

**INVESTIGATION OF NOVEL PKC TARGETS IN THE REGULATION OF  
AUTOPHAGY**

by

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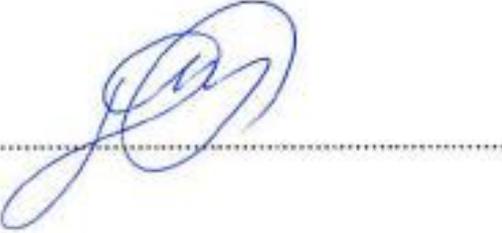
**INVESTIGATION OF NOVEL PKC TARGETS IN THE REGULATION OF  
AUTOPHAGY**

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## **ABSTRACT**

### **INVESTIGATION OF NOVEL PKC TARGETS IN THE REGULATION OF AUTOPHAGY**

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Thesis Supervisor: Assoc. Prof. Ozlem Kutlu

**Keywords:** Autophagy, protein kinase C, Lentivirus transduction

Protein Kinase C (PKC) isozymes are serine/threonine kinases that are important for activation and/or inactivation of intracellular signaling pathways and therefore regulate cellular metabolism. Autophagy is a degradation mechanism functioning under basal conditions and activating under cellular stress including nutrient limitation, oxidative stress or abnormal protein accumulation. It is initiated by formation of double or multi-membrane vesicles in the cytoplasm. These vesicles engulf the cargo and carry it to the lysosome. After the fusion of autophagic vesicle with lysosomes, the cargo is degraded, and its constituents are recycled. The signaling pathways regulated by PKC isozymes are also involved in autophagy mechanism. However, the interaction between autophagy and PKC isozymes is still unclear. The aim of the study is to find novel proteins targeted by PKC isozymes during autophagy mechanism. For this aim, lentiviral shRNA library system was used for silencing of the genes in GFP-LC3 stably expressing mouse embryonic fibroblast (MEF) transgenic cells (MEF GFP-LC3). Upon activation of PKC isozymes, autophagic machinery was examined by GFP-LC3 puncta count, LC3 shift assay and p62 accumulation. Then the positive clones were selected, and their genomic DNA was isolated for target gene sequencing. The genes were identified with Sanger sequence analysis and their relationship with PKC isozymes were analyzed by using RT-q-PCR. Consequently, the role of target gene in the regulation of autophagy was determined by commonly used autophagy techniques.

## ÖZET

### OTOFAJİ DÜZENLENMESİNDE YENİ PKC HEDEFLERİNİN ARAŞTIRILMASI

HÜMEYRA NUR KALELİ

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Tez Danışmanı: Doç. Dr. Özlem Kutlu

Anahtar kelimeler: Otofaji, protein kinaz C, Lentivirus gen aktarımı

Protein Kinaz C (PKC) izozimleri, hücre içi sinyal yollarının aktivasyonunda ve/veya inaktivasyonunda önemli olan serin/treonin kinazlardır ve bu nedenle hücrel metabolizmayı düzenler. Otofaji bazal koşullar altında çalışan ve besin kıtlığı, oksidatif stres veya anormal protein birikimi dahil olmak üzere hücrel stres altında aktive olan bir öğütme mekanizmasıdır. Oluşumu sitoplazma içinde çift veya çok membranlı veziküller ile başlar. Bu veziküller kargoyu içine alır ve onu lizozoma taşır. Otofajik vezikülün lizozom ile birleşmesinden sonra, kargo öğütülür ve bileşenleri geri dönüştürülür. PKC izozimleri tarafından düzenlenen sinyal yolları ayrıca otofaji mekanizmasında da rol oynar. Buna rağmen, otofaji ve PKC izoenzimleri arasındaki ilişki hala belirsizdir. Bu çalışmanın amacı, otofaji mekanizması sırasında PKC izozimlerinin hedeflediği yeni proteinleri bulmaktır. Bu amaçla, GFP-LC3 proteinini stabil olarak eksprese eden fare embriyonik fibroblast (MEF) transgenik hücrelerinde (MEF GFP-LC3) lentiviral shRNA kütüphanesi gen susturmak için kullanılmıştır. PKC izozimlerinin aktifleşmesi ile, otofajik sistem GFP-LC3 nokta sayısı, LC3 değişim analizi ve p62 birikimi ile incelenmiştir. Daha sonra pozitif klonlar seçildi ve genomik DNA'ları hedef gen dizilimi için izole edildi. Genler Sanger dizi analizi ile tanımlandı ve bunların PKC izozimleri ile ilişkileri RT-q-PCR yöntemi kullanılarak analiz edildi. Sonuç olarak, hedef genin otofaji düzenlemesindeki rolü, yaygın olarak kullanılan otofaji teknikleri ile belirlendi.

*“To my mother, father, and brother...”*

*“Canım anneme, babama ve kardeşime...”*

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## LIST OF SYMBOLS AND ABBREVIATIONS

|                  |   |
|------------------|---|
| $\alpha$         | Alpha   |
| $\beta$          | Beta  |
| $\gamma$         | Gamma   |
| $\delta$         | Delta   |
| $\varepsilon$    | Epsilon   |
| $\zeta$          | Zeta  |
| $\eta$           | Eta   |
| $\theta$         | Theta   |
| $\iota$          | Iota  |
| $\kappa$         | Kappa   |
| $\lambda$        | Lambda  |
| $\mu$            | Micro   |
| $\mu\text{L}$    | Microliter  |
| $\mu\text{M}$    | Micromolar  |
| AMPK             | AMP activated protein kinases                       |
| ATG              | Autophagy-related genes                             |
| $\text{Ca}^{+2}$ | Calcium   |
| CMA              | Chaperone mediated autophagy                        |
| DAG              | Diacylglycerol                                      |
| DMEM             | Dulbecco's modified eagle medium                    |
| DMSO             | Dimethylsulfoxade                                   |
| DPBS             | Dulbecco's phosphate-buffered saline                |
| EBSS             | Earl's balanced salt solution                       |
| EDTA             | Ethylenediaminetetraacetic acid                     |
| ER               | Endoplasmic reticulum                               |
| FBS              | Fetal Bovine Serum                                  |
| GFP              | Green Fluorescent Protein                           |
| HSP70            | Heat Shock Protein 70                               |
| IP3              | Inositol-1,4,5-Trisphosphate                        |
| JNK              | C-Jun N-terminal kinase                             |
| LAMP2            | Lysosomal membrane protein-2                        |
| LC3              | Microtubule-associated protein 1 light chain 3      |
| MAPK             | Mitogen-activated protein kinases                   |
| MC               | Monoclones including RFP-shRNA expression vector    |
| mTOR             | Mammalian target of rapamycin                       |
| NEAA             | Non-essential Amino Acid                            |
| PDK1             | Phosphatidylinositol-trisphosphate-dependent kinase |
| PE               | Phosphotidylethanolamine                            |
| RFP              | Red fluorescent protein                             |

|       |  |
|-------|--|
| PI3K  | Class-III-Phosphatidylinositol-3-Kinase                          |
| PIP3  | Phosphatidylinositol-3-phosphate                                 |
| PKC   | Protein kinase C   |
| PMA   | Phorbol 12-myristate 13-acetate                                  |
| PS    | Phosphatidylserine   |
| RAB23 | RAB Oncogene Family Small GTP Binding Protein                    |
| RC    | Monoclones including RFP control vector                          |
| ULK1  | Unc-51 like autophagy activating kinase 1                        |
| TRPV6 | Transient receptor potential cation channel subfamily V member 6 |

