetic acid glacial 99.7-100.5% (VWR Chemicals, BDH3094, USA), Methanol (VWR Chemicals, BDH2018, USA), A/G Magnetic beads (BIO-RAD, 161-4013, USA).

## **2.4. KITS AND SOLUTIONS**

Bovine Endofree Plasmid Maxi Kit (Qiagen, 12362, Germany), Silver Stain Kit (Thermo Scientific, 24612, USA), Effectene Transfection Reagent (Qiagen, 30147, Germany) Mitochondria Isolation Kit ((Thermo Scientific, 89874, USA), Intracellular Nitric Oxide Assay (Cell Biolabs, STA-800, USA), Chemiluminescent Substrate, (Thermo Scientific, 34580, USA), Trypan Blue Solution 0.4 % (w/v) (Corning, 25-900-CI, US), LaemmLi SDS sample buffer (6X) (Alfa Aesar, J60015, USA), RIPA Buffer (Thermo Scientific, 89900, USA), IP Lysis Buffer (Thermo Scientific, 87788, USA), Transfer Buffer 10X (Lonza, 00200309, USA), 10X Tris/Glycine/SDS Buffer (BIO-RAD, 1610772, USA), Stripping Buffer (Thermo Scientific, 21059, USA), XFe96 Extracellular Assay Kit (Agilent, 103592-100, USA).

## **2.5. ANTIBODIES**

ACO2 (Cell Signaling, 6922S, USA), LC3 (Cell Signaling, 2775, USA), Actin (Sigma-Aldrich, 476693, USA), DLP1 (BD Transduction Lab, 611112, USA), Phospho-DRP1 (Ser637) (Cell Signaling, 4867, USA), Phospho-DRP1 (Ser616) (Cell Signaling, 3455, USA), Cysteine S-nitrosylated (SNO-Cys) (U.S. Biological Life Sciences, C9002-75, USA), Tom20 (Santa Cruz Biotechnology, 628381, USA), Ubiquitin (Santa Cruz Biotechnology, P4D1, USA), Anti-phospho-Ubiquitin (Ser65) (Sigma-Aldrich, ABS1513-I, USA), V5 (Invitrogen, R96025, USA), Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson Immuno Research, 115-035-003, USA), Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson Immuno Research, 111-035-003, USA).

### **3. METHODS**

UM-SCC-1A cells were used in this study's experiments with different chemicals and equipment.

#### **3.1. CELL CULTURE**

##### **3.1.1. Cell Growth Conditions**

UM-SCC-1A is a HNSCC cell line isolated from surgical resection of a tumor on the mouth's floor. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, 10 % fetal bovine serum (FBS), 1 % penicillin, and streptomycin prophylactic antimycoplasma reagent. All cells were cultured at 37°C with a 5 % CO<sub>2</sub>-humidified air incubator. UM-SCC-1 cells were generated by stable transduction with pLKO-shSCR, pLKO-shpLC3, pLKO-shp17, sh-iNOS plasmids. All cells were treated either with the vehicle, 10 µM LCL768, for indicated periods.

##### **3.1.2. Thawing Cells From The Storage**

Cryopreserved cells were taken from liquid nitrogen and incubated in a 37°C water bath until they melted. As soon as the cells were thawed, the suspension was transferred into the 15 mL conical, and 9 mL of growth media was added dropwise into the tube immediately. Next, the cells were centrifuged at 300 g for 5 minutes, and the supernatant was discarded. Then, the cell pellet was resuspended in 5mL complete media, transferred into the cell culture flask, and incubated in an incubator at 37°C.

### **3.1.3. Cell Passaging**

When cells reached a confluency of 80-85 %, the contained medium was discarded. The cells were washed with 3 mL PBS, and it was discarded. 2-4 mL of 0.25 % trypsin-EDTA was added to the cells and the flask with attached cells was incubated at 37°C for 5 minutes. After 5 minutes, cells were monitored for detachment with a light microscope. 1:2 ratio of complete media was added to unattached cells and transferred into a 15 mL centrifuge tube. The cells were centrifuged at 300 g for 5 minutes to get rid of trypsin-EDTA. After centrifugation, the supernatant was discarded, and the pellet was resuspended with the complete media. The resuspended cells were transferred into the new culture flask and placed into the CO<sub>2</sub>-humidified air incubator at 37°C.

### **3.1.4. Cryopreservation Of The Cells**

The cells were resuspended with trypsin-EDTA. After centrifugation, the cell pellet was resuspended in the FBS and counted using a hemocytometer. Next,  $1 \times 10^6$  cells were transferred into a cryovial within a freezing mix with the content of 10 % dimethyl sulfoxide (DMSO) and 90 % FBS and placed at the -80°C for freezing. The next day, the cryovial was transferred into the liquid nitrogen -196°C.

### **3.1.5. Determination Of Cell Number**

The cells were resuspended with trypsin-EDTA, centrifuged, and resuspended in the complete media. 10  $\mu$ L aliquot was taken from the cell suspension and loaded into the hemocytometry slide. Four different squares were counted on the hemocytometry slides under the light microscope. The cell number in the suspension was calculated with the equation:

$$\text{Total cell } \frac{\text{number}}{\text{ml}} = \text{The average number of the cells counted for four squares} \\ \times \text{dilution factor} \times 10^4$$

### 3.1.6. Treatment Of Cells With LCL768

LCL768 was prepared in sterile ethanol with a stock concentration of 10 mM. UM-SCC-1A cells were plated 18 hours before treatment. Then, the cells were treated with ethanol as vehicle control or LCL768 (10  $\mu$ M, 20  $\mu$ M, 30  $\mu$ M, 40  $\mu$ M) for 3, 6, and 24 hours.

### 3.1.7. Treatment Of Cells With Nitric Oxide Donor And Inhibitor

UM-SCC-1 cells were treated for nitrosylation studies with a vehicle, eNOS inhibitor N $\omega$ -Nitro-L-arginine methyl ester hydrochloride or NO- and superoxide donor SIN-1 chloride. SIN-1 (chloride) was prepared in sterile ethanol with a stock concentration of 50 mM. N $\omega$ -Nitro-L-arginine methyl ester hydrochloride (L-NAME) was prepared in sterile dH<sub>2</sub>O with a stock concentration of 200 mM. UM-SCC-1A cells were plated 18 hours before treatment. The cells were pre-treated with SIN-1 as a nitric oxide donor or L-NAME as a nitric oxide inhibitor 30 minutes before LCL768 treatment.

## 3.2. CELL VIABILITY ASSAY

Cell viability of UM-SCC-1A cells was analyzed by trypan blue exclusion assay. Cells were cultured and treated with appropriate treatment concentration and time. Media containing dead cells were collected from the well into a 15 mL falcon tube. The remaining media and cells were washed with 1X PBS and added into the same falcon tube with old media. Trypsin-EDTA was added to wells and incubated for 5 minutes at 37°C. Complete media was added to unattached cells to stop the reaction and collected into the same conical tube.

The cells were centrifuged at 300 g for 5 minutes. After centrifugation, the media was discarded, and the pellet was rinsed in 2 mL PBS and centrifuged at 300 g for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 1 mL PBS. In order to determine the number of viable and non-viable cells in the sample, 0.4 % trypan blue and the aliquots of cell suspension were mixed in an Eppendorf tube with a ratio of 1:1 and incubated for 5 minutes at room temperature. The non-viable cells stained with trypan blue and unstained viable cells were counted separately with a hemocytometer. The percentage of viability was calculated:

$$Viability (\%) = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

### 3.3. CELL LYSIS

Cellular lysates were collected in RIPA Lysis Buffer with a 1 % protease phosphatase inhibitor cocktail and 1 % phosphatase inhibitor cocktail. For ubiquitination experiments, PR-619 was used as a general inhibitor of deubiquitylating enzymes (DUBs) with a concentration of 15  $\mu$ M. The cell pellet was rinsed with lysis buffer (2 times the pellet volume) and homogenized with using a 26-G insulin syringe. The cell pellets were incubated on ice for 20 minutes and centrifuged at 13,000 g for 15 minutes at 4°C. The supernatant was transferred into a new tube, and the lysate was stored at -80°C.

### 3.4. PROTEIN CONCENTRATION MEASUREMENT

The Bradford Assay was used to measure protein concentration. Protein standards were prepared using bovine serum albumin with the stock solution of 1 mg/mL. The standards were prepared in a 1.5 mL Eppendorf tube as follows:

Table 3.1. Sample protocol for the Bradford assay.

| Add BSA (1mg/mL stock; $\mu$ L) | Add MilliQ dH <sub>2</sub> O ( $\mu$ L) | Final BSA Concentration (mg/mL) |
|---------------------------------|-----------------------------------------|---------------------------------|
| 0                               | 800                                     | 0                               |
| 2                               | 798                                     | 2                               |
| 4                               | 796                                     | 4                               |
| 6                               | 794                                     | 6                               |
| 8                               | 792                                     | 8                               |
| 12                              | 788                                     | 12                              |
| 16                              | 784                                     | 16                              |
| 20                              | 780                                     | 20                              |

The samples were prepared with the addition of 2  $\mu$ L of lysate into 798  $\mu$ L of MilliQ dH<sub>2</sub>O in the 1.5 mL Eppendorf tube. 200  $\mu$ L protein assay dye reagent was added to the lysates and mixed. 200  $\mu$ L of samples and the standards were put into 96-well-plate triplicate, and the absorbance was measured at 595 nm. The standard curve was prepared using standards' absorbance values, and the cell lysates' protein concentration was calculated by using standard curve.

### **3.5. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)**

Protein samples were separated by electrophoresis. The samples were mixed with 6x Laemmle buffer and denatured by heating at 95°C for 5 minutes. After heat block, the samples and a molecular weight marker were loaded into 4-20 % Mini-PROTEAN Precast Protein Gels in a Mini PROTEAN Tetra Cell Electrophoresis system filled with 1X running buffer. The gel was run until the dye in the sample reached the lower limit.

### **3.6. WESTERN BLOTTING**

After running the gel, it was separated from the glasses and incubated in the transfer buffer following the SDS-PAGE procedure. First, the PVDF membrane was incubated in 100 % methanol for 3 minutes for activation and transferred into the transfer buffer. Next, proteins were transferred from gel to the PVDF membrane using a semi-dry transfer system, Trans-Blot Turbo Transfer System. The blocking was the next step when the membrane with proteins was incubated in 5 % BSA for 1 hour at room temperature. After blocking, PBST washing was applied to the gel three times and incubated in primary antibody in 5 % BSA (ACO2, LC3B, ACO2, cysteine-S-nitrosylated, DLP1, Actin, Lamin, COXIV) overnight. The next day, PBST washing was applied to the gel three times for a 5-minute interval. Next, the membrane was incubated with a secondary antibody prepared in a blocking buffer for 60 minutes at room temperature. After 1 hour, the secondary antibody has removed, PBST washing was applied to the gel three times, and the HRP substrate was added to the membrane and incubated for 5 minutes. Finally, the excess substrate was removed, and the image was captured by Bio-Rad Chemidoc X.

### **3.7. CELL TRANSFECTION**

#### **3.7.1. Stable Transfection**

293T cells were cultured in a 6-well plate and incubated overnight. 293T cells were co-transfected with pCMV-psPAX2 and pMD2 plasmids, and the plasmid of interest are LC3, Drp1, p17, and nontargeting shRNA. The cells with plasmids were incubated for 48 hours. Viral media was collected from 293T cells into the centrifuge tube. The media was centrifuged at 200 g for 5 minutes and filtered into the new falcon tube with a 0.45  $\mu$ m filter. Next, 8  $\mu$ g/mL polybrene was added to filtered media, and the viral media was added to the 20-50 % confluent UM-SCC-1A cells. The UM-SCC-1A cells with viral media were

incubated for 72 hours at 37°C, and antibiotic selection was started by replacing viral media with new growth media, including puromycin, for 2 weeks. Western blotting was used to confirm the transfection, and finally, cryo-stocks of stably transfected cells were prepared for further experiments.

### **3.7.2. Transient Transfection**

Effectene Transfection Reagent was used for transient transfection of UM-SCC-1 cells. The cells were seeded the day before transfection into a 100 mm dish. The cell must be 40-80 % confluent on the transfection day. To start the transfection, 2 µg of DNA was diluted in 298 µl EC buffer, and 16 µl of Enhancer was added to the diluted DNA. The sample was vortexed for a second and incubated at room temperature for 5 minutes. Then, 60 µl Effectene Reagent was added to the DNA-Enhance mixture. The mixture was vortexed for 10 seconds and incubated at room temperature for 10 minutes. While the mixture was incubated, the media on UM-SCC-1 cells was aspirated, 1x PBS was added to the cells for washing and 7 mL growth media was added to the cells. After incubation, 3 mL of growth media was added to the transfection complexes and pipetted for equally distributing. The transfection complexes were added dropwise to the target cells and swirled gently. UM-SCC-1 cells with transfection complexes were incubated for 24-96 hours. The transfection efficiency was confirmed by western blotting.

### **3.8. CYTO-ID**

The autophagic response was analyzed using the Cyto-ID Autophagy Detection kit by staining the lipidated form of LC3 with Cyto-ID Green dye. The cells were cultured in 35-mm glass-bottomed dishes and treated with vehicle or LCL768. After treatment, cells were washed and stained with Cyto-ID Green dye. The staining was visualized by Olympus

FluoView FV10i LIV laser scanning confocal microscope in the MUSC Cell and Molecular Imaging Core Facility.

### **3.9. MEASUREMENTS OF OXYGEN CONSUMPTION RATE (OCR)**

UM-SCC-1A cells were cultured in XF96 96-well plates overnight. The cells were treated either with vehicle or 10  $\mu$ M LCL768. Seahorse Bioscience XF96 instrument was used to measure the rate of O<sub>2</sub> with the automatic addition of various inhibitors (1  $\mu$ M oligomycin, 1  $\mu$ M FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazine, 1  $\mu$ M antimycin A, 2  $\mu$ M rotenone) to the cells.

### **3.10. IMMUNOPRECIPITATION**

Equal amounts of cellular lysates were incubated with 5  $\mu$ g of corresponding antibodies at 4°C overnight. The next day, the antibody-containing samples were added to the tubes, including pre-washed Protein A/G magnetic beads, and incubated for 1 hour at room temperature. Then, the beads were washed three times, and proteins were eluted from the beads by adding loading buffer and heat block. SDS-PAGE and Western blotting were used to analyze the immunoprecipitated proteins.

### **3.11. SAMPLE PREPARATION FOR LIPIDOMICS ANALYSIS**

UM-SCC-1A cells, cultured in 100 mm cell culture dishes, were collected in 15 mL conical tubes. The cells were lysed with RIPA buffer and prepared for analyses of endogenous sphingolipids on a Thermo Fisher TSQ Quantum liquid chromatography/triple-stage quadrupole mass spectrometer by the Analytical Unit in the Lipidomics Shared Resource at MUSC. Lipid levels were normalized by the protein level, including in samples (pmol/mg protein).

### 3.12. METABOLITE PROFILING AND TARGETED METABOLIC FLUX ANALYSIS

UM-SCC-1A cells with different transfection: shSCR, shLC3, shDrp1, and shp17 were grown and treated with either vehicle control or LCL768. The medium of the cells was changed 2 hours before metabolite extraction. Then, the medium was discarded, and the cells were washed with 5-7 mL of ice-cold normal saline solution (not PBS) twice and aspirated again. Next, the plates were rested on dry ice, and the cell scraping was performed with the cell scraper right after the addition of 1 mL of 80 % (vol/vol) methanol. Then the cell containing the methanol mixture was transferred to a 1.5 mL conical tube and frozen in liquid nitrogen. Three freeze-thaw cycles were applied on the cells. The cells were vortexed after each cycle, and stored overnight in the  $-80^{\circ}\text{C}$  freezer for protein precipitation. The next day, the lysates were centrifuged at about 20,000 g for 15 minutes at  $4^{\circ}\text{C}$ . Finally, the supernatants were transferred to new conical tubes and transferred to the metabolomics core facility. Normalized peak areas of metabolites were measured by LC-MS/MS. Data were analyzed by the MetaboAnalyst V5.0 platform (<https://www.metaboanalyst.ca>) (Pang et al., 2021). For the flux experiment, the cells were labeled with  $^{13}\text{C}_3$ -pyruvate or  $^{13}\text{C}_5$ -glutamine for the final hour of LCL768 or vehicle treatment.  $^{13}\text{C}_3$ -pyruvate was prepared at a concentration of 110 mg/L in a DMEM medium without pyruvate, and  $^{13}\text{C}_5$ -glutamine was prepared at a concentration of 584 mg/L in a DMEM medium without glutamine. Untreated cells were used as a control, and they were cultured in a complete DMEM medium without any tracer. The hydrophilic metabolites were collected by cold methanol extraction. Targeted LC-MS/MS measured normalized peak areas of  $^{13}\text{C}$ -labeled metabolites. Data are expressed as the ratio of the newly synthesized metabolite generated from the metabolization of the tracer over the unlabeled metabolite generated from endogenous pyruvate or glutamine.

### **3.13. PROTEOMIC ANALYSIS**

UM-SCC-1A cells were grown in a six-well plate and incubated for 18 hours. The cells were treated either with LCL768 or vehicle for 24 hours. The cells were collected, lysed, and proteins were separated in SDS-PAGE gel. After separation, the gel was washed 3 times with ddH<sub>2</sub>O for 5 minutes. Next, Coomassie stain was added to the gel and incubated at room temperature on a shaker for 1 hour. After staining, destaining was performed with ddH<sub>2</sub>O for at least 30 minutes. The image of the gel was captured by Bio-Rad Chemidoc X. The targeted bands were excised and transferred into a micro centrifuge tube with ddH<sub>2</sub>O. The samples were analyzed with LC-MS/MS for protein identification.

### **3.14. NITRIC OXIDE (NO) MEASUREMENT**

UM-SCC-1A cells were grown in a black 96-well tissue culture plate. After treatment with NO inducer, NO inhibitor, and LCL768, media was discarded from the wells. Next, the cells were incubated with 10 mM DAF-FM diacetate for 30 minutes at room temperature. The cells were washed with 1xPBS after 30-minute incubation. Then, fresh media was added into the plate and the cells were incubated for another 30 minutes to complete the de-esterification of the intracellular diacetates. Next, 10 mM of DCFH-DA was applied to the cells to quantify peroxynitrite formation, which is oxidized by peroxynitrite to fluorescent dichlorofluorescein (DHF) with Ex/Em 502/523. After 45 minutes of incubation at room temperature, the media was discarded. Then fresh growth media was added into plates and incubated for another 45 minutes at room temperature. Lastly, fluorometric measurement was performed by microplate reader.

### **3.15. SUBCELLULAR FRACTIONATION**

UM-SCC-1A cells were scraped and washed with cold PBS. Next, the cells were centrifuged at 200 g for 7 minutes to get a pellet. Then, the cells were resuspended in STM fractionation buffer and homogenized on ice for 1 minute. The homogenized cell content was incubated on ice for 30 minutes. Then it was vortexed in a centrifuge tube for 15 seconds before centrifugation at 800 g for 15 minutes. This pellet included nucleus and debris, and the supernatant contained the cytosolic and mitochondrial fractions. This new pellet was resuspended in fractionation buffer and vortexed. Then, it was centrifuged at 500 g for 15 minutes. Then, it was vortexed and centrifuged at 1,000 g for 15 minutes. The new pellet was dissolved in NET buffer, vortexed and incubated for 30 minutes on ice. The fraction was lysed with 18-gauge needle and centrifuged at 9,000 g for 30 minutes for nuclear fraction. Thereafter, supernatant of the first centrifugation was centrifuged at 800 g for 20 minutes. This supernatant was collected and centrifuged at 11,000 g for 10 minutes, and the new supernatant was precipitated in cold 100% acetone at -20°C for 1 hour and centrifuged at 12,000 g for 5 minutes. The pellet was resuspended in STM buffer, and pellet from this cytosolic fractionation was resuspended in fractionation buffer and centrifuged at 11,000 g for 10 minutes. The pellet was resuspended in SOL buffer, and the fraction was processed as a mitochondrial content. Immunoblotting was performed to assess the purity levels of the subcellular fractions.

### **3.16. IMMUNOFLUORESCENCE**

UM-SCC-1A cells were plated on 4-well dishes for 18 hours. Then the cells were treated with LCL768 with corresponding time and conditions. First, washing the cells was carried out with 1x PBS and fixed with 4 % paraformaldehyde for 20 minutes. Then, permeabilization was performed by using 0.1 % Triton X-100 in 1× PBS (pH 7.4) for 10 min. Next, blocking was applied on the cells with 1% BSA / PBS (pH 7.4) for 1 hour. Then,

the antibody incubation was applied with corresponding antibodies in a blocking solution (1:50) for 18 hours at 4°C. Thereafter, the samples were washed with 1x PBS and the cells were stained with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (1:1000) with an hour incubation period. Immunofluorescence was completed by using an Olympus FV10i microscope involving the channels of 543- and 488-nm. Images were taken at three random fields at ×63 magnification.

### **3.17. PROXIMITY LIGATION ASSAY**

UM-SCC-1A cells were plated on 4-well dishes for 18 hours. Then the cells were treated with LCL768 with corresponding time and conditions. First, cells were fixed with 4 % paraformaldehyde for 20 minutes. Then, permeabilization was performed by using 0.1 % Triton X-100 in 1× PBS (pH 7.4) for 10 min. Next, the cells were blocked with a drop of Duolink Blocking Solution for 60 minutes at 37°C. Then cells were incubated with corresponding antibodies in Duolink Antibody Diluent overnight in a heated humidity chamber at 37°C. Next, the slides were washed with Wash Buffer A, and ligation solution was added to each sample and incubated for 30 minutes at 37°C. Then, the cells were washed with Buffer A and the amplification solution was added to each sample and incubated for 100 minutes at 37°C. Finally, the slides were washed with Wash Buffer B and waited to be dried in the dark. The slides were mounted with mounting media and visualized using an Olympus FV10i microscope. Images were taken at three random fields at ×63 magnification and quantified by DuOlink Image Tool Software (Olink BioScience).

### **3.18. EX VIVO CULTURE OF TUMOR-DERIVED 2D ORGANOID FROM HNSCC PATIENTS**

The surgical removal of HNC and adjacent normal tissue explants was obtained to culture 2D Organoids from patients using an IRB-approved protocol. Tissues were cut in 1 mm<sup>3</sup>

cube in growth media containing dish by razor and placed on top of an absorbable gelatin sponge in complete DMEM media with an addition of a vehicle and LCL768 for 3 days. After 3 days, tissues were collected and processed for further experiment.

### **3.19. VERTEBRATE ANIMALS**

Institutional Animal Care and Use Committee at MUSC approved animal experiment for this study. Female NSG mice were get from The Jackson Laboratory with the age of 8 to 12 weeks, weighing 18 to 22 g. The SCID mice were injected with UM-SCC-1A human HNSCC cells into both flanks to establish human tumor xenografts. Tumor development has waited for 2 weeks and xenograft-derived tumor growth in the with/without LCL768 for 21 days (3-6 mg/kg, 3 injections/week via intraperitoneal injections) compared to vehicle-treated controls followed by euthanasia. Tumor size and condition of animals are measured every other day until the experiment's termination. Caliper measurement was used for calculating tumor size.

### **3.20. STATISTICAL ANALYSIS**

Graphpad Prism 8 was for the graphs of data, which were revealed as mean standard deviation or standard error as signified. The data were compared using the 2-sided Student t-test or analysis of variance (ANOVA).

## **4. RESULTS**

The results of this study demonstrated a novel mechanism for LCL768 in the regulation of ceramide-mediated mitophagy in HNSCC.

#### 4.1. ANALYSIS OF ACO2 AND LC3 PROTEIN LEVELS UPON LCL768 TREATMENT

ACO2 degradation and lipidated LC3 for UM-SCC-1A cells were analyzed upon LCL768 treatment with different incubation times and concentrations. Separated proteins were probed for ACO2 and LC3 antibodies normalized to the levels of actin. The ACO2 degradation and the lipidated and active form of LC3 were increased in time and dose-dependent manner in UM-SCC-1A cells (Fig 4.1).

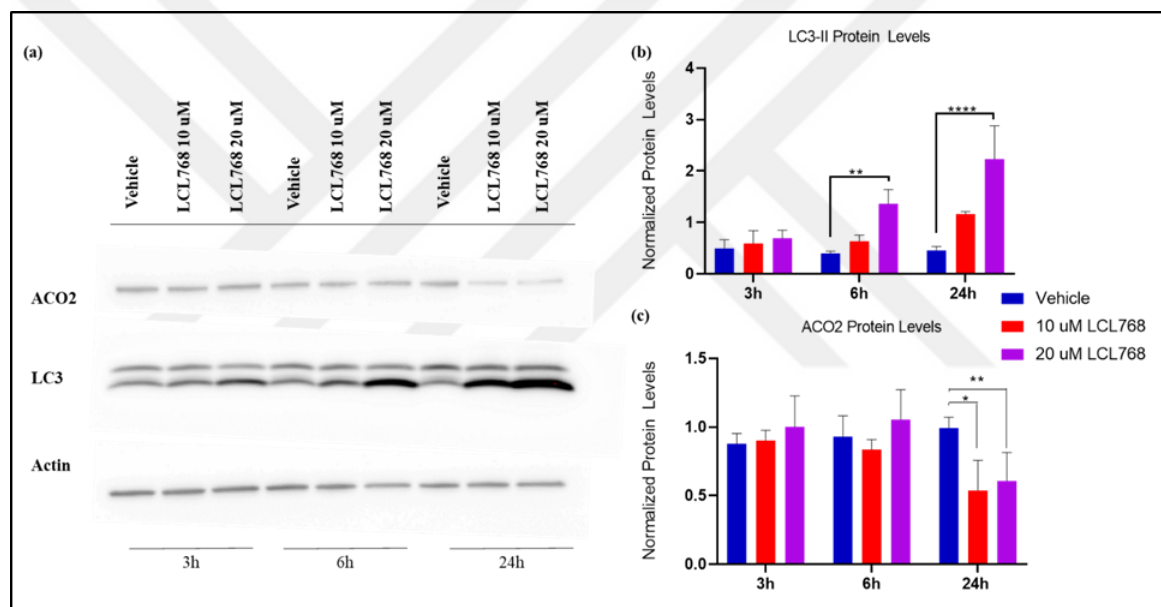


Figure 4. 1. ACO2 and LC3 protein levels in UM-SCC-1A cells upon LCL768 treatment. (a) Western blot for ACO2 and LC3B proteins in UM-SCC-1A cells treated with 20, 30, and 40 uM LCL768 for 3,6, and 24 hours. Actin was used as a western blot loading control (b), (c), and Quantifications were performed using Fiji software. Data are means  $\pm$  SD (n=3 independent experiments, \*\*p <0.05).

#### 4.2. ANALYSIS OF PROTEIN EXPRESSION IN SHRNA-MEDIATED TRANSFECTED UM-SCC-1A CELLS

shRNA-mediated stable knockdown (described in Section 2.7.1) of LC3, Drp1, and p17 in UM-SCC-1A was confirmed with western blot analysis (described in Sections 2.5 and 2.6). As a result, LC3 (Figure 4.2.a), Drp1 (Figure 4.2.b), and p17 (Figure 4.2.c) protein levels decreased compared to wild-type or shscr UM-SCC-1A cells.

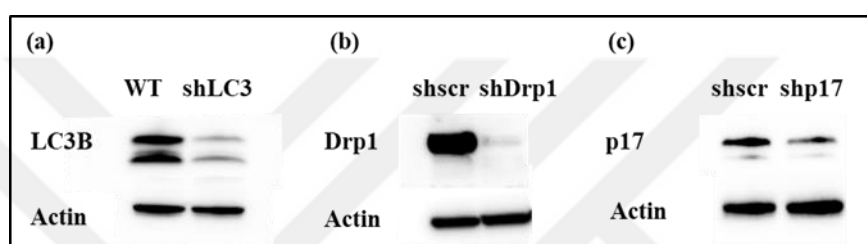


Figure 4. 2. LC3, Drp1, and p17 protein levels in UM-SCC-1A cells upon shRNA-mediated stable transfection. (a) Western blot for LC3B-I and LC3B-II, Drp1 (b), and p17 (c) in UM-SCC-1A cells stably transfected with LC3, Drp1, and p17 shRNAs. Actin was used as a western blot loading control.

#### 4.3. ANALYSES OF ACO2, LC3-II, AND PHOSPHO-UBIQUITIN PROTEIN LEVELS OF SHRNA-MEDIATED TRANSFECTED UM-SCC-1A CELLS UPON LCL768 TREATMENT

ACO2 degradation, lipidated LC3, and phosphorylated ubiquitin levels for UM-SCC-1A cells were determined by Western Blotting analysis (described in Sections 2.5 and 2.6) upon LCL768 treatment in shscr and shRNA-mediated transfected cells. Separated proteins were probed for ACO2, Drp1, LC3, and phosphor-ubiquitin antibodies. In addition, the protein levels of actin were used to determine the equal loading of proteins. As a result, in Figure 4.3.a LCL768 treatment-induced ACO2 degradation and LC3 activation in a dose-dependent response in all cells. Though, the knockdown of LC3 and Drp1 had a preventive role in this

process when their protein levels were compared to shscr. In Figure 4.3.b, LCL768 induced ubiquitin phosphorylation in shscr and shLC3 cells, but shDrp was largely protected.

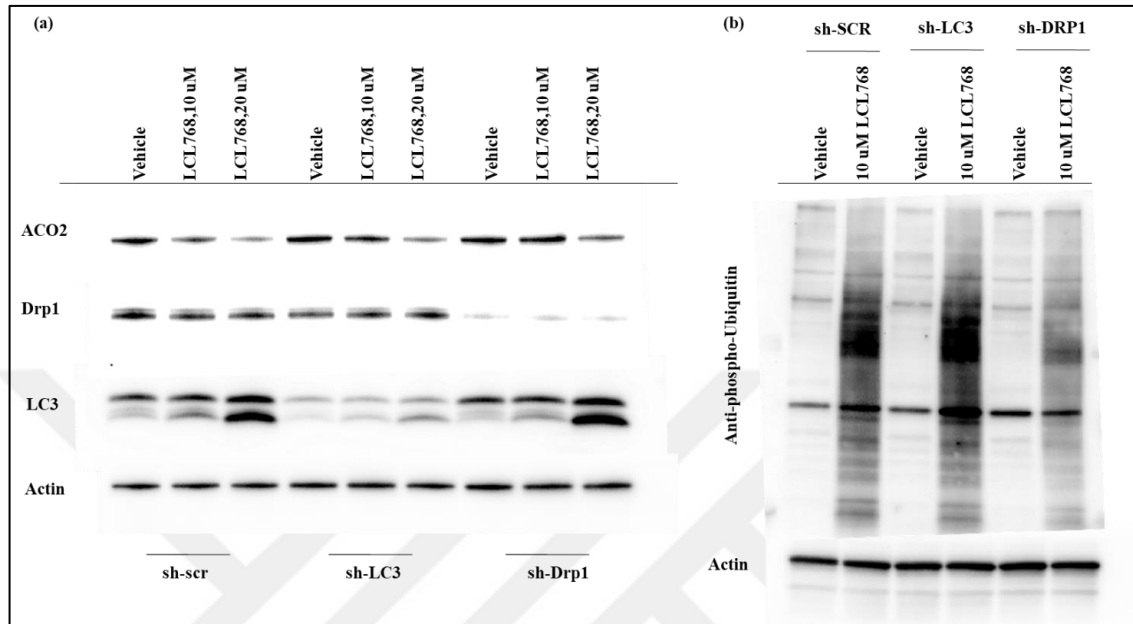


Figure 4.3. Altered protein levels in shscr and shRNA-mediated knockdown UM-SCC-1A cells upon LCL768 treatment. Western blot for (a) ACO2, Drp1, LC3B, and (b) phospho-ubiquitin proteins in UM-SCC-1A cells transfected either with shscr or shLC3 or shDrp1 were treated with 10,20  $\mu$ M LCL768 for 24 hours. Actin was used as a western blot loading control.

#### 4.4. AUTOPHAGY DETECTION UPON LCL768 TREATMENT IN UM-SCC-1A CELLS TRANSFECTED WITH SHSCR, SHLC3, OR SHDRP1

The autophagic response of wild-type, shLC3, and shDrp1 UM-SCC-1A cells was evaluated by Cyto-ID autophagy detection analysis. The 488 nm-excitable green fluorescent was detected to observe autophagosomes responding to LCL768 treatment with different incubation times. According to the results, autophagosome formation was increased 67 times in wild-type cells in a time-dependent manner, whereas autophagosome formation was prevented mainly in shDrp1 and shLC3 cells in Figure 4.4.b.

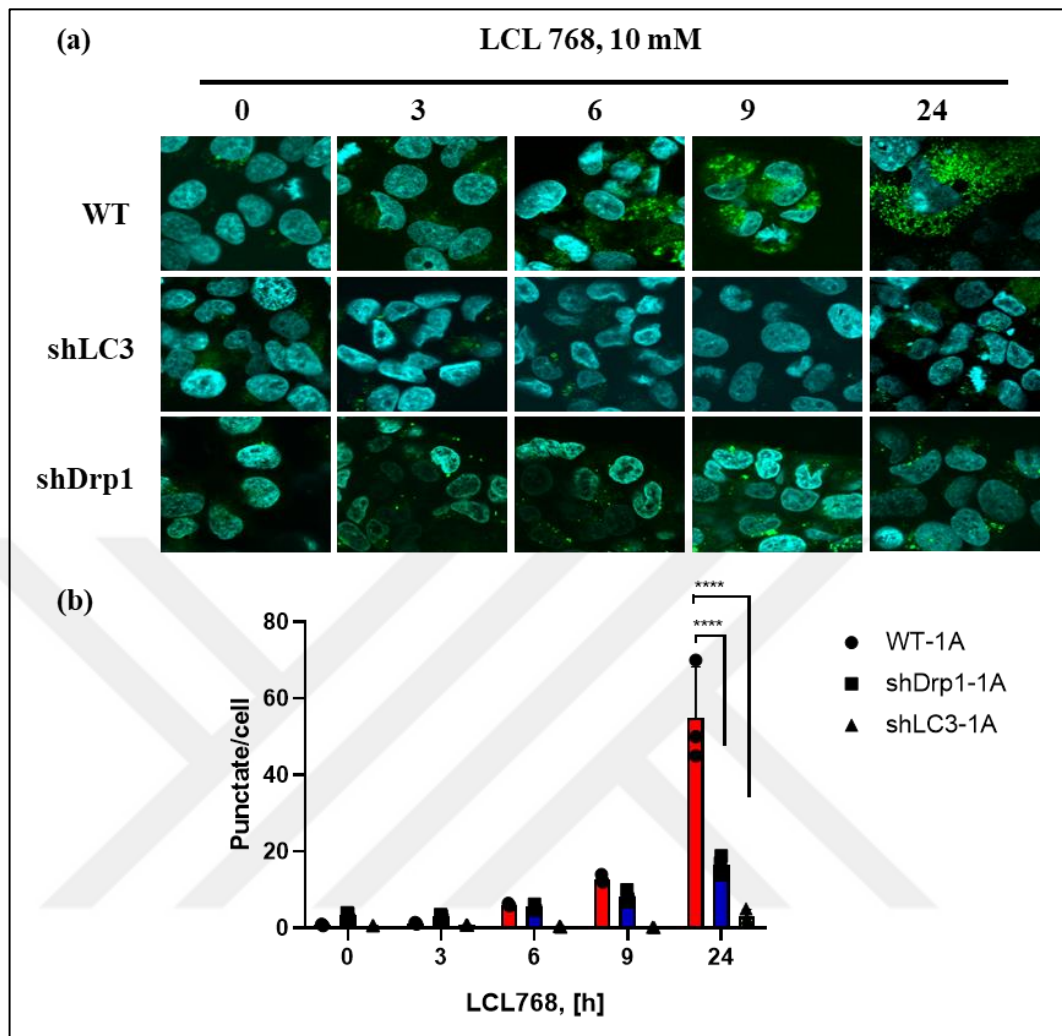


Figure 4.4. Autophagic response detected by Cyto-ID assay in UM-SCC-1A cells in response to LCL768 treatment. (a) Confocal images of Cyto-ID green staining in UM-SCC-1A cells treated either with vehicle or LCL768 (b) Quantifications were performed using Fiji software. Data are means  $\pm$  SD (n=3 independent experiments, \*\*p < 0.001).