# 1. INTRODUCTION

## 1.1. Autophagy

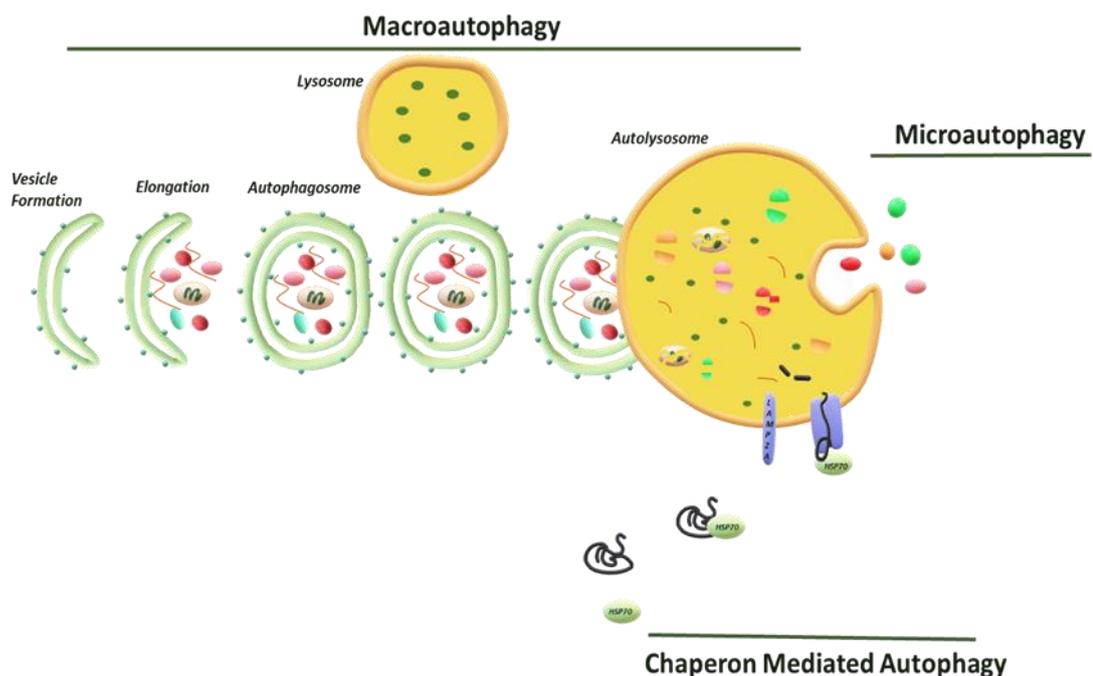
The discovery of lysosomes by C. de Duve in the 1950s brings along crucial cellular system questions including what the fate across the regulation of lysosome is and how its regulation affects the protein- enzyme function (De Duve, Pressman, Gianetto, Wattiaux, & Appelmans, 1955). During the investigation of those questions, another research team, Marilyn Farquhar and her associates focused on electron microscopy images of cells and discovered the existence of several membrane-covered vesicles. Initially, they define them as lytic bodies, known as phagophore today. The vesicles engulfed mitochondria, ER and ribosomes are called autophagic bodies (Smith & Farquhar, 1966). Investigations have been accelerating and autophagosomes, autolysosomes, and even different types of autophagy mechanisms were discovered.

Autophagy is a well-conserved recycling mechanism in the cell to remove cellular waste and toxins in order to maintain energy homeostasis. The mechanism promotes survival by delaying senescence under stress conditions such as nutrient starvation or oxidative stress. In this mechanism, double-membrane vesicles surround non-functional organelles, protein aggregates, and cytoplasmic compartments and then these complexes transport toward lysosomes for degradation. Cooperation of different autophagy-related proteins known as ATG proteins maintain the autophagy stages, which include initiation, elongation, maturation, lysosomal fusion and degradation (Figure 2.) (Glick, Barth, & Macleod, 2010). Autophagy serves as a house cleaner because both unnecessary proteins and cellular organelles are swept to make room for newly built functional materials.

Insufficiency of autophagy results in disease progression due to the accumulation of waste materials (Levine & Kroemer, 2008).

mTOR mediated PI3K/Akt activation is well studied signaling pathway and negative regulation of mTOR pathway promotes formation of ULK1 complex with ATG proteins and initiates phagophore formation (Akers, Löffler, Wesselborg, & Stork, 2012; Jung, Ro, Cao, Otto, & Kim, 2010). Lipidation of LC3 results in the conversion of LC3-I to LC3-II protein that leads to the expansion of phagophore around recycling materials. Efficient fusion of autophagosome with lysosome creates autolysosome, where the waste materials are degraded (Nath et al., 2014).

Autophagy is classified into three main groups: Macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In macroautophagy, larger molecules, such as organelles, protein aggregates that could not be degraded by the proteasomal system are recycled. In microautophagy, smaller molecules are directly transported to the lysosome and degraded. In chaperone-mediated autophagy, proteins having the KFERQ motif are first recognized by Heat Shock Protein 70 (HSP70) and then directed towards lysosome. These proteins are further identified by lysosomal membrane protein-2 (LAMP2) and engulfed in lysosomes for degradation.



**Figure 1.1.** Illustration of autophagy mechanism

## 1.2. Type of Autophagy

### 1.2.1. Macroautophagy

Macroautophagy is the best characterized type of autophagy (Levine & Klionsky, 2017). Two main kinase systems known to regulate autophagy namely the mTOR–ULK1 and the BECN1 complex. In detail cell's energy sensor mTOR or TOR is an important kinase that regulates cell growth, proliferation, survival, death, and energy metabolism. It consists of two sets of proteins, mTORC1 and mTORC2. mTORC1 is involved in cellular events such as cell growth, proliferation, and death, while mTORC2 provides the regulation of the cellular skeleton. The mTORC1 protein cluster, which plays a role in autophagy activation in starvation conditions, consists of proteins called Raptor, mLST8, PRAS40, and DEPTOR, which bind to mTOR. When nutrient or growth factors are abundant, autophagy protein Ulk1 is phosphorylated by mTOR and this phosphorylation results in inactivation of autophagy. The ULK complex contains the ULK1 or ULK2 kinase, ATG13, FIP200 (focal adhesion kinase-family interacting protein of 200 kDa) and ATG101. However, when nutrients and growth factors are limiting in the environment or under ER stress, AMP-Kinase (AMP-activated kinase) activated by LKB1 (liver kinase B1) via the phosphorylation of the Raptor directly on the mTORC1 cluster. Alternatively, AMP-Kinase inhibits mTORC1 by blockade of Rheb (Ras-related small G protein) pathway via TSC2 inhibition in the upregulated signaling pathway of mTORC1. Thus, the formation of autophagosomes is triggered. When activated, mTORC1 favors cell growth by promoting translation via the phosphorylation of p70S6K (70 kDa polypeptide 1 ribosomal protein S6 kinase) and of 4E-BP1, an inhibitor of translation initiation, therein inactivating it (Y. Chen & Klionsky, 2011).