#### 4.5. ANALYSIS OF OXYGEN CONSUMPTION RATE OF WILD-TYPE AND SHLC3 UM-SCC-1A CELLS UPON LCL768 TREATMENT

In order to analyze the effects of LCL768 on mitochondrial function by determining mitochondrial respiration and oxygen consumption rate Sea Horse was used in wild-type and shLC3 cells. As a result, LCL768 treatment reduced OCR in wild-type UM-SCC-1A

cells. However, knockdown of LC3 prevented the inhibition of OCR upon LCL768 treatment.

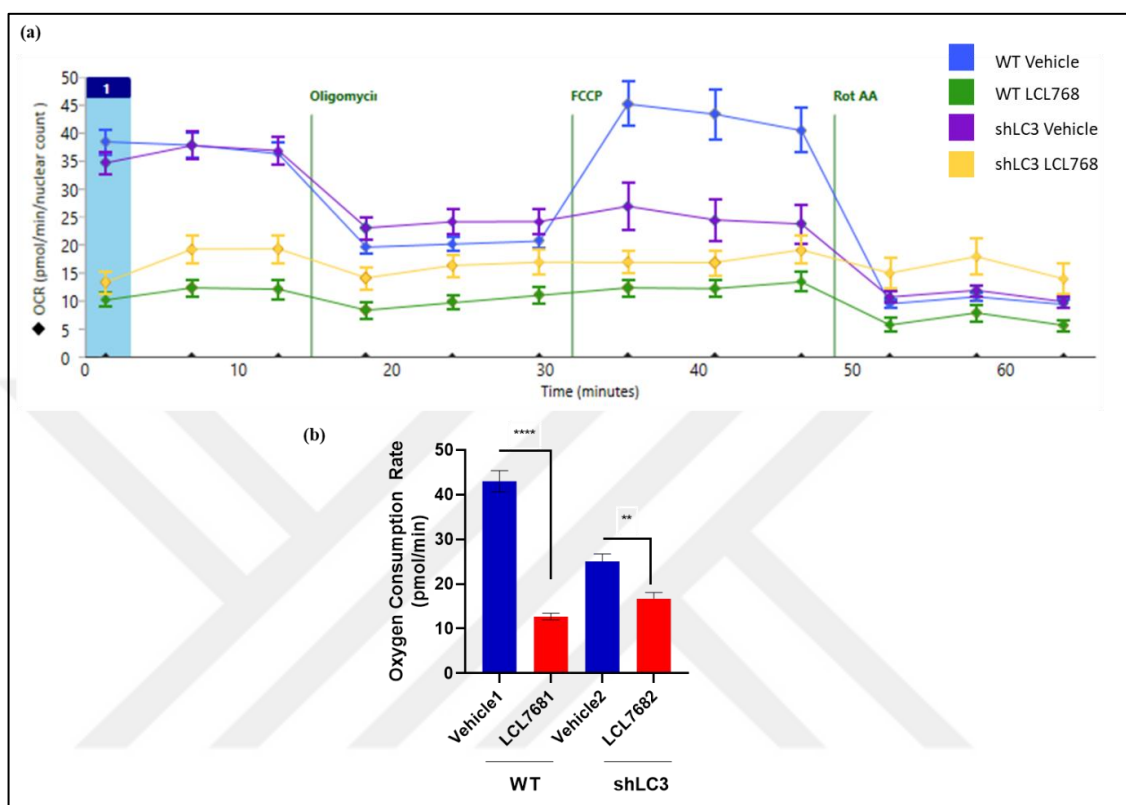


Figure 4.5. Relative changes in oxygen consumption rate in WT and LC3-shRNA transfected UM-SCC-1A cells in response to LCL768 treatment. (a) Metabolic responses of WT and LC3-shRNA transfected UM-SCC-1A cells were used to analyze OCR of basal respiration and the effects of the electron-transport chain inhibitors by Sea Horse upon LCL768 treatment. (b) Quantifications of OCR rate. Data are means  $\pm$  SD (n=3 independent experiments, \*\*p < 0.01 or \*\*\*p < 0.001).

#### 4.6. LIPIDOMICS ANALYSIS OF LCL768 TREATED UM-SCC-1A CELLS

In order to detect subcellular accumulation of LCL768 and its results on endogenous ceramide metabolism in shscr, shDrp1, shLC3, and shp17 cells, mass spectrometry-based lipidomics analyses were performed in nuclear, mitochondrial and cytoplasmic fractions

which were confirmed with western blot analysis. As a result, LCL768 mainly accumulated in mitochondria of shscr cells, whereas this accumulation was prevented in cells with shRNA-mediated knockdown of Drp1, LC3, or p17/PERMIT (Figure 4.6.a and Figure 4.6.b). Also, LCL768 induced endogenous C18-ceramide accumulation in mitochondria in a time-dependent way (Figure 4.6.d).

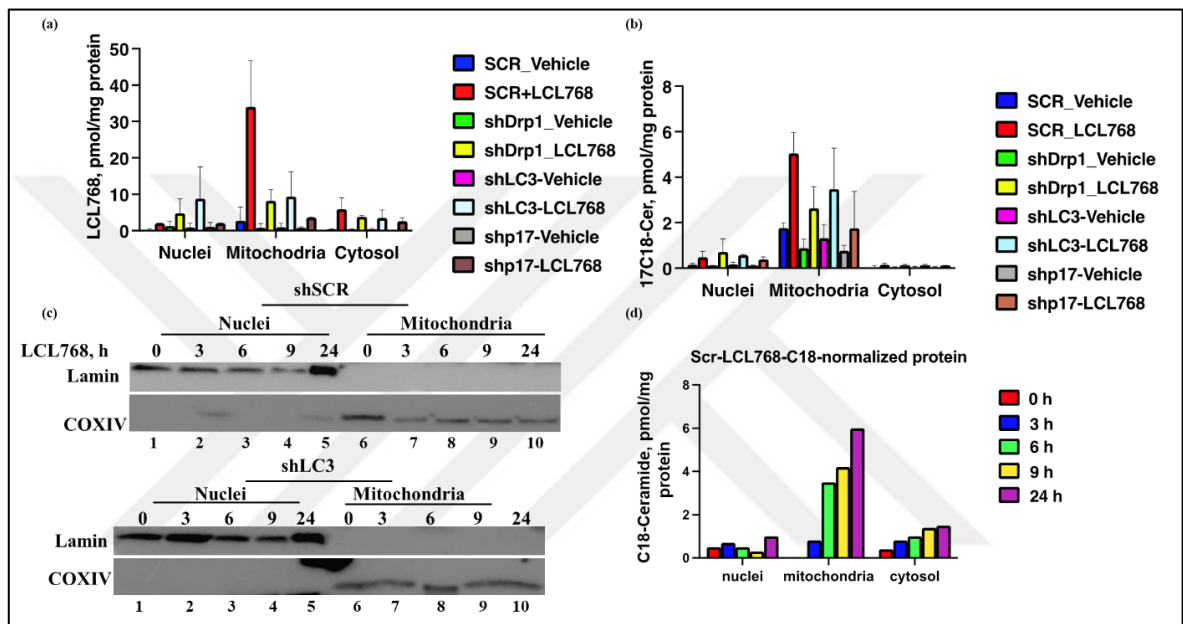


Figure 4.6. Lipidomic analysis of LCL768 treated shscr and shRNA-mediated knockdown UM-SCC-1A cells. (a) Subcellular accumulation of LCL768 in shscr, shDrp1, shLC3, and shp17 UM-SCC-1A cells was measured by lipidomics measurement by LC/MS/MS. (b) Endogeneous C18-ceramide accumulation in response to LCL768. (c) Confirmation of isolated nuclei and mitochondria by western blotting using anti-lamin (nucleus) or COXIV (mitochondria). (d) The mass of C18-ceramide in nuclear, mitochondrial, and cytosolic fractions of shscr, shDrp1, shLC3, and shp17 UM-SCC-1A cells was measured using mass spectrometry lipidomics.

#### 4.7. ANALYSES OF ACO2, LC3-II, AND CERS1 PROTEIN LEVELS OF SHRNA-MEDIATED TRANSFECTED UM-SCC-1A CELLS UPON LCL768 TREATMENT

ACO2 degradation, lipidated LC3, and mitochondrial CerS1 levels of shscr and shDrp1 UM-SCC-1A cells were determined by Western Blotting analysis upon LCL768 treatment. Separated proteins were probed for ACO2, LC3, and CerS1 antibodies. As a result, the knockdown of Drp1 decreased ACO2 degradation and LC3 activation. Also, knockdown of Drp1 attenuated mitochondrial CerS1 levels in response to LCL768 treatment (Figure 4.7).

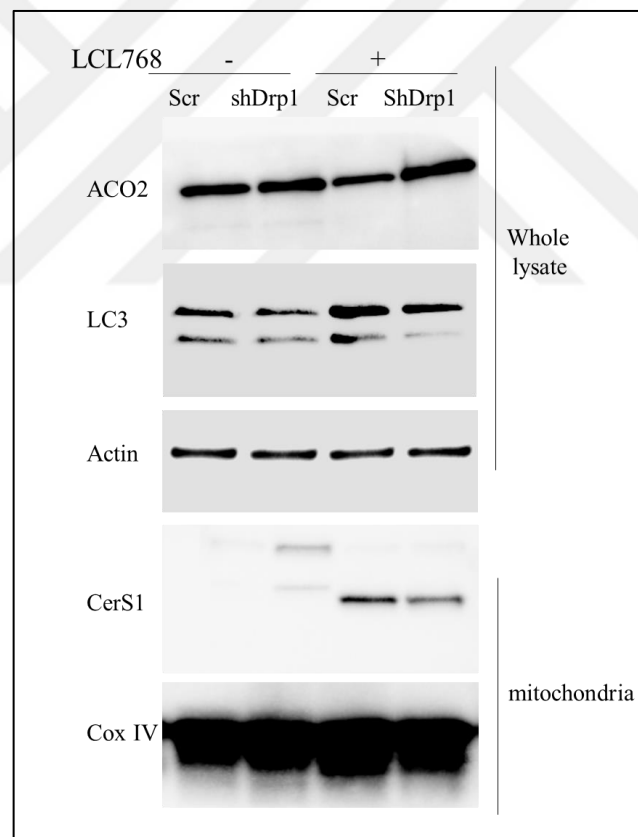


Figure 4.7. Altered protein levels in shscr and shDrp1-mediated knockdown UM-SCC-1A cells upon LCL768 treatment. Western blot for ACO2, Drp1, LC3, and CerS1 proteins in UM-SCC-1A cells stably transfected with shscr or shDrp1 were treated with 10  $\mu$ M LCL768 for 24 hours. Both actin and CoxIV were used as a western blot loading control.

#### **4.8. PROTEOMIC ANALYSIS OF UBIQUITINATED PROTEINS IN LCL768 TREATED UM-SCC-1A CELLS**

To detect ubiquitinated proteins in LCL768 treated UM-SCC-1A cells, co-immunoprecipitation was performed via ubiquitin antibody. The pulled-down proteins were loaded into SDS-PAGE gels, separated and visualized with Coomassie Blue and Silver Staining. The pair of bands which was increased upon LCL768 treatment were cut and analyzed via a mass spectrometry-based approach (Figure 4.8.a). As a result, three different proteins were founded as candidate proteins: Eukaryotic Translation Elongation Factor 1 Alpha 1 (EEF1A1), catalase, and Pyruvate kinase (PKM) (Figure 4.8.b-c).

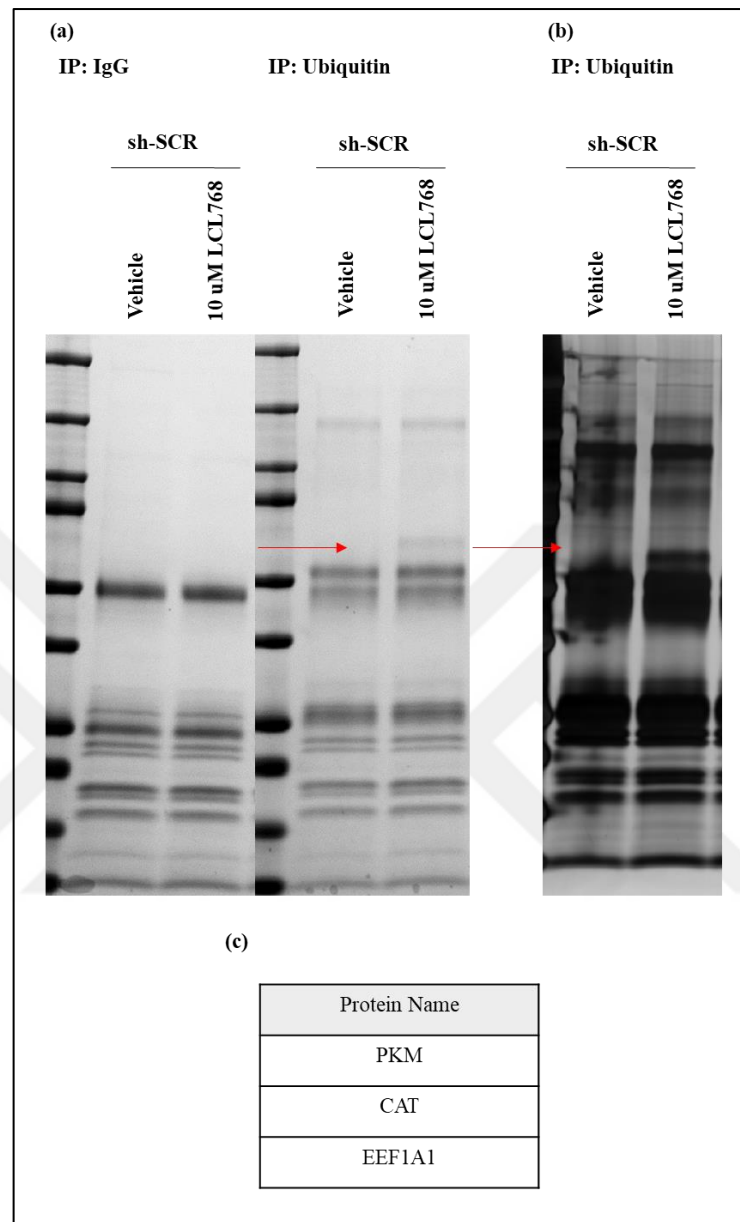


Figure 4.8. MS-based proteomics of LCL768 treated UM-SCC-1A cells. (a) SDS-PAGE gel visualized with Coomassie brilliant blue for ubiquitinated proteins of UM-SCC-1A cells upon 10  $\mu$ M LCL768 treatment for 24 h, (b) SDS-polyacrylamide gel stained with silver staining, (c) List of the proteins identified with mass spectrometry-based proteomics.

#### 4.9. ANALYSES OF MITOCHONDRIAL PROTEIN LEVELS OF UM-SCC-1A CELLS UPON LCL768

In order to detect the effects of LCL768 on mitochondrial protein levels, Western blot analysis was performed. In addition, separated proteins were probed for candidate proteins from proteomics analysis and also for Leucine Rich Pentatricopeptide Repeat Containing (LRPPRC), Tom40, Tom20, COX17, and COXIV antibodies. As a result, LCL768 treatment decreased EEF1A1, catalase, and other mitochondrial protein levels, whereas PKM1/2 levels did not change in UM-SCC-1A cells (Figure 4.9.a-b).

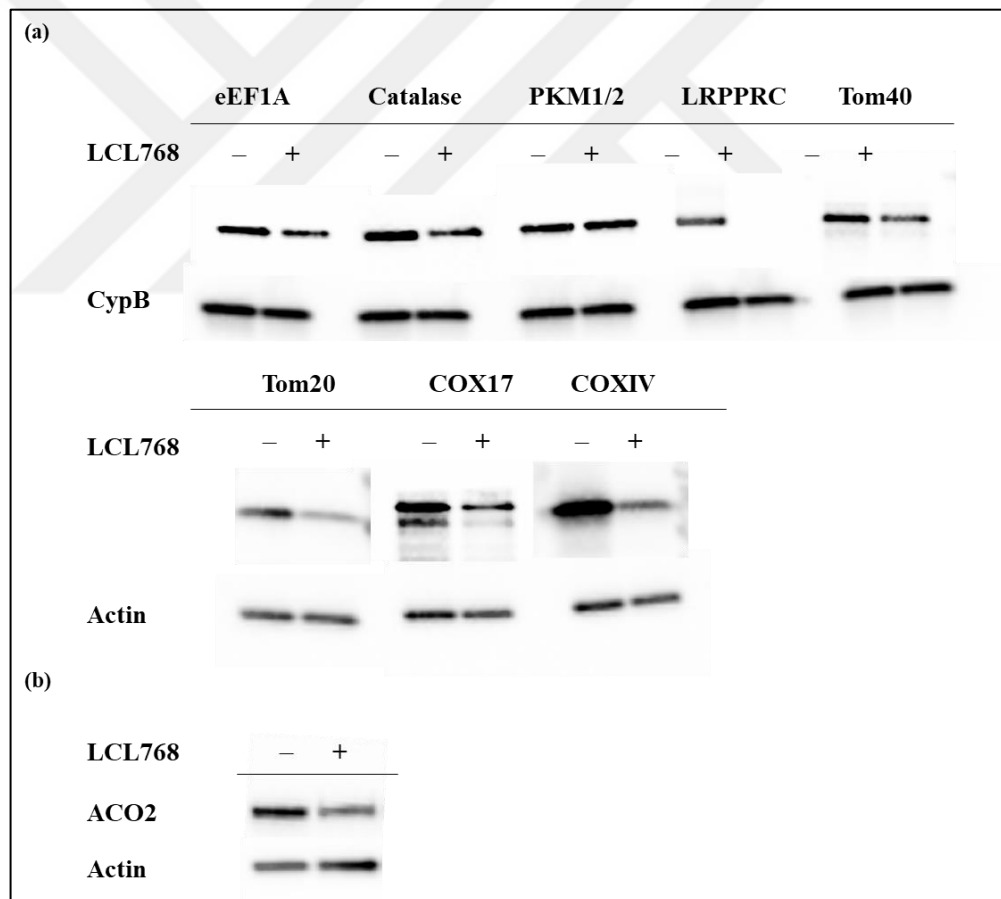


Figure 4.9. Altered protein levels in shscr UM-SCC-1A cells upon LCL768 treatment. (a)

Western blot for EEF1A1, catalase, PKM1/2, LRPPRC, Tom40, Tom20, COX17, and COXIV 1 proteins in UM-SCC-1A cells treated with 10  $\mu$ M LCL768 for 24 hours. Both

actin and cyclophilin B were used as western blot loading control (b) Confirmation of the LCL768 treatment efficiency by western blotting with ACO2 and actin antibodies.

#### 4.10. IMMUNOFLUORESCENCE ANALYSIS OF COLOCALIZATION OF MITOCHONDRIA AND DRP1 IN RESPONSE TO LCL768

The effects of LCL768 treatment on colocalization of Drp1 and mitochondria were measured by immunofluorescence with the Alexa Fluor 488 and 594 attached secondary antibodies for Tom20 and Drp1 antibodies. According to the result, LCL768 induced colocalization between Tom20 and Drp1 by 23.2-fold ( $p < 0.05$ ) (Figure 4.10).

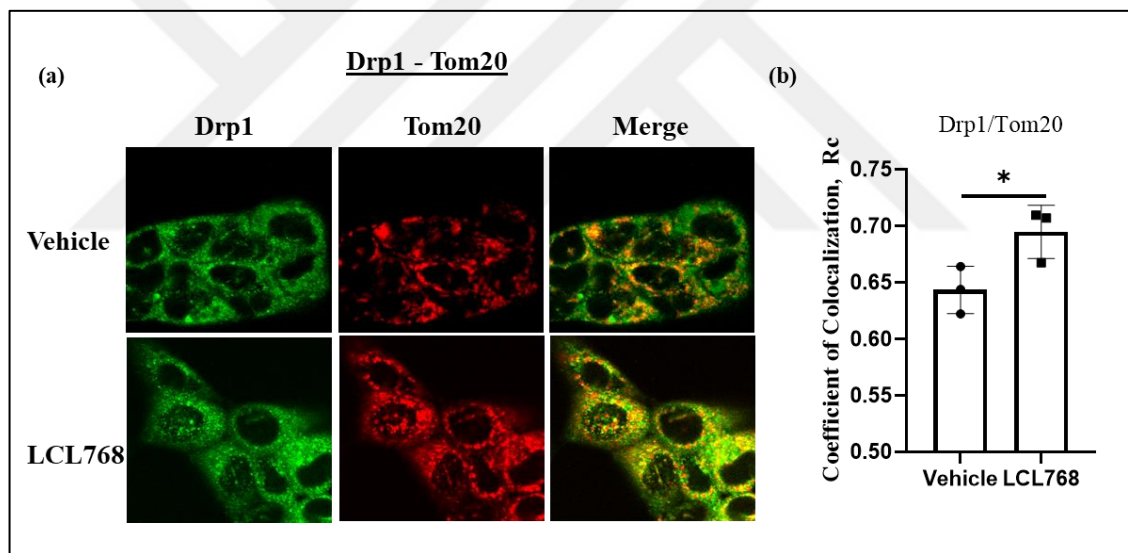


Figure 4.10. Immunofluorescence analysis for LCL768 treatment. (a) Confocal images of immunofluorescence staining of Drp1 (green) and mitochondrial protein Tom20 (red) in UM-SCC-1A cells treated either with vehicle or LCL768. Yellow signal demonstrates colocalization. The images represent one of three independent experiments. (b) Quantification of confocal images of immunofluorescence in (a). Data are means  $\pm$  SD ( $n = 3$  different optical fields,  $*p < 0.05$ ).

#### 4.11. IMMUNOFLUORESCENCE ANALYSIS OF COLOCALIZATION OF ER AND DRP1 IN RESPONSE TO LCL768

The effects of LC768 treatment on colocalization of Drp1 and ER were measured by immunofluorescence (described in Section 2.16) with the Alexa Fluor 488 and 594 attached secondary antibodies for Drp1 and PDI antibodies. According to the result, LCL768 induced colocalization between Drp1 and ER by 50-fold ( $p < 0.01$ ) (Figure 4.11.a-b).

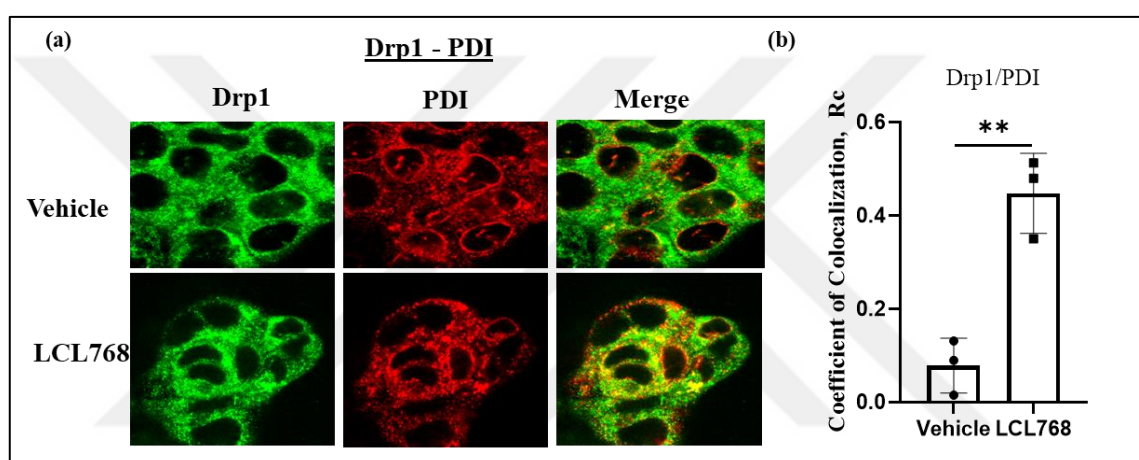


Figure 4.11. Immunofluorescence analysis for LCL768 treatment. (a) Confocal images of immunofluorescence staining of Drp1 (green) and PDI (red) in UM-SCC-1A cells treated with LCL768. Yellow signal demonstrates colocalization. The images represent one of three independent experiments. (b) Quantification of confocal images of immunofluorescence in (a). Data are means  $\pm$  SD ( $n = 3$  different optical fields,  $**p < 0.01$ ).

#### 4.12. IMMUNOFLUORESCENCE ANALYSIS OF COLOCALIZATION OF ER AND MITOCHONDRIA IN SHRNA-MEDIATED KNOCKDOWN UM-SCC-1A CELLS UPON LCL768

In order to analyze the roles of Drp1 and LC3 in the colocalization of Drp1 and mitochondria in response to LC768 treatment, colocalization was measured by immunofluorescence with

the Alexa Fluor 488 and 594 attached secondary antibodies for Tom20 and PDI antibodies. According to the result, LCL768 induced colocalization between Tom20 and Drp1 in shscr cells, whereas the colocalization was prevented in shLC3 and shDrp1 cells ( $p < 0.01$ ) (Figure 4.a-b).

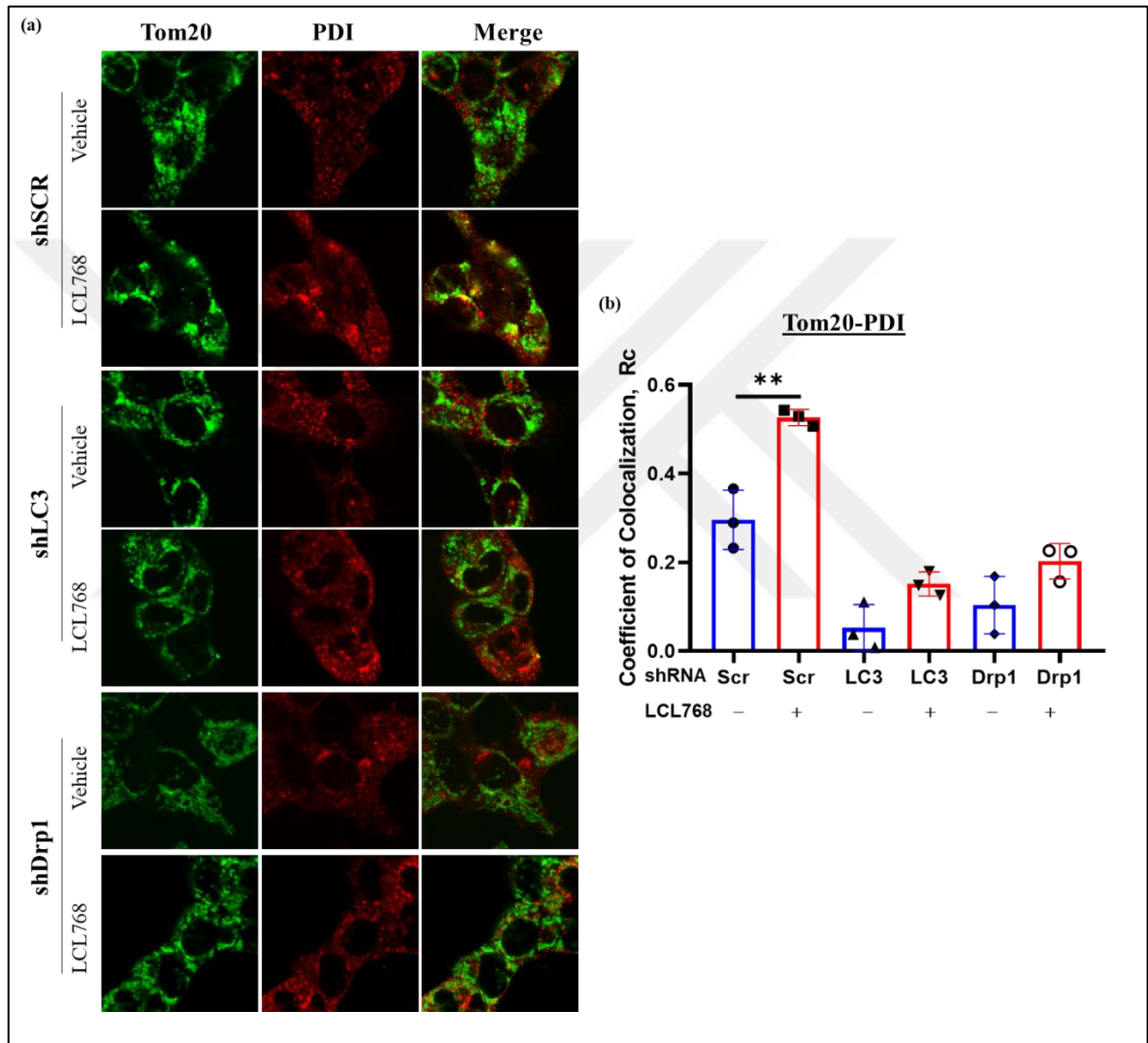


Figure 4.12. Immunofluorescence analysis for LCL768 treatment in shscr, shLC3, and shDrp1 UM-SCC-1A cells. (a) Confocal images of immunofluorescence staining of Tom20 (green) and PDI (red) in UM-SCC-1A cells treated with LCL768. Yellow signal demonstrates colocalization. The images represent one of three independent experiments. (b) Quantification of confocal images of immunofluorescence in (a). Data are means  $\pm$  SD ( $n = 3$  different optical fields,  $**p < 0.01$ ).

#### 4.13. IMMUNOFLUORESCENCE ANALYSIS OF COLOCALIZATION OF CERS1 AND MITOCHONDRIA IN SHRNA-MEDIATED KNOCKDOWN UM-SCC-1A CELLS IN RESPONSE TO LCL768

In order to analyze the roles of Drp1 and LC3 in colocalization of CerS1 and mitochondria in response to LC768 treatment, colocalization was measured by immunofluorescence (described in Sections 2.16) for Tom20 and CerS1 antibodies. According to the result, LCL768 induced colocalization between Tom20 and CerS1 in shscr and shLC3 cells, whereas the colocalization was prevented in shDrp1 cells ( $p < 0.001$ ) (Figure 4.13.a-b).

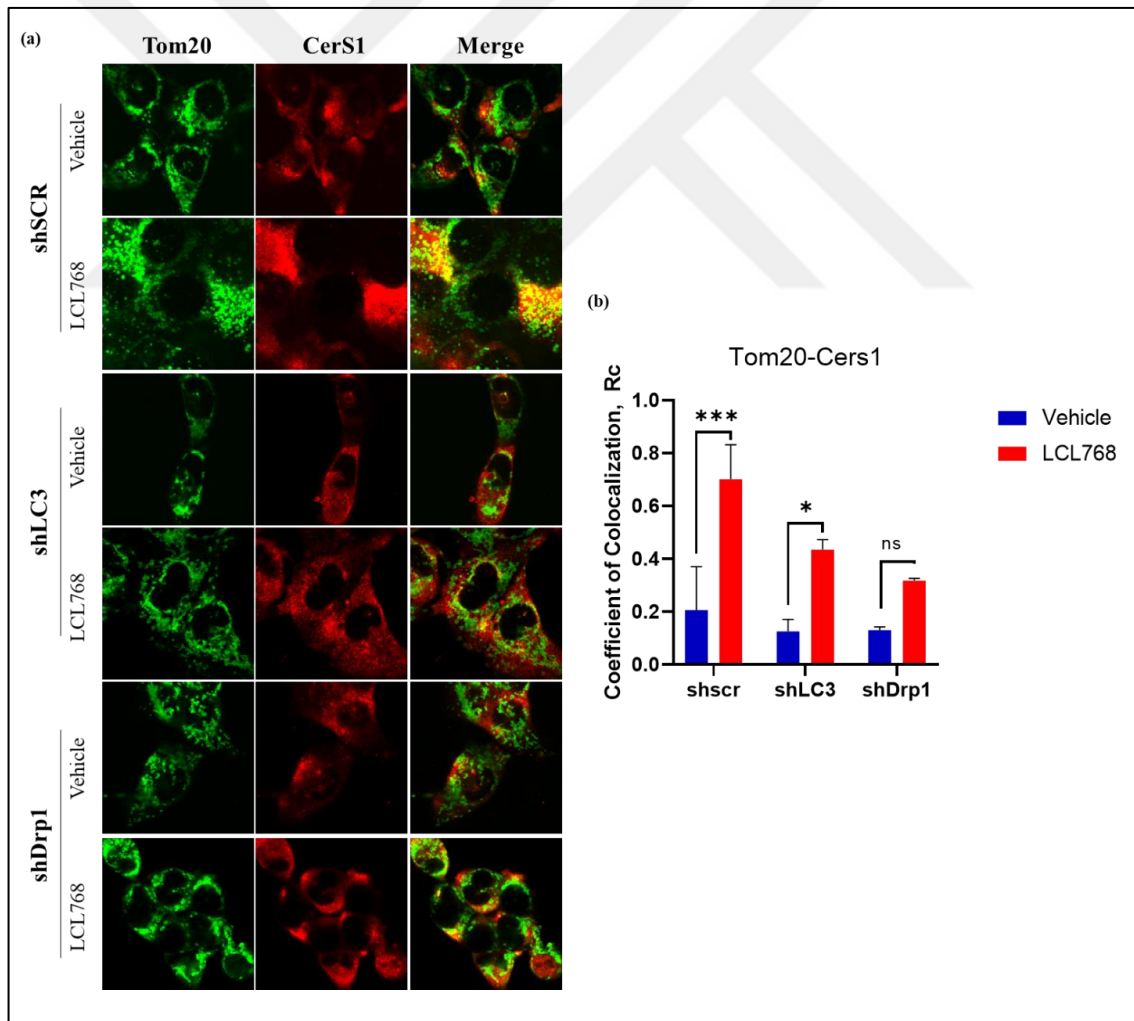


Figure 4.13. Immunofluorescence analysis for LCL768 treatment in shscr, shLC3, and shDrp1 UM-SCC-1A cells. (a) Confocal images of immunofluorescence staining of

Tom20 (green) and CerS1 (red) in UM-SCC-1A cells treated with LCL768. Yellow signal demonstrates colocalization. The images represent one of three independent experiments.