Another protein complex needed for the formation of autophagosomes is a complex of class III phosphatidylinositol 3-kinases (PI3K). The core components of PI3K complex are also responsible for the catalytic activity of the complex and these are VPS34 (vacuolar protein sorting 34), VPS15 and Beclin-1 (BECN1) proteins. PI3P plays a crucial role in the clustering of proteins required for autophagic vesicle formation in vesicle budding regions and provides a fusion platform for many proteins.

Phosphatidylinositol-3-phosphate (PI3P) constitutes an essential membrane component of the elongating isolation membrane and it is generated by VPS34 (Devereaux et al., 2013). In mammalian cells, PI3P molecules function as recruiting point for several autophagy-related proteins to the isolation membrane such as WIPI1/2, DFCP1, and Alfy. In addition to that, ATG9 (ATG9L1 in mammals) is also one of the crucial transmembrane proteins, plays a role in the lipid delivery and establishes a platform for recruiting effectors for the phagophore (Young et al., 2006).

The autophagic sac elongation involves two separate protein-protein interactions which resemble ubiquitination. The first pathway enables the binding of the ubiquitin-like ATG12 protein to ATG5 and then the ATG5-ATG12 complex recruits ATG16L to form the ATG12-ATG5-ATG16 complex. Here, the ATG7 and ATG10 proteins, which regulate the addition of ATG12 in a manner similar to ubiquitination, play the E1 and E2-like role, respectively (Hanada et al., 2007). The second pathway is the covalent binding of phosphatidylethanolamine (PE) to the protein LC3. LC3B protein is synthesized as precursor and is a critical component of autophagosome formation. During this formation, cysteine protease ATG4 cleaves LC3 from its C-terminus and a glycine residue is exposed. Cleaved form of LC3, is activated by ATG7 then transferred to ATG3 through covalent binding and linked to an amino group of phosphatidylethanolamines (PE). Conjugation of LC3-PE to both sides of the isolation membrane enables them to act as surface receptor for the specific recruitment of other proteins (Nakatogawa, Ichimura, & Ohsumi, 2007). The autophagosomal closure results in a typical double-membraned vacuole formation.

Apart from AMP-Kinase, PTEN which is one of the tumor suppressors converts PIP3 to PIP2 and inhibits mTOR via PI3K / Akt / TSC1-2 pathway so that has a role in autophagy regulation. Also, ERK1 / 2 and c-Jun N-terminal kinase1 (JNK1) in Ras / MAPK pathway have been found as inducers of autophagy (Obara, Noda, Niimi, & Ohsumi, 2008; Xie & Klionsky, 2007). Some of oncogenes such as Class I PI3K, Akt, TOR, Bcl-2 suppresses autophagy. Studies have shown that p53, one of the most important tumor suppressor genes, plays a dual role in autophagy (Pattingre & Levine, 2006; Tasdemir, Maiuri, Morselli, et al., 2008). A number of studies have shown that p53, particularly to the cell nucleus, induces autophagy dependent or independent of the effect of transcription (Levine & Abrams, 2008; Tasdemir, Maiuri, Galluzzi, et al., 2008). On the other hand,

some studies suggest that wild and mutant forms of p53 located in the cytoplasm suppress autophagy (Crichton et al., 2006; Feng, Zhang, Levine, & Jin, 2005). In addition, TNF- $\alpha$  (Tumor necrosis factor), which plays a fundamental role in many disease mechanisms, including cancer, has been shown to activate mTORC1 by phosphorylating the TSC1 complex (IKK $\beta$ ) (Lee et al., 2007).

### **1.2.2. Microautophagy**

Microautophagy involves direct engulfment of the proteins/cytoplasm into the lysosome (mammalians) or vacuole (plants and fungi) by invagination. Dynamin-related GTPase Vps1p plays an active role to regulate the invagination of microautophagic process. Importantly, these invaginations grow and shrink rapidly, and their frequency depends on the nutritional conditions. Starvation induces the initiation and extension of invagination respectively (Uttenweiler, Schwarz, & Mayer, 2005). During extension, this formation specializes into a characteristic tubular shape termed as “autophagic tube”, depending on its unique structure and autophagy-related function (Müller et al., 2000).

The soluble substrates of microautophagy can be induced by N-starvation or rapamycin through the regulatory signaling complexes. The maintenance of organelle size, membrane homeostasis, and cell survival under N-restriction are considered as main functions of microautophagy (W.-w. Li, Li, & Bao, 2012). As a basic form of autophagy, microautophagy-dependent lysosomal/vacuolar degradative process is either non-selective or selective. The non-exclusive microautophagy engulfs soluble intracellular substrates by tubular invaginations. Thus, the selective microautophagy sequesters specific organelles with arm-like protrusions. The non-selective microautophagy is regularly observed in mammalian cells, while the three forms of selective microautophagy are frequently induced in yeasts (micropexophagy, piecemeal microautophagy of the nucleus (PMN), micromitophagy).

### **1.2.3. Chaperon mediated autophagy**

Chaperone mediated autophagy (CMA) is a selective form of autophagy and it has a distinctive way to recognize cargo molecules. In CMA, well known chaperone protein heat shock protein 70 (hsp70) recognizes proteins with specific pentapeptide motif KFERQ and translocates them to the lysosomal lumen for degradation via lysosomal-associated membrane protein 2A (LAMP2A) (Kaushik & Cuervo, 2018). CMA activation is much crucial for some cellular events such as control of cell cycle (Park, Suh, & Cuervo, 2015), CD4+ T cell activation (Valdor et al., 2014), protein quality control (Schneider et al., 2015), and response to starvation (Finn & Dice, 2005). In addition, link between CMA mechanism and neurodegenerative diseases has been reported. It is known that, CMA contributes to the degradation of misfolded proteins, which prone to become aggregates.

### **1.2.4. Selective Autophagy**

For cellular homeostasis, proper clearance of organelles or specific molecules is critical in living organisms. In selective autophagy, receptor proteins recognize specific cargo such as mitochondria, lipid droplets, invading pathogens etc. These receptor proteins are responsible for carrying the cargo to the site of autophagosomal engulfment. So far, several specific receptors or adaptor proteins, which regulate the selective degradation of specific cargo have been identified and partially characterized. Selective autophagy has drawn the attention of researchers because of its potential importance in clinical diseases; however, the physiological roles are not yet fully understood.