(b) Quantification of confocal images of immunofluorescence in (a). Data are means  $\pm$  SD (n = 3 different optical fields, \*p < 0.05 or \*\*\*p < 0.001).

#### **4.14. ANALYSIS OF INTERACTION BETWEEN ER AND MITOCHONDRIA IN SHRNA-MEDIATED KNOCKDOWN UM-SCC-1A CELLS IN RESPONSE TO LCL768**

Proximity Ligation Assay was performed to analyze the roles of Drp1, LC3, and p17 in the association between ER and mitochondria in response to LC768 treatment (described in Section 2.17). According to the result, LCL768 induced interaction between Tom40 and PDI in shscr cells about 4-fold, whereas the interaction was attenuated in shp17 and shCerS1 cells and almost completely prevented in shDrp1 cells (Figure 4.14.a-b). Also, interaction between mitochondria and ER was confirmed by co-IP assay by using Tom20 (mitochondria) and PDI (ER) antibodies. According to the result, sh-Drp1 knockdown cells prevented the interaction between ER and mitochondria which confirmed the results of PLA assay (Figure 4.14.c).

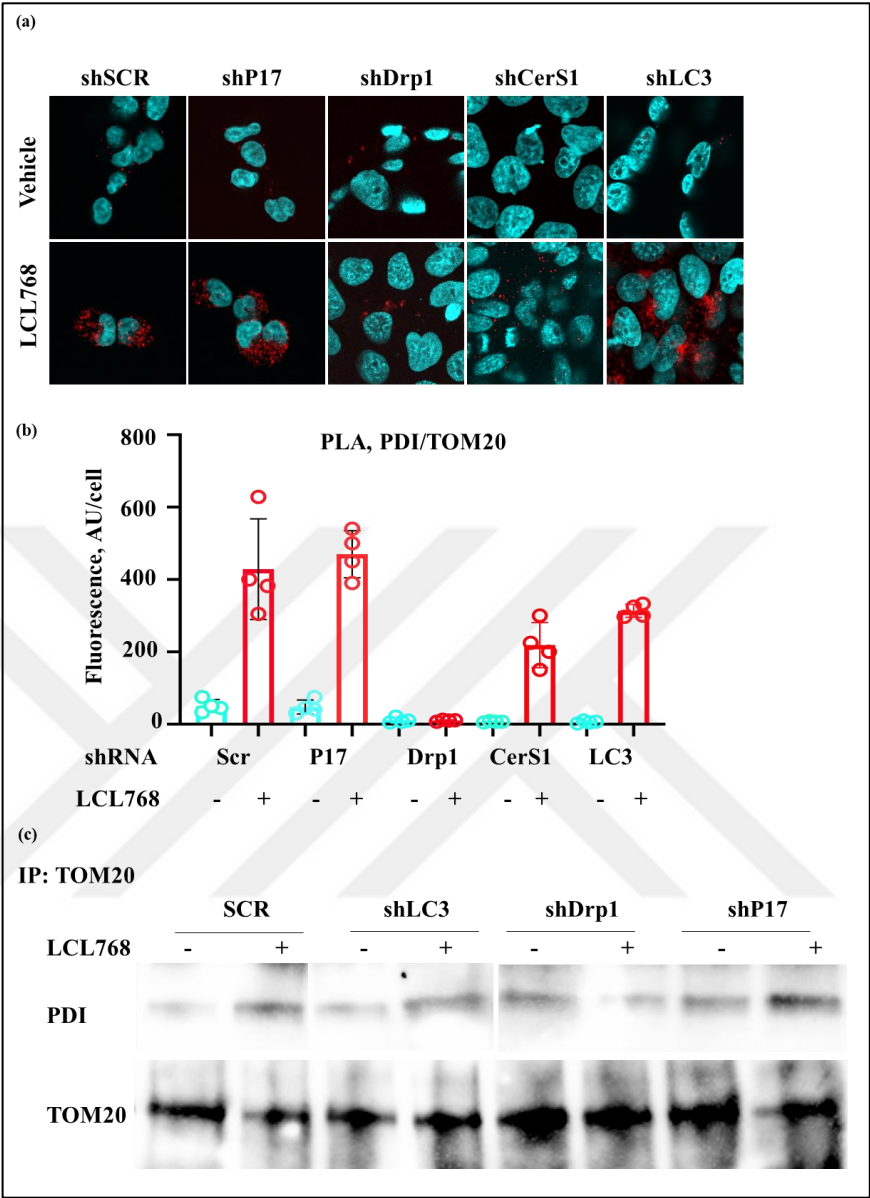


Figure 4.14. PLA analysis of LCL768 treatment in shscr, shLC3, shDrp1, and shp17. (a) Confocal images of PLA fluorescence signals of UM-SCC-1A cells treated with LCL768. The images represent one of three independent experiments. (b) Quantification of PLA signals by PLA analysis software. (c) Confirmation of interaction between Tom20 and PDI by co-IP assay.

#### 4.15. DETERMINATION OF REACTIVE NITROGEN SPECIES LEVELS IN UM-SCC-1A CELLS UPON LCL768 TREATMENT

RNS species (NO and ONOO<sup>-</sup>) were measured (described in Section 2.14) in response to LCL768 treatment with different treatment periods and concentrations. According to the result, LCL768 induced RNS generation in a dose and time-dependent way (Figure 4.15).

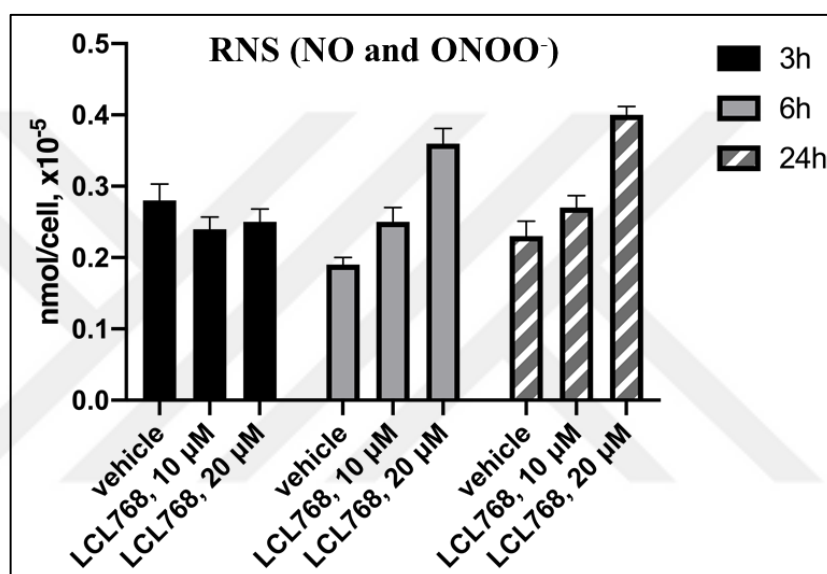


Figure 4.15. RNS accumulation in response to 3,6 and 24-hour LCL768 treatment in UM-SCC-1A cells.

#### 4.16. DETERMINATION OF CELL NUMBERS OF UM-SCC-1A CELLS UPON LCL768 TREATMENT WITH NO-DONOR AND NOS INHIBITOR

The changes in the viable cell numbers upon 10 μM LCL768 treatment, which were pre-treated either with SIN-1 (NO donor) or L-NAME (NO-scavenger), were analysed by Trypan blue assay (described in Section 2.2). As a result, SIN-1 increased the growth inhibition by 54 percent ( $p < 0.001$ ), L-NAME whereas prevented the growth inhibition effect of LCL768 on UM-SCC-1A cells (Figure 4.16.a-b).

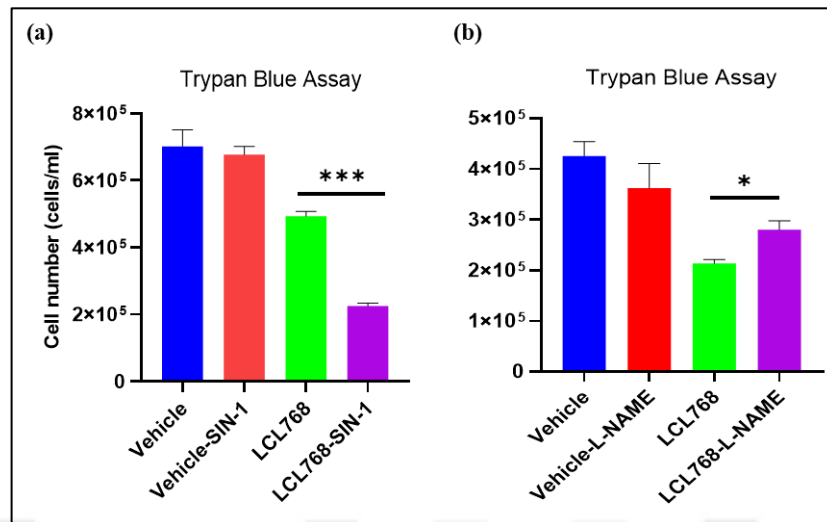


Figure 4.16. The effect of LCL768 treatment on UM-SCC-1A cells treated either with NO donor or NOS inhibitor. Viable cell numbers were measured using trypan blue assay. Data are means  $\pm$  SD (n=3 independent experiments, \*p < 0.05 or \*\*\*p < 0.001).

#### 4.17. DETERMINATION OF THE EFFECTS OF SHRNA-MEDIATED KNOCKDOWN OF INOS ON UM-SCC-1A CELLS UPON LCL768 TREATMENT

In order to analyze the effect of iNOS on LCL768-mediated RNS accumulation and S-nitrosylation of Drp1, shRNA-mediated knockdown cells were used for RNS measurement and co-IP experiment for S-nitrosylation of Drp1 followed by Western blotting. According to the result, RNS generation and S-nitrosylation of Drp1 were both decreased in knockdown of iNOS by more than 75 percent for UM-SCC-1A cells (p < 0.01) (Figure 4.17.a-b).

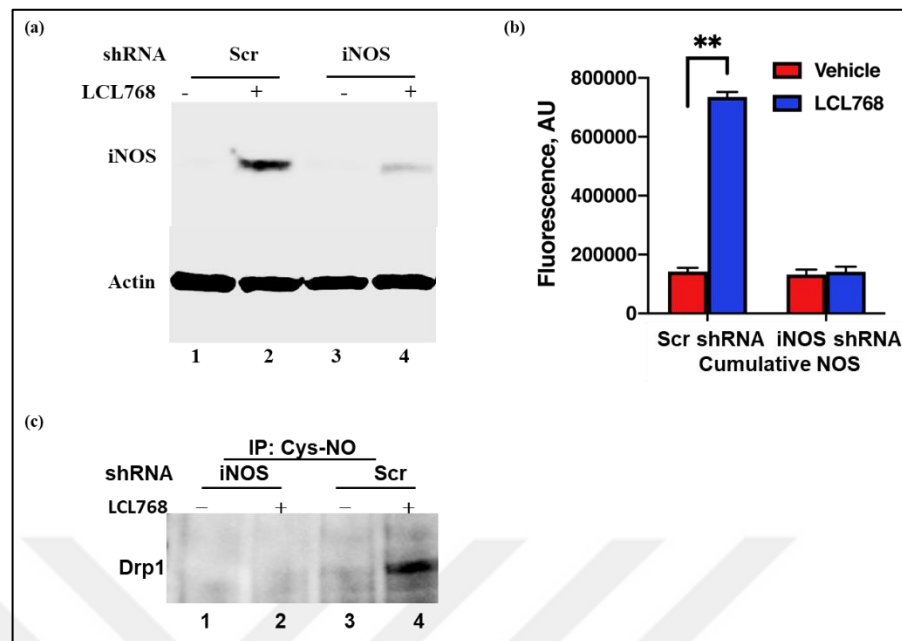


Figure 4.17. RNS accumulation and S-nitrosylation in response to LCL768 treatment in shscr and shiNOS UM-SCC-1A cells. (a) iNOS protein levels in UM-SCC-1A cells upon shRNA-mediated stable transfection. Western blot to detect iNOS upon LCL768 treatment. Actin was used as a western blot loading control. (b) RNS accumulation upon LCL768 treatment in shscr and shiNOS UM-SCC-1A cells. (c) S-nitrosylation of Drp1 measurement by co-IP and then Western blotting using anti-Cys-NO and Drp1 antibodies.

#### 4.18. ANALYSIS OF INTERACTION BETWEEN ER AND MITOCHONDRIA IN SHRNA-MEDIATED DRP1 KNOCKDOWN UM-SCC-1A CELLS IN RESPONSE TO LCL768

PLA and co-IP were performed to analyze the roles of Drp1 in the association between ER and mitochondria in shDrp1, and shDrp1 cells reconstituted WT and two different mutants in response to LC768 treatment. Stably transfected shDrp1 cells were used for transient transfection of WT-Drp1, C644A-Drp1 (non-S-nitrosylated mutant), and C644W-Drp1 (S-nitrosylation mimicking mutant). According to the results, Tom40 and PDI association was restored in WT-Drp1 expressing cells, but not in empty vector and C644A expressing cells.

Also, Tom40 and PDI association was sustained in the absence or presence of LCL768 for C644W cells (Figure 4.18.a-c).

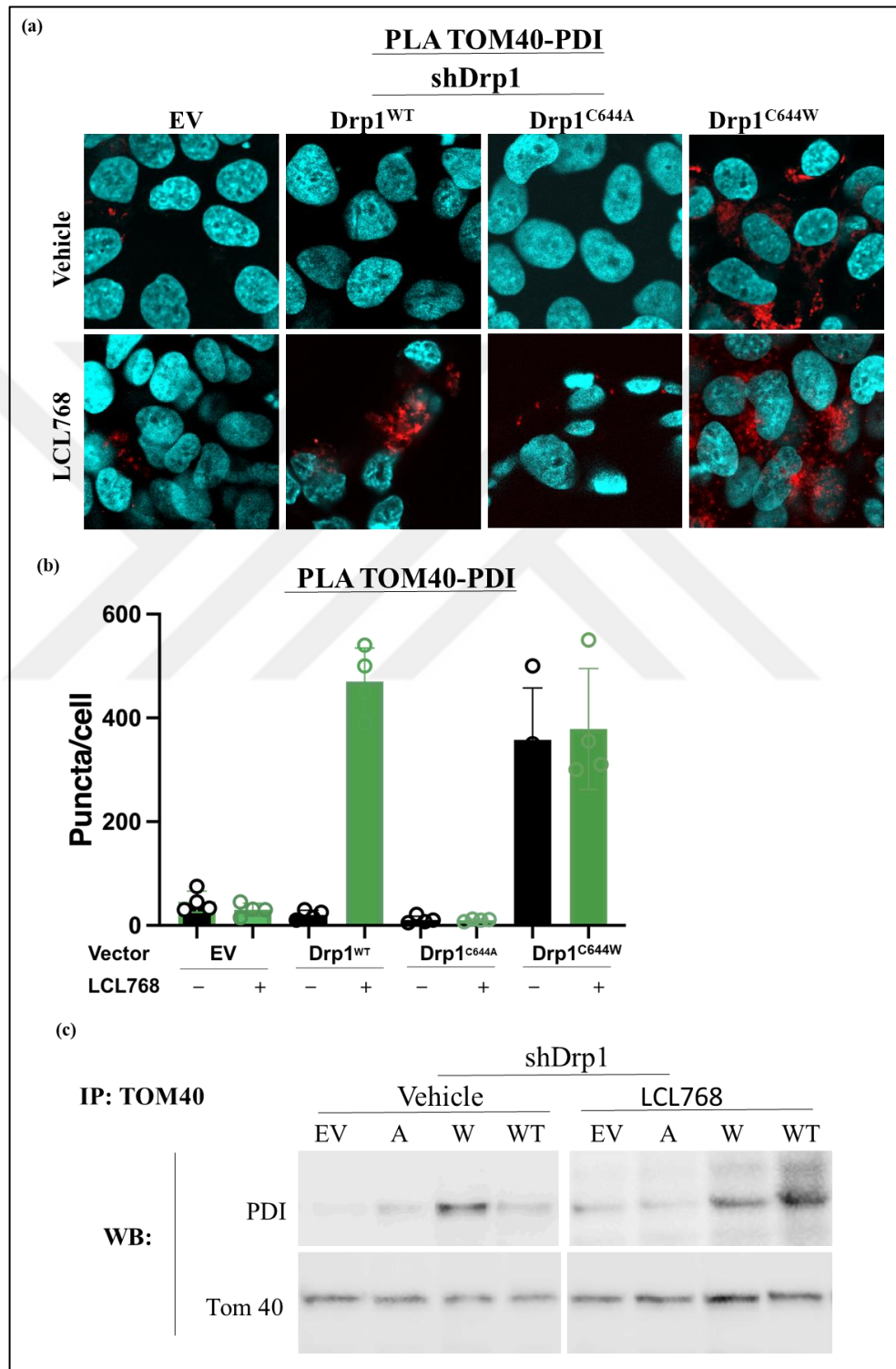


Figure 4.18. PLA analysis of shDrp1 cells reconstituted with WT-Drp1, C644A-Drp1 (non-S-nitrosylated mutant), and C644W-Drp1 (S-nitrosylation mimicking mutant) upon

LCL768 treatment. (b) Quantification of discrete signals by PLA analysis software. (c) Confirmation of interaction between Tom40 and PDI by co-IP assay.