#### **1.2.4.1. Mitophagy**

Mitochondria are highly dynamic, double-membrane surrounded organelles, which have a major function in energy production within the eukaryotic cells. They are involved in a variety of cellular functions within eukaryotic cells and have an ancient bacterial origin. Besides energy production, they are involved in amino acid synthesis, fatty acid

production, heme synthesis, innate immunity (Tait & Green, 2012) as well as programmed cell death processes (Green & Kroemer, 2004).

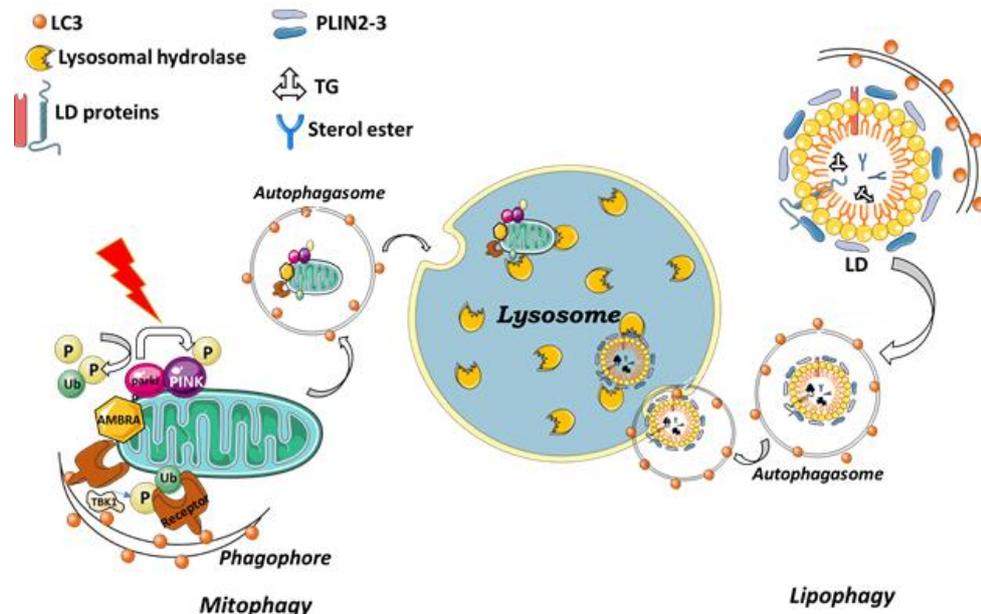
Considering all these missions of mitochondria, to control its balance according to cell's demand is essential for disease progression. The selective degradation to remove damaged mitochondria by autophagy, is called mitophagy (K. Wang & Klionsky, 2011). Parkin and PINK1-dependent mitophagy is one of the best-studied forms of mitophagy (Matsuda et al., 2010; Narendra, Tanaka, Suen, & Youle, 2008). Mainly, mitophagy is maintained by these two famous genes and their loss-of-function mutations are linked to early-onset of Parkinson's Disease. PTEN-induced putative kinase 1 (PINK1), encodes for a mitochondrially localized kinase, and PARK2, encodes a cytosolic E3 ubiquitin ligase (Narendra et al., 2008). Under normal conditions, after being synthesized as a precursor in the cytoplasm, PINK1 is imported to mitochondria through translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM) complexes. When PINK1 is imported, it is post-translationally modified within mitochondria by mitochondrial proteases. PINK1 is first cleaved through its N-terminal matrix targeting sequence (MTS) by matrix processing peptidases (MPP) and resulting cleavage followed by another cleavage by Presenilin-associated rhomboid-like protease (PARL) in the matrix (Deas et al., 2011). PARL-mediated N-terminal cleavage results in destabilizing Phe104 residue and therefore, when retranslocation from mitochondria to cytoplasm emerges, degrades by proteasome through recognition of its N-terminus (Yamano & Youle, 2013). Under stress conditions, PINK1 import to mitochondria is blocked, PINK1 proteins on OMM get dimerized and this dimerization is necessary for autophosphorylation events. This accumulated PINK1 phosphorylates various targets, including ubiquitin, and recruits the cytoplasmic E3 ubiquitin ligase, Parkin protein. After this moment, Parkin acts as an amplifier of the PINK1-generated mitophagy signal (Lazarou, Jin, Kane, & Youle, 2012).

Additionally, AMBRA1 is another key mitophagy regulator that allows proper Parkin-dependent and independent mitochondrial clearance. It is ubiquitously expressed in adult midbrain and found in complex with Parkin but AMBRA1 has no effect on Parkin recruitment to mitochondria, its activation effect on PI3K suggested to be critical for PINK/Parkin-mediated mitophagy (Strappazzon et al., 2015).

### 1.2.4.2. Lipophagy

Lipid droplets (LDs) are specialized organelles composed of lipids essential for cellular energy (metabolism) and membrane production (Walther & Farese, 2012). LDs consist of a neutral lipid namely triglyceride (TG) and cholesterol esters and are coated by a phospholipid monolayer and various proteins such as the perilipins (PLINs). Under certain conditions like nutrient deprivation, cellular lipids stored as triglycerides in LDs are hydrolyzed into fatty acids for energy. As expected second cellular response to starvation is the induction of autophagy. Interrelationship between autophagy and lipid metabolism was shown in mouse hepatocytes for first time in 2009 by Mochida et al. This study revealed that inhibition of macroautophagy, pharmacologically or by silencing of the ATG genes, leads to the accumulation of TGs and LDs in serum-starved hepatocytes. Thus, nutrient limitation triggers LD degradation by macroautophagy in hepatocytes (Mochida et al., 2015).

Microlipophagy has been detected in yeast in response to nitrogen starvation, glucose depletion, survival during stationary phase, and phospholipid imbalance. Under these conditions, LDs are taken up into the vacuole at sites of vacuolar membrane invagination (Seo et al., 2017; van Zutphen et al., 2014).



**Figure 1.2.** Illustration of selective autophagy mechanism

### **1.3. Protein Kinase C Family**

Protein Kinase C (PKC) protein family is a phospholipid-dependent serine/threonine kinase that are discovered by Nishizuka and his colleagues in the 1970s. This protein family is initially defined as PKM due to their  $Mg^{+2}$  dependent activations, but later on, they are called as PKC due to their  $Ca^{+2}$  dependent activations. Protein kinase family consists of over 15 subgroups with more than 500 kinases, each of which is involved in the regulation of gene expression therefore, downregulation or upregulation of these kinases induces severe consequences in the progression of disorders such as cancer or neurodegenerative diseases.

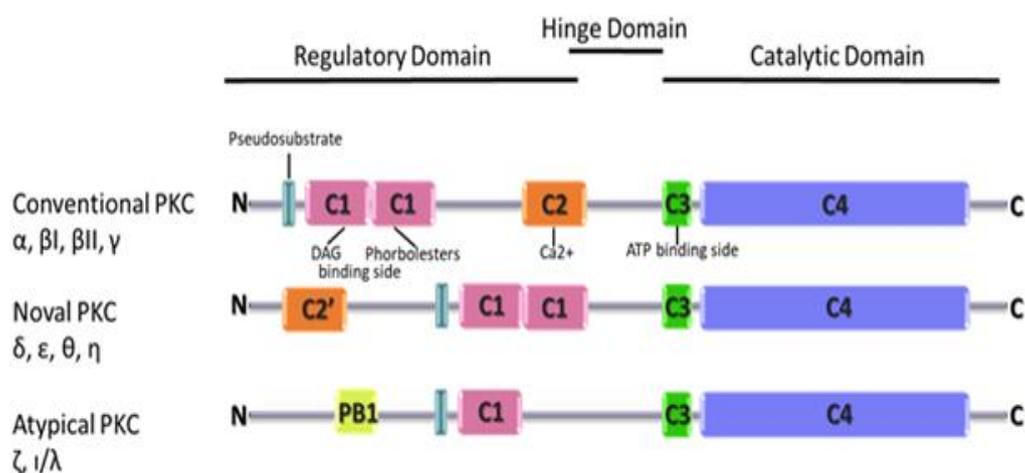
Due to the regulatory role in cellular activities through interaction of other signaling proteins, PKCs have a critical role in disease progression. To date, the involvement of PKCs in disease progression is being studied in both in vitro and in vivo models as well as patient-derived samples. In vivo animal models were designed to analyze the development of cancer (Hafeez et al., 2011; Leitges, 2007; Spitaler & Cantrell, 2004), neurodegenerative diseases (W. M. Lau et al., 2018; Russo et al., 2018; Sanna, Quattrone, Ghelardini, & Galeotti, 2014), metabolic disorders (Jha et al., 2016; Sajan et al., 2018) and muscular defects (Madaro et al., 2012; Quack et al., 2011; G.-S. Wang et al., 2009).

#### **1.3.1. Structure and Classification of PKC isozymes**

Protein kinase C (PKC) is the subgroup of the kinase family and comprises ten members. The distinguishing feature of PKCs is that they include N-terminal regulatory domain connected to C-terminal catalytic domain through a hinge domain (K.-P. Huang, 1989; Newton, 1995). Each PKC isozymes has common structural characteristics since they have four conserved domains, C1, C2, C3 and C4. C1 and C2 locate on N-terminal regulatory domain while C3 and C4 reside on C-terminal catalytic domain. C1 domain is composed of 50 amino acid sequence and has C1A and C1B domains. Cysteine and histidine residues on C1 domain associate with zinc ions.  $Zn^{+2}$  association is important because of protection of the conservation of tertiary structure. C1 domain structure shows that it has hydrophilic ligand binding site enclosed with hydrophobic amino acids. On hydrophilic region, there are diacylglycerol (DAG) and phorbol esters binding sites (Schultz, Ling, & Larsson, 2004). When DAG and phorbol esters bind on that region, C1

domain becomes completely hydrophobic and this configuration enables C1 domain to translocate and bind to the membrane (Schultz et al., 2004). C1 domains are activated by different lipids and the situation causes difference in activation of some PKCs within same subgroup. C2 is consist of 130 amino acids which include secondary messenger,  $\text{Ca}^{+2}$ , binding site (Johnson, Giorgione, & Newton, 2000). Among protein kinases, sequences of C2 domain do not show homology and have diverse differences. Because of the differences, C2 domain has distinctive functions as protein phosphorylation or lipid modification. There are three  $\text{Ca}^{+2}$  binding sites on C2 domain facilitating interaction with phosphatidylserine at the membrane and resulting in conformational change of PKCs (Bolsover, Gomez-Fernandez, & Corbalan-Garcia, 2003; Edwards & Newton, 1997). C3 has ATP binding site and C4 has protein substrate binding sites (Ramos, Reyes-Reyes, & Nanez, 2018). All PKC isozyme has pseudo-substrate region that is a substrate-mimicking short amino acid sequence that binds the substrate-binding site in the catalytic domain, inhibiting the enzyme activity (Yang, Langston, Tang, Kiani, & Kilpatrick, 2019) In the latent form, the N-terminal pseudo-substrate (PS) region which is acting as autoinhibitory region on PKC renders the active site, which is located between the two lobes of the catalytic domain (Igumenova, 2015).

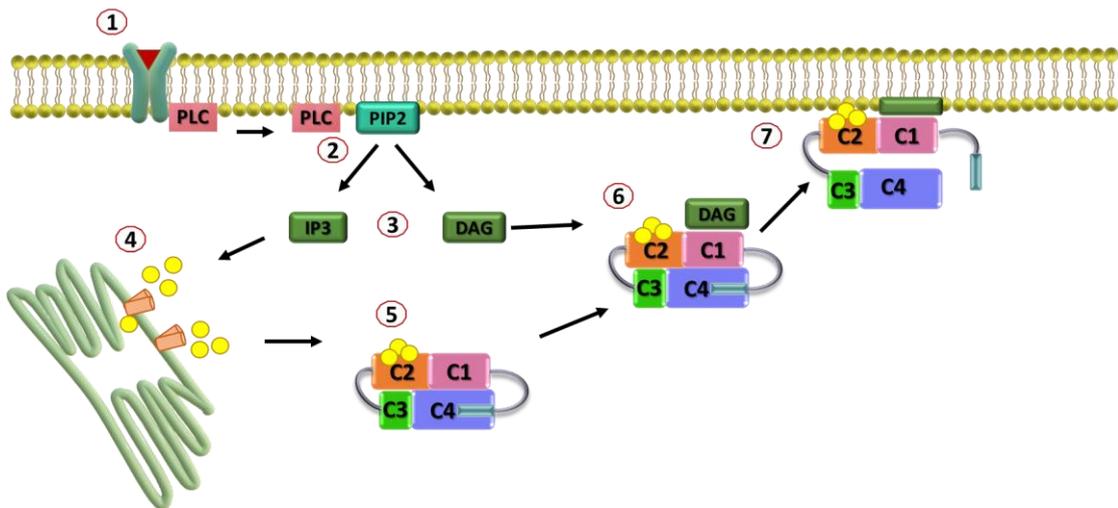
Based on structural features and activators, PKCs classify split into three categories. Conventional PKCs consist of PKC $\alpha$ , PKC $\beta$ I, PKC $\beta$ II, and PKC $\gamma$ . Novel PKCs consist of PKC $\delta$ , PKC  $\eta$ , PKC  $\theta$ , and PKC  $\epsilon$ . Atypical PKCs consist of PKC  $\zeta$  and PKC  $\iota/\lambda$  (Steinberg, 2008).