#### **4.19. IMMUNOFLUORESCENCE ANALYSIS OF COLOCALIZATION OF ER AND MITOCHONDRIA IN SHRNA-MEDIATED DRP1 KNOCKDOWN UM-SCC-1A CELLS IN RESPONSE TO LCL768**

Immunofluorescence analysis was performed to analyze the roles of Drp1 in the association between ER and mitochondria in shDrp1, and shDrp1 cells reconstituted WT, C644A-Drp1, and C644W-Drp1. The colocalization was measured by immunofluorescence with the Alexa Fluor 488 and 594 attached secondary antibodies for Tom20 and PDI antibodies. According to the results, Tom40 and PDI association was restored in WT-Drp1 expressing cells ( $p < 0.01$ ), but not in empty vector and C644A expressing cells. In addition, Tom40 and PDI association was sustained in the with/without of LCL768 in C644W cells (Figure 4.19.a-b). The overexpression of Drp1 mutants were confirmed with immunoblotting (Figure 4.19.c).

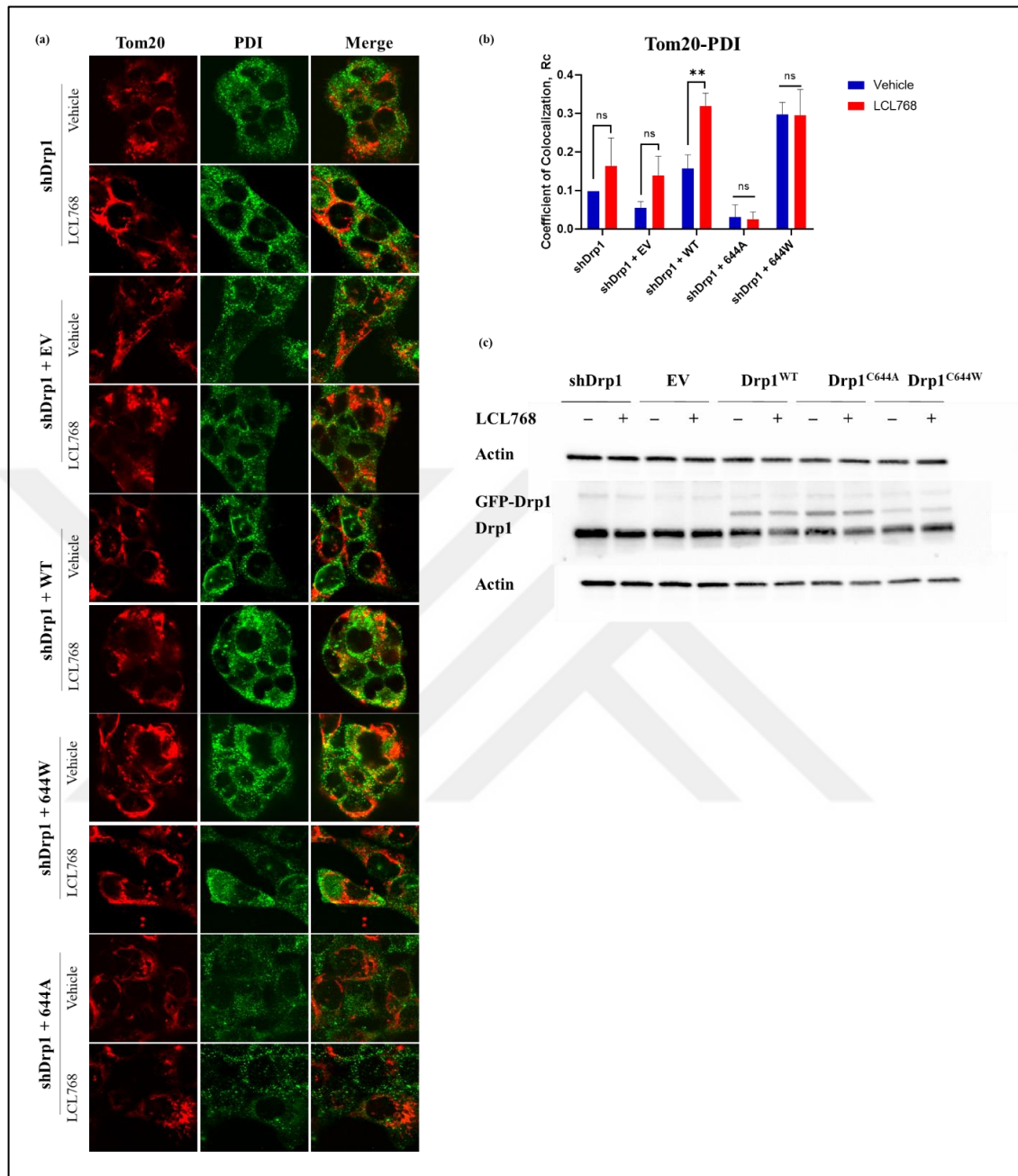


Figure 4.19. Immunofluorescence analysis of shDrp1 cells reconstituted with WT-Drp1, C644A-Drp1, and C644W-Drp1 upon LCL768 treatment. (a) Confocal images of immunofluorescence staining of PDI (green) and Tom20 (red) in UM-SCC-1A cells treated with LCL768. Yellow signal demonstrates colocalization. The images represent one of three independent experiments. (b) Quantification of confocal images of immunofluorescence in (a). Data are means  $\pm$  SD (n = 3 different optical fields, \*\*p < 0.01).

**4.20. IMMUNOFLUORESCENCE ANALYSIS OF THE INTERACTION BETWEEN MITOCHONDRIA AND CERS1 IN SHRNA-MEDIATED DRP1 KNOCKDOWN UM-SCC-1A CELLS IN RESPONSE TO LCL768**

Immunofluorescence analysis was performed to analyze the roles of Drp1 in the association between CerS1 and mitochondria in shDrp1 and shDrp1 cells reconstituted WT, C644A-Drp1, C644W-Drp1, and two more mutants; Drp1-ER (cannot associate with ER) and Drp1-MT (cannot associate with mitochondria). The colocalization was measured by immunofluorescence for Tom20 and CerS1 antibodies. According to the results, LC768 induced Tom20 and CerS1 association in shscr cells ( $p < 0.01$ ). Furthermore, Tom20 and CerS1 association was restored in WT-Drp1 expressing cells ( $p < 0.001$ ) and sustained with/without LCL768 in C644W cells (ns). However, the association was prevented in empty vector, C644A, Drp1-ER, and Drp1-MT expressing cells (ns) (Figure 4.20.a-b). The overexpression of Drp1 mutants were confirmed with immunoblotting (Figure 4.20.c).

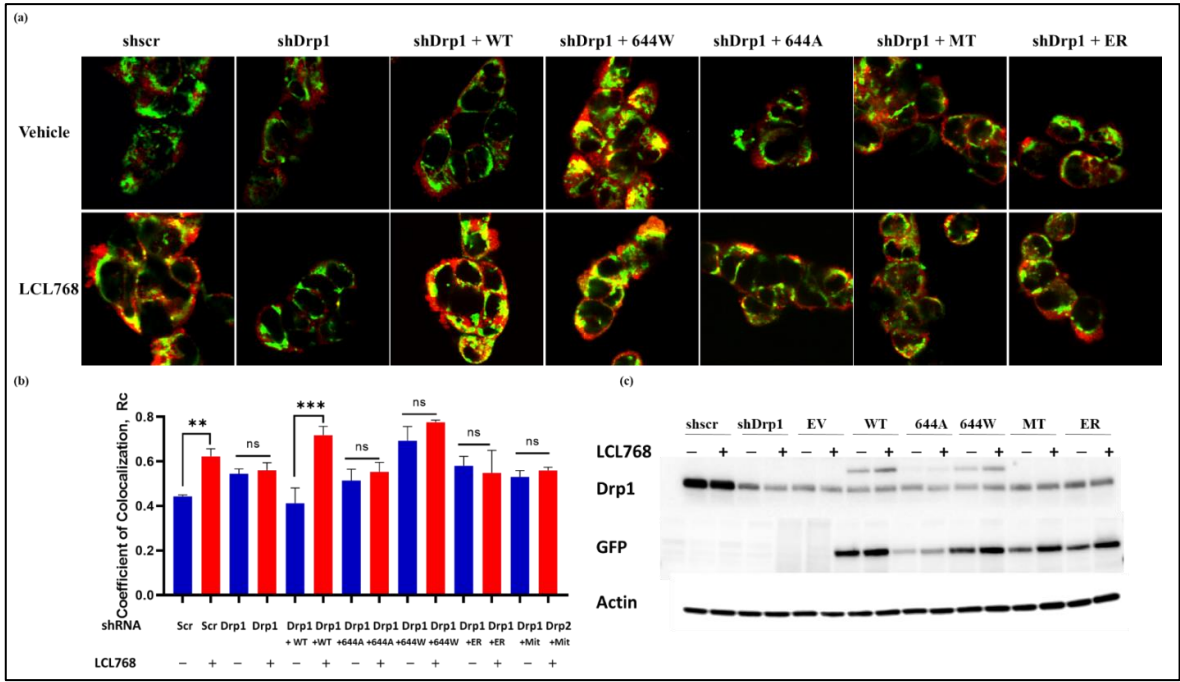


Figure 4.20. Immunofluorescence analysis of shDrp1 cells reconstituted with WT-Drp1, C644A-Drp1, C644W-Drp1, Drp1-MT, and Drp1-ER upon LCL768 treatment. (a)

Confocal images of immunofluorescence staining of Tom20 (green) and CerS1 (red) in UM-SCC-1A cells treated with LCL768. Yellow signals demonstrate colocalization. The images represent one of three independent experiments. (b) Quantification of confocal images of immunofluorescence in (a). Data are means  $\pm$  SD (n = 3 different optical fields, \*\* p < 0.01 or \*\*\* p < 0.001).

#### **4.21. ANALYSIS OF INTERACTION BETWEEN ER AND MITOCHONDRIA IN SHRNA-MEDIATED DRP1 KNOCKDOWN UM-SCC-1A CELLS IN RESPONSE TO LCL768**

PLA was performed to analyze the roles of Drp1 in the association between ER and mitochondria in shscr, shDrp1, and shDrp1 cells reconstituted WT, Drp1-ER, and Drp1-MT. According to the results, LC768 induced Tom20 and CerS1 association in shscr cells (p < 0.0001). Furthermore, Tom20 and CerS1 association was restored in WT-Drp1 expressing cells (p < 0.0001), whereas the association was prevented in shDrp1, Drp1-ER, and Drp1-MT expressing cells (ns) (Figure 4.21.a-b).

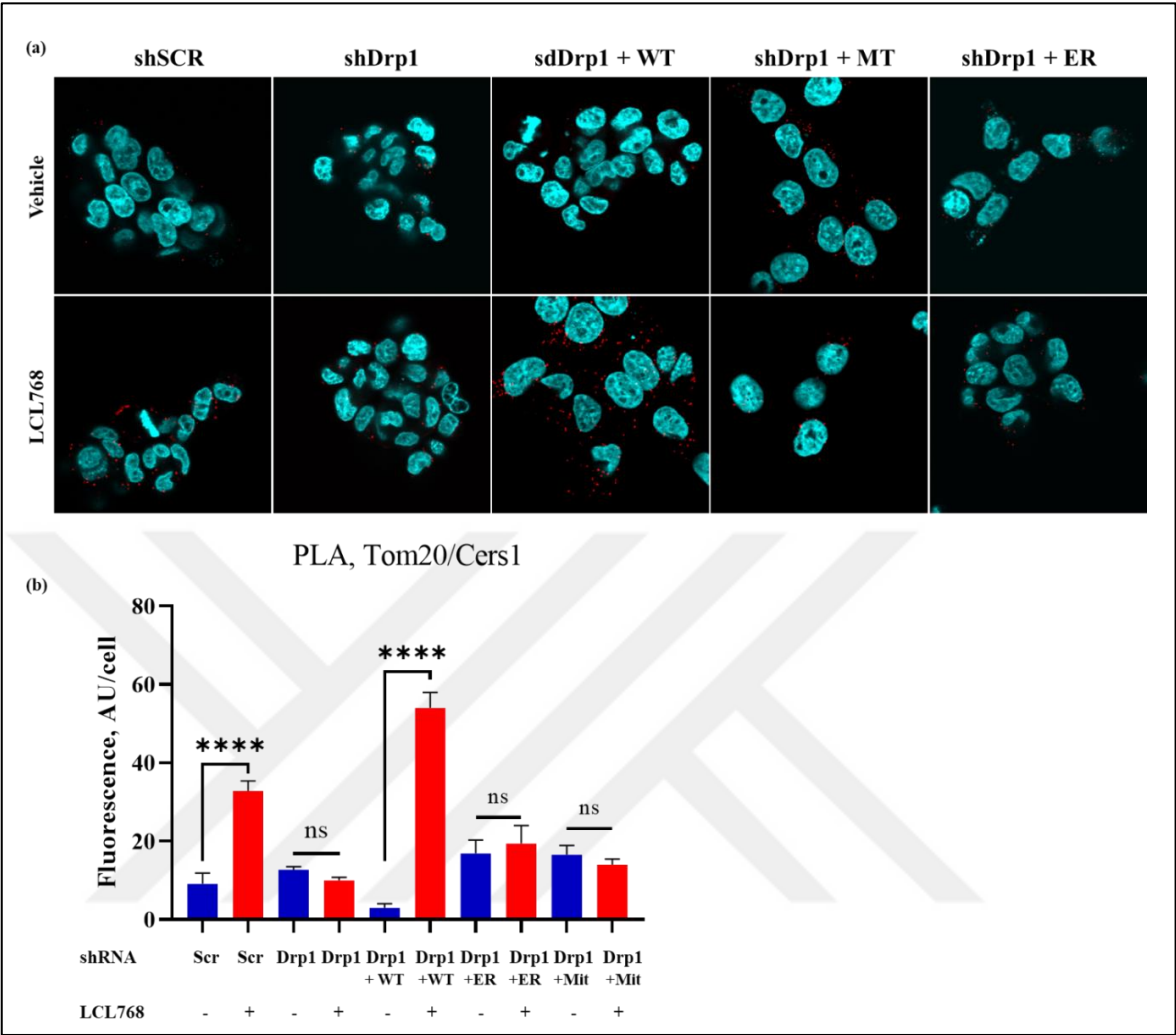


Figure 4.21. PLA analysis of shscr and shDrp1 cells reconstituted with WT-Drp1, Drp1-ER, and Drp1-MT upon LCL768 treatment. (b) Quantification of discrete signals by PLA analysis software.

**4.22. ANALYSIS OF ACO2 AND LC3 PROTEIN LEVELS IN 3D-HNSCC ORGANOIDs UPON LCL768 TREATMENT**

3D-HNSCC organoids obtained from a patient with HNSCC tumor tissues ex vivo were either treated with vehicle, SoSe, or LCL768. Separated proteins were probed for ACO2 and LC3 antibodies. In addition, the actin levels were used to compare loading amount of proteins. According to the result, the ACO2 degradation and the lipidated form of LC3 were

increased in response to SoSe and LCL768. Also, the interaction between mitochondria and LC3 was analyzed with co-IP. As a result, Tom40 and LC3 association was increased upon SoSe and LCL768 in human HNSCC organoids.

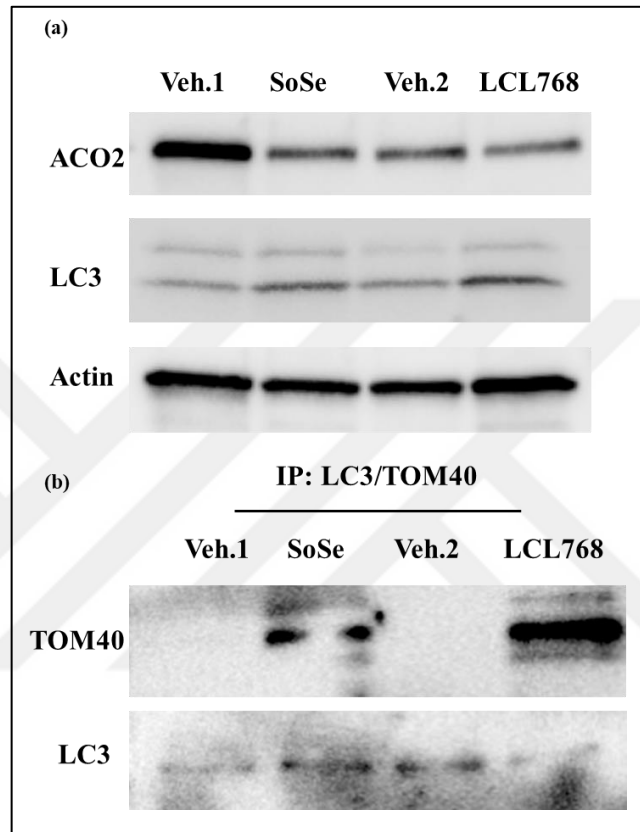


Figure 4.22. ACO2 and LC3 protein levels in HNSCC organoids upon SoSe and LCL768 treatment. (a) Western blot for ACO2 and LC3B proteins in response to SoSe and LCL768.

Actin was used as a western blot loading control (b), co-IP analysis of Tom40 and LC3 upon SoSe and LCL768 treatment in HNSCC organoids.

#### **4.23. IMMUNOFLUORESCENCE ANALYSIS OF THE INTERACTION BETWEEN CERS1-P17 AND CERS1-TOM20 IN 3D-HNSCC ORGANOIDs UPON LCL768 TREATMENT**

Immunofluorescence analysis was performed to analyze the association between CerS1-p17 and CerS1-Tom20 in HNSCC organoids. The colocalization was measured by

immunofluorescence for CerS1, p17, and Tom20 antibodies. According to the results, LCL768 induced CerS1-p17 and CerS1-Tom20 association more than 2 folds in HNSCC organoids ( $p < 0.001$ ) (Figure 4.23.a-b).