**Figure 1.3** Domain structures of PKC isozymes.

### 1.3.2. Activation and Function of PKC isozymes

PKCs stay at the resting state without secondary messengers. Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) leads to diacylglycerol (DAG) and inositol 1,4,5- trisphosphate (IP<sub>3</sub>) formation. IP<sub>3</sub> translocates to ER and initiates intracellular Ca<sup>+2</sup> release (Spitaler & Cantrell, 2004). This secondary messenger mobile to C2 domain of PKC and bind on “region”. Binding of Ca<sup>+2</sup> changes affinity of C2 domain for membrane. Conformational change and translocation to the membrane happen. When PKCs reach to membrane, they interact with PS to enable strong membrane penetration. Interaction with Ca<sup>+2</sup> is not enough to activate conventional PKCs because they also need DAG and phorbol for activation. When DAG and phorbol esters bind to C1 domain, PKCs become active and stimulate downstream signaling pathways. Foremost, phosphatidylserine interacts with C1 domain and provide suitable circumstance for binding of DAG (Steinberg, 2008). When Ca<sup>+2</sup>, DAG and phosphatidylserine collaborate, conformational change PKCs at membrane occurs in order to remove pseudo-substrate sequence. In comparison to conventional PKCs, novel PKCs do not require Ca<sup>+2</sup> for activation by reason of their C2 domain sequence variation on Ca<sup>+2</sup> binding site. So that their activation via Ca<sup>+2</sup> does not proceed. Accordingly, novel PKCs only require DAG and PS for the initiation of signaling cascades. At last, atypical PKCs consist of PKC ζ and PKC ι/λ, which only need DAG for activation.



**Figure 1.4** Activation of Protein Kinase C

### 1.3.3. PKC in Autophagy Mechanism

Activation of Protein kinase C isozymes is involved in extensive cellular mechanisms, including the autophagy pathway. Each isozyme has a diverse role in the pathway due to its phosphorylation abilities. Various functions of PKCs in main autophagy pathways such as PI3K / Akt / mTOR have been reported in several studies.

It has been described that, PKC $\alpha$  suppresses autophagy by inducing expression of miR-129-2 in maternal diabetes and provokes neural tube defects (F. Wang et al., 2017). In another study, tetrandrine, which is a PKC $\alpha$  inhibitor, causes autophagy induction in breast cancer cells through the AMPK independent and mTOR dependent mechanism (Wong et al., 2017). On the other hand, Xue et al. showed that the inhibition of PKC $\alpha$  causes lysosomal dysfunction and abnormalities in autophagosome-lysosome fusion in NRK-49F cells. Thus, the recovery of autophagic flux by PKC $\alpha$  activation resulted in kidney fibrosis through TGF $\beta$ -1 induction (Xue et al., 2018). Cisplatin, a chemotherapeutic agent, has a role for initiating several signaling pathways in order to activate cell death. The expression of PKC $\beta$  is promoted by cisplatin treatment in HeLa cells. When PKC $\beta$  is silenced, suppression of cisplatin-induced apoptosis was observed while formation of cisplatin-induced autophagy was promoted (N. Li & Zhang, 2017). Another drug, clozapine used in schizophrenia therapy, has a role in autophagy regulation. PKC $\beta$  restriction by ruboxistaurin increase clozapine-induced lipophagy and causes the recycling of lipid accumulation both in vitro and in vivo (Rimessi et al., 2017). Conventional PKC $\gamma$  reduces neuron-specific autophagy via phosphorylation of mTOR on serine 2448 and serine 2481 (Hua et al., 2018). It has been reported that cPKC $\gamma$  provokes the inhibition of ubiquitin C-terminal hydrolase L1 (UCHL1) and is involved in the ERK-mTOR mediated autophagy during ischemic neural injury (Dan Zhang et al., 2017).

Moreover, inhibition of PKC $\delta$  has a role in the correction of nephrotoxicity in kidneys by upregulating autophagy through blockage of cisplatin-induced mTOR, AKT, and ULK1 pathway (Dongshan Zhang et al., 2017b). Similarly, PKC $\delta$  was used to phosphorylate GSK3 $\alpha\beta$  and suppress autophagy under Cadmium-induced Heme oxygenase-1 (HO-1) expression (So & Oh, 2016). Inhibition of PI3K/Akt/mTOR pathway through PKC $\delta$  results in impairment of autophagic flux in neural retina cells (S.-P. Huang, Chien, & Tsai, 2015). PKC $\epsilon$  has a role in lipid metabolism by participating in hepatocyte autophagy (Yan, Niu, & Tian, 2018). Reversely, PKC $\epsilon$  suppresses autophagy in glioblastoma cells

(Toton et al., 2018). Another isozyme, PKC $\theta$ , activation in Epstein-Barr virus (EBV)-infected cells causes phosphorylation of p38 and MAPK where it leads to autophagy induction (Gonnella et al., 2015). Hypoxic-induced autophagy is enhanced under calcium-dependent PKC $\theta$  activation (Jin et al., 2016). Interestingly, under pathogenic infection, autophagy mechanism is upregulated due to an increase in the pathogen's phagosomal escape, and PKC $\eta$  silencing results in suppression of ATG7, an essential protein in autophagosome formation. Even though ATG7 levels decreased in the absence of PKC $\eta$ , the number of phagosomes is not changed. Therefore, PKC $\eta$  in autophagy regulation may have indirect effects and is subjected for further research (Micheva-Viteva, Shou, Ganguly, Wu, & Hong-Geller, 2017).

PKC $\iota$  downregulates autophagic flux by repressing LC3 conversion. Accordingly, PKC $\iota$  knockdown resulted in autophagic degradation of Hsc70 through CMA independent degradation (B.-S. Wang et al., 2013). Also, downregulation of PKC $\iota$  promotes autophagic degradation of  $\beta$ -catenin, independent of CMA (B.-S. Wang et al., 2014). In another study, PKC $\iota$  knockdown induces autophagy via the restriction of PI3K/Akt/mTOR pathway. Overexpression of mutant PKC $\iota$  protein induces autophagosome formation and depletion in p62 protein. So, they suggested that overexpression of mutant PKCs may be used as antagonists of wild type PKC $\iota$  for the upregulation of autophagy (Qu et al., 2016).

## **2. AIM OF THE STUDY**

In the literature, studies showed that some PKC isozymes have role in the regulation of autophagy; yet, the exact mechanism of the relationship between autophagy and PKC isozymes is still unclear. In this project, we aimed to find novel proteins targeted by PKC isozymes during autophagy mechanism by shRNA-based target gene screening.

### 3. MATERIALS

#### 3.1. Cell Culture

**Table 3.1** Cell Culture Solutions & Reagents

| <b>Cell Culture Reagents</b>                  | <b>Supplier</b>                   |
|---|-----------------------------------|
| Dulbecco's Modified Eagle's<br>Medium         | PAN Pan-P04-03500                 |
| Fetal Bovine Serum (FBS)                      | PAN P30-3304                      |
| Penicillin/Streptomycin (100X)                | PAN-P06-07100                     |
| L-Glutamine                                   | PAN P04-80100                     |
| 1x MEM Non-Essential Aminoacid                | Gibco 11140-35                    |
| EBSS  | Biological Industries BI02-010-1A |
| Lipofectamine 2000                            | Invitrogen 11668-19               |
| Trypsin-EDTA<br>(0.5 mM EDTA, 0.025% Trypsin) | PAN P10-019100                    |
| Phosphate Buffer Saline                       | PAN PAN-P04-036503                |
| FuGENE 6                                      | Promega E2691                     |
| PEI   | Polysciences, Inc. 23966          |
| Opti-MEM                                      | Gibco 31985-047                   |
| G-418   | Roche 04727894001                 |

### 3.2. Plasmids for Transfection

**Table 3.2** List of Plasmid

| <b>Plasmids</b>                          | <b>Supplier</b>       |
|--|-----------------------|
| PKC $\delta$                             | Addgene               |
| shPKC $\delta$                           | Addgene               |
| siTRPV6                                  | Dharmacon SO-2747429G |
| pCDNA3.1                                 | Addgene               |
| pMAX-GFP                                 | Lonza vpd-1001        |
| psPAX2                                   | Addgene12260          |
| pMD2.G                                   | Addgene12259          |
| pRSI9-U6-(sh)-UbiC-TagRFP-2A-Puro        | Addgene 28289         |
| DECIPHER Lentiviral shRNA Library<br>MM1 | Addgene 28287         |

### 3.3. Antibodies for Immunouorescence and Western Blotting

**Table 3.3** List of Antibodies

| <b>Antibody</b>       | <b>Company</b>               |
|-----------------------|------------------------------|
| Actin                 | Sigma A5441                  |
| SQSTM1                | BD Transduct Lab 610832      |
| LC3                   | Sigma 7543                   |
| TRPV6                 | Alomone Lab A036             |
| PKC $\delta$          | Santa Cruz sc213             |
| Secondary anti-mouse  | ImmunoResearch Lab 115035003 |
| Secondary anti-rabbit | ImmunoResearch Lab 111035144 |

### 3.4. General Kits

**Table 3.4** List of Kits

| <b>Kit</b>                    | <b>Company</b>   |
|-------------------------------|------------------|
| QIAquick PCR purification kit | QIAGEN 28704     |
| ECL                           | Roche 1201520001 |

|   |                       |
|---|-----------------------|
| NucleoBond™ Xtra Midi Plus                                  | Macherey-Nagel 740420 |
| SYBR Green  | Roche 04887352001     |
| Genomic DNA Isolation Kit                                   | QIAGEN 69504          |
| Amaxa Nucleofector™ Kits for Human Dermal Fibroblast (NHDF) | Lonza vpd-1001        |

### 3.5. Chemicals and Reagents

**Table 3.5** Chemicals & Reagents

| <b>12</b>                                      | <b>Brand</b>               |
|--|----------------------------|
| 2-Mercaptoethanol                              | Neofroxx 1414              |
| 2-Propanol                                     | Neofroxx 1496              |
| LB Broth Agar                                  | Caisson Labs LBP03         |
| Agarose  | Sigma A9539                |
| Albumin Fraction V (Bovine Serum Albumin, BSA) | Sigma A3059                |
| Ammonium persulfate (APS)                      | Neofroxx 1610              |
| Ampicillin                                     | Neofroxx 1728GR010         |
| Bradford Reagent                               | Sigma B6916                |
| Bromophenol blue                               | PanReac Applichem A3640    |
| Calcium chloride-dihydrate                     | Merck A1229282             |
| Ceramide                                       | Santa Cruz Sc3527          |
| Chloroform                                     | Sigma C2432                |
| DMSO   | Sigma D2650                |
| dNTP-Mix, 20mM                                 | GeneOn                     |
| Ethanol  | InterLAB                   |
| Ethylene diamine tetra acetate (EDTA)          | Neofroxx 1108              |
| 50bp DNA Ladder                                | New England BioLabs N3236S |
| Glycerol                                       | Neofroxx 1280LT001         |
| Glycine  | Neofroxx 1154              |
| HCl  | Sigma 7102                 |
| HEPES  | Sigma H6147                |
| Methanol                                       | InterLAB                   |
| Kanamycin                                      | Neofroxx 1162gr010         |
| Milk powder                                    | Neofroxx 1172              |
| Nonidet P40                                    | Applichem A1694            |
| Nitrocellulose Membrane                        | Milipore IPVH00010         |
| Nuclease free water                            | Roche 03315843001          |

|  |                      |
|--|----------------------|
| PageRuler Prestained Protein Ladder    | ThermoFisher 26616   |
| Paraformaldehyde                       | Sigma 158127         |
| PMA                                    | Milipore 524400      |
| PMSF                                   | Sigma P7626          |
| Potassium chloride                     | Neofroxx 1197        |
| Protease Inhibitors                    | Sigma P8340          |
| RNase Inhibitor                        | ThermoFisher EO0382  |
| SDS                                    | Neofroxx 3250        |
| Sodium Azide                           | Riedel de Haen 13412 |
| Sodium chloride                        | Neofroxx 1236        |
| Tetramethylethylenediamine (TEMED)     | Sigma T7024          |
| Tris (tris-hydroxymethyl-aminomethane) | Sigma T1503          |
| GENEzol RNA Isolation Reagent          | Geneaid GZR100       |
| Tween 20                               | Neofroxx 8506        |
| Dream Taq DNA polymerase               | ThermoFisher EP0701  |
| DNase I                                | ThermoFisher EN0521  |
| Revertaid reverse transcriptase        | ThermoFisher EP0441  |
| Random hexamer                         | ThermoFisher SO142   |
| Ribolock RNase                         | ThermoFisher EO0381  |
| PVDF WB Membrane                       | Roche 03010040001    |
| RNase Exitus Plus                      | Applichem A7153      |

### 3.6. Buffers and Solutions

**Table 3.6** Preparation of Buffer and Solutions

| Buffer/Solution       | Component  |
|-----------------------|--|
| RIPA Buffer           | 25mM Tris<br>125 mM NaCl<br>1% NP40<br>0.5% Sodium deoxycolate<br>0.1% SDS<br>0.004% sodium azide<br>pH 8.0  |
| 4XProtein Loading Dye | 200mM TrisHCl pH 6.8<br>8% SDS<br>50% Glycerol<br>4% mercaptoethanol<br>50 mM EDTA<br>0.08% Bromophenol Blue |