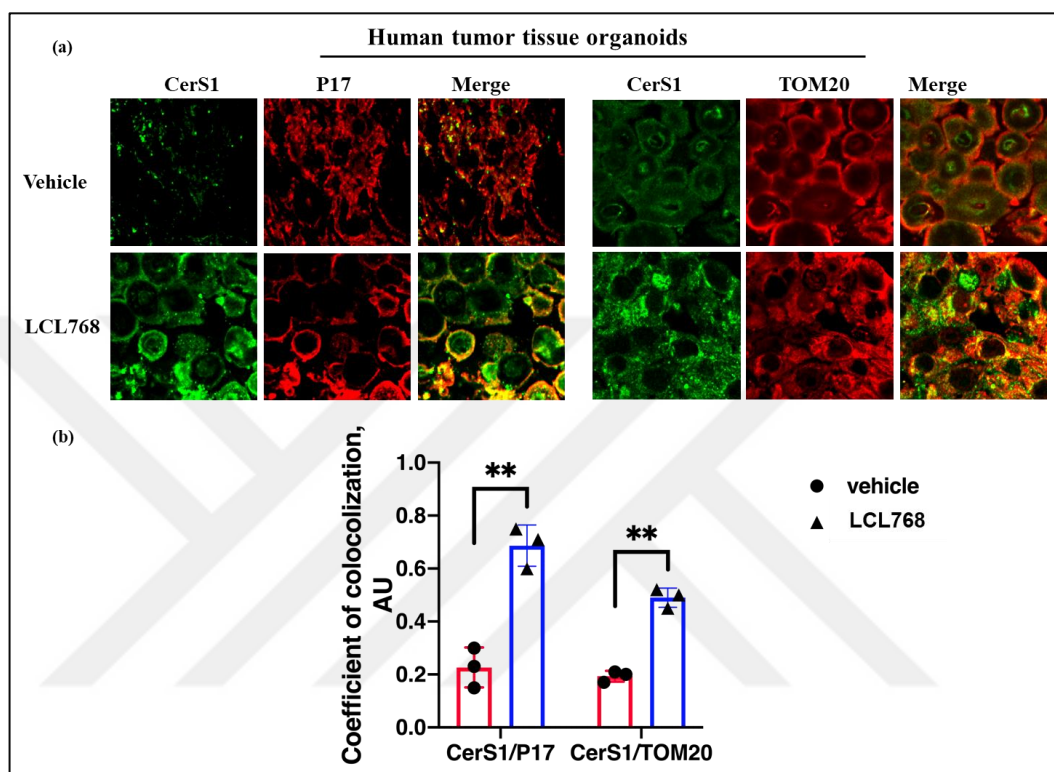


Figure 4.23. Immunofluorescence analysis for LCL768 treatment in HNSCC organoids. (a) Confocal images of immunofluorescence staining of CerS1 (green), p17, and Tom20 (red) in organoids treated with LCL768. Yellow signals demonstrate colocalization. The images represent one of three independent experiments. (b) Quantification of confocal images of immunofluorescence in (a). Data are means  $\pm$  SD ( $n = 3$  different optical fields,  $**p < 0.01$ ).

#### 4.24. METABOLOMICS ANALYSIS OF *IN VITRO* AND *EX VIVO* HNSCC ORGANIDS TREATED BY LCL768

To analyze the metabolic results of LCL768 treatment on HNSCC, hydrophilic metabolites were measured by mass spectrometry and metabolomics in 3D HNSCC organoids and UM-

SCC-1A cells expressing shscr, shLC3, shDrp1, shDrp reconstituted with WT or C644W. According to the results, LCL768 reduced the amounts of metabolites: citrate, succinate, fumarate, and malate involved in the TCA cycle. LCL768 also decreased glutamine, arginine, and lactic acid levels, whereas LCL768 also increased the glucose levels in shscr UM-SCC-1A cells. In addition, the knockdown of LC3 and Drp1 mostly prevented the reduction in mitochondrial metabolism. However, C644-Drp1 expressed shDrp1 cells sustained the decrease in mitochondrial metabolites and the increase in glucose metabolism in both the absence and presence of LCL768. In 3D-HNSCC organoids, both SoSe and LCL768 reduced the mitochondrial metabolism. However, they did not affect glucose levels compared to in vitro cell culture levels (Figure 4.24.a-b).

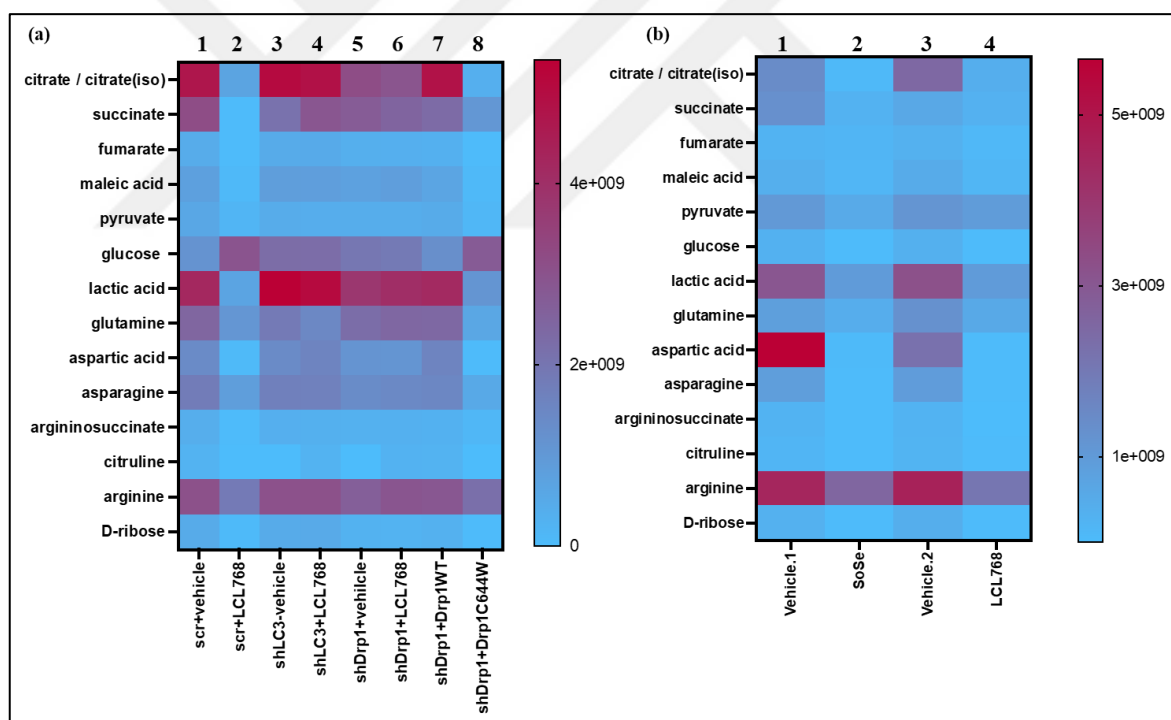


Figure 4.24. Levels of metabolites in UM-SCC-1A cells (a) stably expressing shscr, shLC3, shDrp1, shDrp1 shDrp reconstituted with WT or C644W and 3D-HNSCC organoids (b). Each group were subjected to methanol extraction with three biological replicates and sent for metabolomics analysis. The data were analyzed by using MetaboAnalyst 5.0 software.

#### 4.25. DETERMINATION OF THE EFFECT OF LCL768 ON TUMOR GROWTH *IN VIVO*

In order to assess the impact of LCL768 on HNSCC tumor growth *in vivo*, UM-SCC-1A cells were injected into both flanks of SCID mice. After 2 weeks, the intraperitoneal injection of vehicle or LCL768 and xenograft-derived tumor growth measurements were performed by using caliper for 21 days. According to the results, LCL768-treated mice showed a reduction in xenograft-tumor growth by about 50 % compared to the vehicle control mice (Figure 4.25).

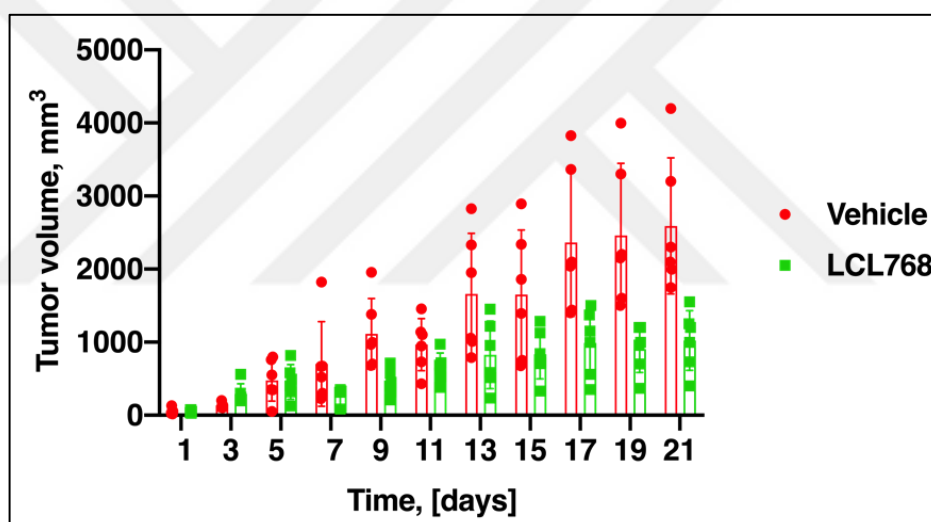


Figure 4.25. The volumes of HNSCC xenograft tumors in SCID mice. Female NSG mice at the age of 8 to 12 weeks were implanted with UM-SCC-1A cells and treated with vehicle or LCL768 for 21 days.

#### 4.26. ANALYSIS OF PROTEIN LEVELS IN HNSCC XENOGRAFT-DERIVED TUMORS IN RESPONSE TO LCL768

ACO2 degradation, LC3 activation, pDrp1-637, and S-nitrosylated Drp1 were determined by Western Blotting analysis upon LCL768 in HNSCC xenograft-derived tumors.

According to the results, the ACO2 degradation and the lipidated form of LC3 were induced by LCL768 in xenograft-derived tumors and adjacent tissues. Also, LCL768 activated Drp1 activity by decreasing inhibitory P-Drp1 ( $p < 0.05$ ) and increasing nitrosylated Drp1 more than 2 folds ( $p < 0.01$ ) (Figure 4.26 a-d).

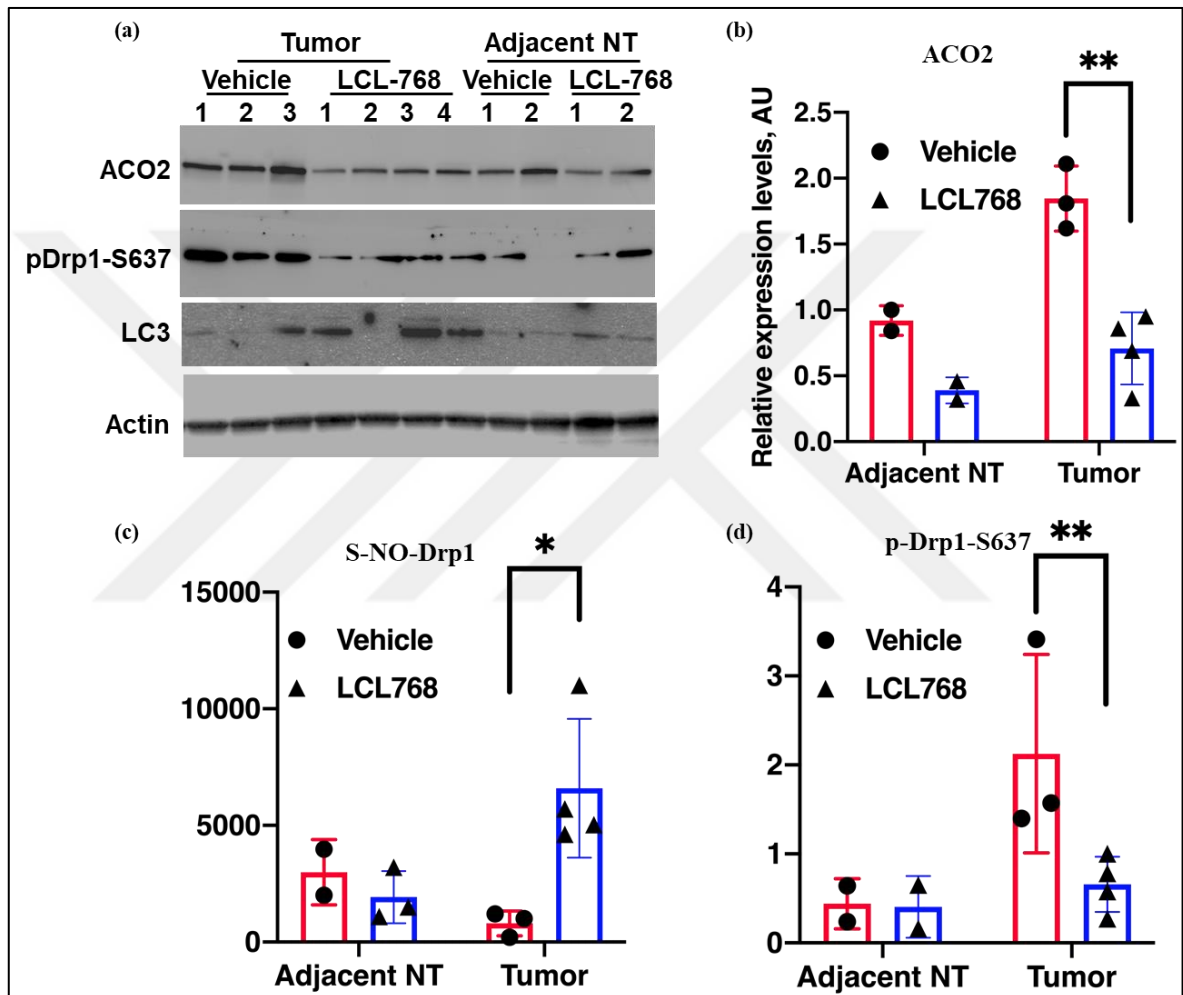


Figure 4.26. Alterations in protein levels related to mitophagy induction in xenograft-derived tumors upon LCL768 treatment. (a) Western blot for ACO2, LC3B, and p-Drp1-S637 proteins (c) Quantification of co-IP by using anti-Cys-No and anti Drp1 antibodies (b), (d), Quantifications of proteins in (a). Quantifications were performed using Fiji software. Data are means  $\pm$  SD ( $n=3$  independent experiments, \*  $p<0.05$  or \*\*  $p<0.01$ ).

#### 4.27. LIPIDOMICS ANALYSIS OF HNSCC XENOGRFT-DERIVED TUMORS IN UPON LCL768 TREATMENT

In order to detect the accumulation of LCL768 and C18-ceramide in tumors and adjacent tissues, mass spectrometry-based lipidomics analysis was performed in HNSCC xenograft-derived tumors. As a result, LCL768 accumulated and increased C18-ceramide levels ( $p < 0.0001$ ) in both tumor and adjacent normal tissues in comparison to vehicle-treated controls, but much more in tumor tissues (Figure 4.27 a-c). Also, LCL768 increased C18-ceramide levels in both xenograft-derived tumors and adjacent tissue ( $p < 0.0001$ ) (Figure 4.27.b).

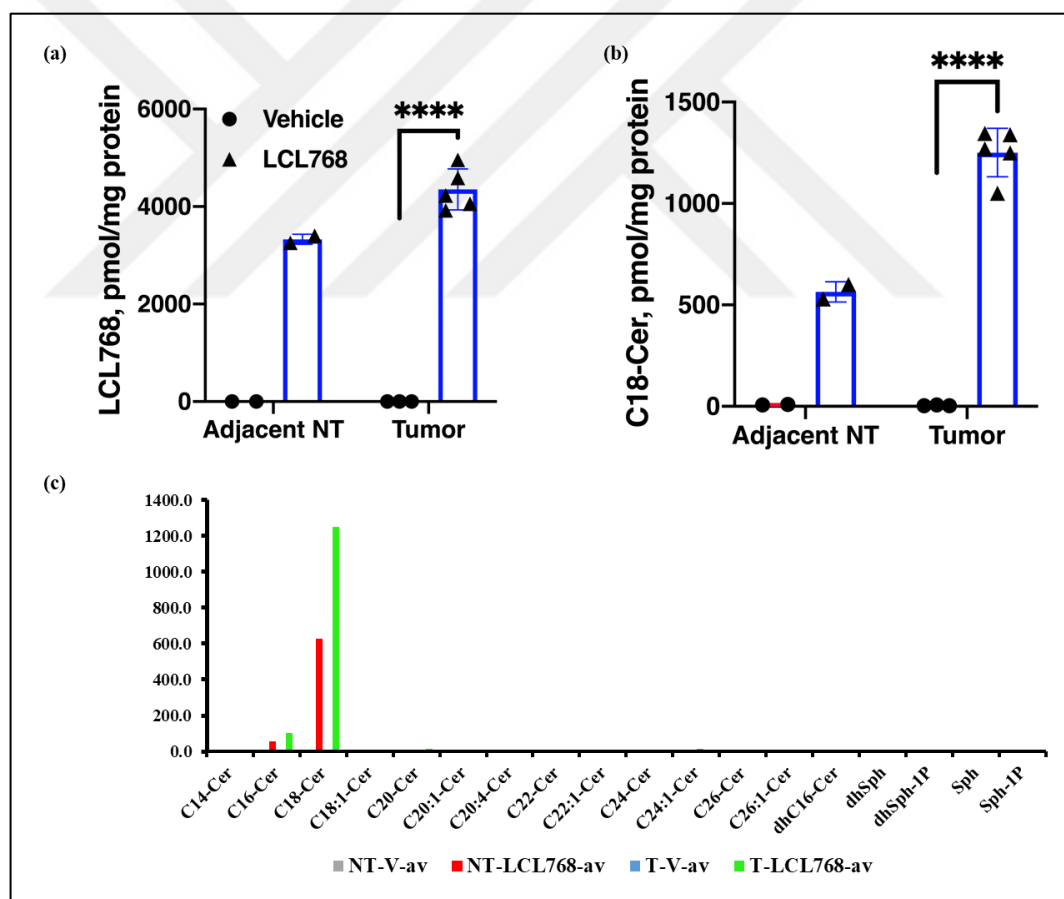


Figure 4.27. Lipidomic analysis of LCL768 treated xenograft-derived tumor and adjacent tissues (a) LCL768 and (b) C18-ceramide accumulations were measured by lipidomics measurement by LC/MS/MS. (c) Lipidomics profile of tumor xenografts and adjacent normal tissues in SCID mice treated with LCL768.

## 5. DISCUSSION

Mitochondria has dynamic activities that uses fission and fusion machineries to maintain its functionality (289). Mitophagy is a cellular process for recycling mitochondria through selective autophagy, which is essential for sustaining the quality and functionality of the mitochondria (290). Mitochondria selected for mitophagy are encapsulated in double membraned autophagosomes and fused with lysosomal degradation (271). Dysfunctional mitochondria have been related with many diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and cancer (291,292).

Sphingolipids are bioactive lipids that are involved in many signal transduction pathways as either pro-survival or pro-tumoral functions that regulate cancer cell death, growth, and migration (36). Besides, sphingolipids are involved in invasion, metastasis, apoptosis, and lethal mitophagy pathways in response to anticancer therapy (46). Ceramide is the central sphingolipid that induces cell death, although S1P facilitates tumor growth and anticancer therapy resistance (46). Endogenous ceramides have different fatty acyl chains generated by ceramide synthases 1-6 (CerS1-6) that also specialize in the function of the ceramide (101). Ceramide synthesis is induced in response to various stress stimuli including chemotherapeutic drugs or oxidative stress, which leads to death in the cancer cell (34). C18-ceramide synthesized mainly by CerS1 has emerged as a tumor suppressor lipid inducing lethal mitophagy in AML and HNSCC, which ends up as a ceramide-mediated cell death where the mitochondria are degraded until the cancer cell dies (125,127). Recent studies have presented that most HNSCC tumors have a reduced level of C18-ceramide (287). Previously, pyridinium ceramide has been demonstrated to induce ceramide-mediated mitophagy in cancer cell death (125). In addition, mitochondrial localization of C18-ceramide in response to SoSe-induced mitophagy requires CerS1 trafficking from ER to mitochondria and Drp1/LC3 activation (127). However, there are major challenges with conventional ceramide analogs due to their low solubility, poor cellular uptake, high toxicity or high intracellular metabolism to complex sphingolipids.

To enhance the effect of pyridinium ceramide on mitophagy induction, selenium was conjugated with pyridinium ceramide (294). In this study, we aimed to unravel the mechanism of LCL768 for tumor suppression by inducing ceramide-mitophagy in HNSCC cells, xenograft-derived tumors, and 3D organoids.

First, the effect of LCL768 on mitophagy induction on HNSCC cells was determined by Western blotting of ACO2 and LC3. ACO2 is a mitochondrial protein that is involved in the TCA cycle that is shown to be susceptible to mitophagic degradation (295), and LC3 is a cytosolic protein whose conjugation with phosphatidylethanolamine induces autophagosome formation (296). Time- and dose-dependent decrease in ACO2 and elevation in LC3-II protein levels showed that LCL768 was effective for mitophagy induction in UM-SCC-1A cells (Figure 4.1.). Drp1 is an essential protein for mitophagy initiation since it is responsible for mitochondrial fission, which is the initial step of mitochondrial degradation (297). Its activation either by dephosphorylation at S637 or nitrosylation at C644 was shown to be involved in CerS1/C18-ceramide mediated mitophagy induction (125,127). LC3 lipidation is an important process for autophagosome formation during the autophagic response (298). Both Drp1 and LC3 have been shown to play vital roles in ceramide-induced mitophagy in cancer cells (125,127). Thus, to detect the effects of LCL768 on mitophagy induction, LC3 and Drp1 were knocked down using sh-RNA in UM-SCC-1A cells (Figure 4.2). These cells were examined with Western blotting to compare their response to LCL768-induced mitophagy. Western blotting of ACO2 showed that LCL768 induced mitophagy in sh-scr cells, whereas this effect was prevented in LC3 and Drp1 knockdown cells (Figure 4.3.a). Moreover, phosphorylated ubiquitin at Ser65, which is induced by PINK1 and functions as a receptor for Parkin recruitment during mitophagy (299), was found to be accumulated in sh-scr and sh-LC3 but not in sh-Drp1 cells (Figure 4.3.b). This suggests that Drp1 might contribute to the process of phosphorylation of Ser65 in mitophagy induction much more significantly than LC3. Also, Cyto-ID data demonstrated that LCL768 induced autophagosome formation in WT cells, and knockdown of both LC3 and Drp1 decreased mitophagic response (Figure 4.4). Furthermore, LCL768 reduced the

mitochondrial function in sh-scr cells upon LCL768 treatment, which was assessed by measuring oxygen consumption rate (OCR) with Seahorse (Figure 4.5). However, the knockdown of LC3 prevented the inhibition of OCR in response to LCL768. These data suggest that LCL768 induces mitophagy by reducing mitochondrial function, which is dependent on LC3 and Drp1 in UM-SCC-1A cells, for the first time in literature.

Exogenous C18-pyridinium analog shown to be involved in mitochondrial stress signaling and mitophagy (125). Hence, lipidomic analysis was performed to detect LCL768 subcellular accumulation and its ability to endogenous ceramide generation. LCL768 mainly accumulated in mitochondria in sh-scr cells and induced endogenous C18-ceramide generation mostly in mitochondria, whose effect was reduced with the knockdown of Drp1 and LC3 (Figure 4.6). That supports the hypothesis that LCL768 comprises a positively charged pyridinium ring to increase its solubility and to selectively target negatively charged mitochondria (300–302) in the presence of a selenium conjugate as mitophagy inducer (303). Trafficking of CerS1 to mitochondria upon the release of stress signaling has been validated (127). According to the CerS1 accumulation in response to LCL768, ACO2 degradation and CerS1 accumulation in mitochondria decreased in Drp1 knockdown cells (Figure 4.7). This is consistent with the reduction in endogenous C18-ceramide generation, which is the outcome of CerS1 accumulation in mitochondria (127). So LCL768 accumulates and induces endogenous C18-ceramide accumulation in mitochondria which is Drp1 dependent, resulting in mitophagy.

The ubiquitin chain on the damaged mitochondria binds autophagy receptors which then induce LC3 lipidation and autophagosome formation (304). Thus, MS-based proteomic analysis was performed on ubiquitinated proteins upon LCL768 treatment. Catalase was the only candidate protein to be mitochondria-related (Figure 4.8.c). The protein levels of catalase and other mitochondrial proteins decreased upon LCL768 (Figure 4.9.a). These data suggest LCL768 reduces the expression level of mitochondria-related proteins and so the mitochondrial function through the induction of LC3/Drp1/ceramide-dependent mitophagy.

To identify the mechanism of Drp1-dependent LCL768-induced mitophagy, immunofluorescence analyses were performed to determine whether Drp1 has a role in gathering ER and mitochondria to mediate CerS1 trafficking that has been shown to induce C18-ceramide generation, recruitment of LC3 II-dependent autophagosomes, and mitophagy (127). According to data, LCL768 induced the colocalization between ER and mitochondria, whereas knockdown of LC3 or Drp1 thoroughly inhibited the ER-mitochondria colocalization in response to LCL768 (Figure 4.10, Figure 4.11, and Figure 4.12). Besides, the colocalization between mitochondria and CerS1 increased in control cells; however, this colocalization decreased in LC3 knockdown cells and was prevented in Drp1 knockdown cells (Figure 4.12). In addition, ER-mitochondria association was measured by PLA assay. LCL768 increased the association between ER and mitochondria scramble control cells which was shown to be involved in CerS1 trafficking to mitochondria during ceramide-mediated mitophagy (Figure 4.14.a-b). shRNA-mediated knockdown of p17 did not affect the association, but knockdown of LC3 and CerS1 decreased the association, and Drp1 knockdown almost completely prevented ER-mitochondria association in response to LCL768. Taken together, Drp1 is significant for trafficking of CerS1 from ER to mitochondria upon LCL768-mediated mitophagy by gathering the ER and mitochondria together.

Our data showed that Drp1 is an essential protein involved in LCL768-induced mitophagy. In order to determine the activation mechanism of Drp1 by LCL78, nitrosylation of Drp1 was measured, which was shown to involve in SoSe-mediated mitophagy (127). First, the effect of LCL768 on RNS generation was measured. The data showed that LCL768 induced RNS generation in a time- and dose-dependent manner (Figure 4.17). To understand the mechanism in detail, HNSCC cells were pre-treated either with NO donor or NOS inhibitor, and their effects on UM-SCC-1A cell number were analyzed with Trypan Blue assay. According to data, NO donor pre-treatment increased the growth inhibition, whereas NOS inhibitor decreased the growth inhibition of LCL768. iNOS is the responsible enzyme for RNS generation (305). Therefore, iNOS was knocked down by using shRNA system in the

cells (Figure 4.17.a). Knockdown of iNOS completely inhibited RNS generation (Figure 4.17.b). Besides, LCL768 increased S-nitrosylation of Drp1, whereas iNOS knockdown prevented the activatory S-nitrosylation of Drp1 (Figure 4.17.c). Drp1 is known to be activated for the induction of mitochondrial fission by nitrosylation at C644 (306). Thus, nitrosylation lacking mutant C644A and nitrosylation mimic mutant C644W of Drp1 were used in shDrp1 cells for reconstitution in addition to WT. The effect of nitrosylation levels of Drp1 on ER-mitochondria association was measured by PLA assay using shDrp1 cells overexpressed with empty vector, WT, and the mutants of 644A and 644W and then confirmed with co-IP and Western blotting. According to the results, ER and mitochondria association was sustained for C644W-Drp1 even without LCL768. WT-Drp1 restored the ER-mitochondria association upon LCL768 but not C644A-Drp1 (Figure 4.18.a-c). The ER-mitochondria association in response to LCL768 was also checked with immunofluorescent analysis, which was consistent with PLA and co-IP analyses (Figure 4.19) that the ER-mitochondria colocalization was restored in WT-Drp1, but not in C644A-Drp1. Also, C644W-Drp1 mutant induced colocalization even without LCL768. These results suggest that S-nitrosylation of Drp1 at C644 plays a vital role in the association between ER and mitochondria. To determine the role of Drp1 in ER-mitochondria association in response to LCL768, which is essential for CerS1 trafficking for CerS1/C18-ceramide-mediated mitophagy induction (306), Drp1-ER and Drp1-MT mutants were generated whose mutated residues were known to be necessary for interaction either with ER or mitochondria (307,308). Then the effect of LCL768 on CerS1-mitochondria association was measured using immunofluorescence and PLA assay. According to the results, LCL768 induced CerS1-Tom20 association in control cells and shDrp1 overexpressed with Drp1-WT, but not in Drp1-C644A, Drp1-MT and Drp1-ER mutants (Figure 4.20). Also, C644W-Drp1 mutant induced colocalization with/without LCL768. These results were also confirmed with the PLA assay, where Drp1 mutants that cannot bind either to ER or mitochondria, did not restore the association in response LCL768 (Figure 4.21). Altogether, these data support that S-nitrosylation of Drp1 at C644, which is essential for Drp1 activation, plays a vital role in

the association between ER and mitochondria, mediating CerS1 trafficking for the induction of ceramide-dependent mitophagy (127).

To assess the effects of LCL768 on 3D-organoids, head and neck cancer and adjacent normal tissue explants were obtained from a biopsy during surgery *ex vivo*, and they were cultured on top of an absorbable gelatin sponge and treated either with the vehicle, SoSe or LCL768. Then, mitophagy induction in 3D HNSCC organoids was confirmed by decreased ACO2 or lipidated LC3 proteins using Western blotting and increased association of LC3-Tom40 by co-IP in the presence or absence of SoSe or LCL768 compared to vehicle-treated controls (Figure 4.23). The effects of LCL768 on CerS1-mitochondria association on 3D-organoids were measured using immunofluorescence. The data showed that LCL768 induced CerS1-mitochondria association in 3D HNSCC organoids (Figure 4.24) as well as *in vitro*. Mitochondria is a center of metabolic energy generation that produce most of the energy essential for cell's biochemical reactions (309). Thus, to determine the metabolic consequences on cell metabolism of LCL768-mediated mitophagy, metabolic analysis was performed using LC-MS/MS with *in vitro* UM-SCC-1A cell with scramble control, LC3 knockdown, Drp1 knockdown, and reconstituted WT-Drp1 and C644W-Drp1. According to the results, LCL768 reduced the TCA cycle metabolites; citrate, succinate, fumarate, and malate; however, treatment increased the glucose levels (Figure 4.25a). Besides, lactic acid, glutamine, asparagine, arginine, citrulline, and argininosuccinate levels were decreased in response to LCL768. C644W-Drp1 mutants showed the same responses in the absence of LCL768, whereas knockdown of LC3 and Drp1 prevented these responses in their metabolite levels. Metabolomic analysis was repeated for *ex vivo* 3D organoids. In this case, SoSe was also used for treatment in addition to LCL768. These data were also consistent with the effects of LCL768 on mitochondrial metabolites *in vitro*. Both LCL768 and SoSe reduced mitochondrial metabolism by decreasing citrate, succinate, fumarate, malate, arginine, and aspartic acid, as well as glucose levels in 3D-organoids (Figure 4.25b). Overall, the results provide that LCL768-induced mitophagy results in the TCA cycle inhibition, resulting in mitochondrial metabolite exhaustion.

In order to analyze the effects of LCL768 on HNSCC tumor growth *in vivo*, UM-SCC-1 cells were implanted into immunodeficient SCID mice, which the mice develop a systemic HNSCC that has the main biological characteristics of the under researched disease (310). LCL768-treated mice showed a reduction in xenograft-tumor growth by about 50 % compared to the vehicle control mice. Mechanistically, LCL768-mediated mitophagy induction *in vivo* was confirmed by decreased ACO2 in tumor tissue rather than adjacent normal tissues. In addition, Drp1 activation in tumor tissues in response to LCL768 was detected via two activity mechanisms: increasing nitrosylation and decreasing inhibitory phosphorylation at S637 (126,127). Besides, lipidomic analysis showed that LCL768 accumulated and increased C18-ceramide levels mostly in tumor tissues. Hence, these data indicate that targeting mitochondria using LCL768 induces C18-ceramide-mediated mitophagy *in vivo* that might be a novel alternative therapeutic strategy for head and neck squamous cell carcinomas.

Overall, the study presented here describes the mechanism of a novel pyridinium-ceramide analog-mediated mitophagy induction in HNSCC in which mitochondria are selectively targeted by an exogenous ceramide analog drug LCL768. Furthermore, this study might have broad inferences as targeting mitochondria by pyridinium ceramide-induced mitophagy (36) derives an anticancer strategy since the mitochondrial function is needed for the production of energy and intermediates for macromolecule synthesis tumor cell proliferation (311).

## 6. CONCLUSION AND FUTURE PERSPECTIVE

Ceramide is a central regulatory sphingolipid essential in regulating selective autophagy, and mitophagy (36). This study reveals that a new Pyr-Cer analog drug, LCL768 conjugated with selenium induces mitochondrial damage and mitophagy in HNSCC as a result of the induction of CerS1/C18-ceramide mediated ceramide signaling, orchestrated by S-nitrosylation and activation of Drp1. This initiated ceramide-dependent mitophagy, which required S-nitrosylation at its C644 residue for the clearance of the mitochondria. Drp1 activation was regulated via iNOS that induced RNS generation in response to LCL768. Thus, LCL768 induced RNS generation by iNOS, leading to S-nitrosylation of Drp1 activation and ceramide-dependent mitophagy. Drp activation regulated CerS1 trafficking to mitochondria for CerS1/C18-ceramide mediated ceramide signaling that induced ER and mitochondria association which involved C644 residue for S-nitrosylation and selective residues for ER/mitochondria interactions. Besides, LCL768 reduces mitochondrial function by decreasing OCR. LCL768-mediated mitophagy brought along the decrease in the TCA cycle metabolites *in vitro*, 3D-organoid HNSCC. Also, this analog drug inhibits the fumarate/malate/aspartate shuttle in HNSCC. Moreover, LCL768 reduced xenograft-tumor growth and induced ceramide-mediate mitophagy in both 3D organoids and xenograft-derived tumors.

Overall, the studies presented here describe the mechanism of the novel ceramide analog drug LCL768, in which ceramide-mediated mitophagy selectively targets the mitochondria. Moreover, these data might have broad implications for understanding the mechanisms of diseases where mitochondrial dysfunction or mitophagy regulation has importance, such as cancer and neurodegenerative diseases. Moreover, LCL768 might provide a novel therapeutic approach against cancer by decreasing mitochondrial function and bioenergetics that are required for ATP production and biomacromolecules synthesis for inducing growth, proliferation, and drug resistance.

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