|                           |  |
|---------------------------|--|
| 15% Separating Gel        | 375 mM TrisHCl pH 8.8<br>0.1% SDS<br>15% Acrylamide:Bisacrylamide<br>0.05% APS<br>0.005% TEMED   |
| 10% Separating Gel        | 375 mM TrisHCl pH 8.8<br>0.1% SDS<br>10% Acrylamide:Bisacrylamide<br>0.05% APS<br>0.005% TEMED   |
| 4% Stacking Gel           | 0.125 mM TrisHCl pH 6.8<br>0.1% SDS<br>4% Acrylamide:Bisacrylamide<br>0.05% APS<br>0.0075% TEMED   |
| PBST                      | 3.2 mM Na <sub>2</sub> HPO <sub>4</sub><br>0,5 mM KH <sub>2</sub> PO <sub>4</sub><br>1,3 mM KCl<br>135 mM NaCl<br>0,05% Tween 20<br>pH 7.4 |
| 2xHBS                     | 140 mM NaCl<br>1,5 mM Na <sub>2</sub> HPO <sub>4</sub><br>50 mM HEPES  |
| 4% Paraformaldehyde (PFA) | 150 mM NaCl<br>1% NP40<br>0.5% Sodiumdeoxycolate<br>0.1% SDS<br>50 mM Tris pH 7.4  |
| Red Solution              | 5% BSA,<br>0.02% Sodium Azide in PBST<br>Phenol red<br>pH 7.5  |
| 3X protein loading dye    | 6% SDS<br>30% Glycerol<br>16% β-Mercaptoethanol<br>0.1% Bromophenol blue<br>in 1 M Tris-HCl pH 6.8   |
| ECL solution              | 25 mM luminol<br>9 mM coumeric acid,<br>70 mM Tris-HCl pH 8.8  |
| 50X TAE                   | 2M Tris<br>0.5M EDTA<br>1M Glycial Acetic Acid<br>ddH <sub>2</sub> O up to 1 lt  |

### 3.7. Software and Websites

**Table 3.7** List of Software and Websites

| <b>Name</b>   | <b>Purpose</b>                      |
|---|-------------------------------------|
| ImageJ  | Image analysis                      |
| <a href="http://www.ensembl.org/index.html">http://www.ensembl.org/index.html</a> | Genome sequence analysis            |
| <a href="https://www.uniprot.org/">https://www.uniprot.org/</a>                   | Genome sequence analysis            |
| <a href="https://primer3plus.com/">https://primer3plus.com/</a>                   | Primer design program               |
| <a href="https://www.addgene.org/">https://www.addgene.org/</a>                   | Vector map analysis                 |
| GPS 2.0   | Phosphorylation Site Identification |
| Phosphomotif  | Phosphorylation Site Identification |
| DISPHOS   | Phosphorylation Site Identification |
| KinasePhos2.0   | Phosphorylation Site Identification |
| NetPhos 3.1   | Phosphorylation Site Identification |
| NetPhorest  | Phosphorylation Site Identification |
| PKIS  | Phosphorylation Site Identification |
| PhosphoPick   | Phosphorylation Site Identification |
| Muside  | Phosphorylation Site Identification |

## 4. METHODS

### 4.1. Cell Culture

Mouse embryonic fibroblast (MEF) GFP-LC3 transgenic cells and HeLa cells stably expressing LC3 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine and 1x MEM non-essential amino acid solution. For MEF GFP-LC3 single cell monoclonal, puromycin (3 µg/ml) was added into complete medium. For HeLa GFP-LC3 stable cells, G-418 (0,6mg/ml) was added into complete medium. Cells were maintained at 37°C with 5% CO<sub>2</sub>. Starvation induced autophagy was established by treatment of cells with Earl's balanced salt solution (EBSS) for 2 hours. PMA (100nM) and ceramide (1 µM) treatments were performed for 30 minutes.

For cryopreservation of cells, cells in 10cm culture dishes were detached with trypsin and counted. Each cryopreservation tubes contained at least 1,5 million of cells. Cryotubes were prepared with 900 µl cell suspension and 100 µl DMSO. Cryotubes including cells were froze in cryobox including isopropanol and stored at -80°C overnight. Next day, cryotubes were transferred to liquid nitrogen tank for long term preservation.

For cell thawing, cells preserved into nitrogen tank were taken and thawed into 37°C water bath for 15 seconds. Then they were transferred into 15 ml falcon tube including 5 ml complete medium. Cell suspension was centrifuged at 300g for 5 minutes and pellet was dissolved into 8 ml complete medium and seed in 10 cm culture plate.

## **4.2. Decipher Lentiviral shRNA library Transduction**

DECIPHER lentiviral shRNA library, Mouse Module 1: Pathway Targets comprises 27,000 shRNAs targeting 5,000 gene sequences and carries a puromycin-resistance cassette. Calcium- phosphate transfection method was used for transduction of lentiviral shRNA library. HEK293T cells were transduced with 10 µg lentivirus vector, 8 µg psPAX2 and 2 µg pmD2.G in DMEM containing 10%FBS, 2mM L-Glutamine and 100U/ml penicillin/streptomycin. Total volume of plasmid mixture was completed to 225 µl with Hyclone water and 25 µl 2.5 M CaCl<sub>2</sub> was added. This mixture was added onto 250 µl of 2xHBS dropwise. 8 hours later, cells were washed with 1X PBS three times, and fresh media was added. After 24 hours, media containing lentiviruses were collected and fresh media were added. 36 hours later, media were harvested again and combined with the previously collected media and centrifuged at 300 × g for 5 minutes. Supernatant was filtered with 0,45 µm filter, aliquoted and stored at -80 °C. MEF GFP-LC3 transgenic cells were infected with 100 µl of the lentiviral supernatant. 6 hours later, cells were washed, and fresh media were added. After 72 hours, transduction efficiency was checked under an Olympus inverted microscope. Transduced cells were selected with 3 µg/mL puromycin for 10 days. Single cell colonies were produced.

## **4.3. Target Gene Sequencing**

### **4.3.1. Genomic DNA Isolation**

Genomic DNA was isolated by QIAamp® DNA Mini Kit as described by the manufacturer. Briefly, MEF GFP-LC3 transgenic monoclonal cells were seeded on 10cm cell culture dishes (2x 10<sup>6</sup> cells/dish) and cultured at 37<sup>0</sup>C with 5% CO<sub>2</sub>. Next day DMEM was aspirated and cells were rinsed with 2 ml 1X PBS. For harvest cells, 2 ml 1X PBS were put on culture dish and gently scraped with using cell scraper. Cell suspension were collected in 15 ml centrifugation tube and centrifuged at 300 × g for 5 minutes at room temperature (15–25°C). Supernatant was aspirated and cells were resuspended in 200 µl 1X PBS. They were transferred into 1,5 ml microcentrifuge tube. 20 µl QIAGEN proteinase K was added and vortexed. 200 µl Buffer AL was added to the suspension and

mixed by up&down and vortexed for 15 seconds. The suspension were incubated at 56<sup>0</sup>C for 10 minutes. After incubation, 200  $\mu$ l ethanol was added and vortexed for 15 seconds. Then the mixture was transferred to mini spin column and centrifuged at 6000  $\times$  g for 1 minute at room temperature. The column was located on new 2 ml collection tube. 500  $\mu$ l Buffer AW1 was added and centrifuged at 6000  $\times$  g for 1 minute at room temperature. The column was located on new 2 ml collection tube. 500  $\mu$ l Buffer AW2 was added and centrifuged at 14000  $\times$  rpm for 3 minute at room temperature. The column was located on new 1.5 ml microcentrifuge tube and centrifuged at 15000  $\times$  rpm for 1 minute in order to eliminate remaining buffer. The column was located on new 1.5 ml microcentrifuge tube and 50  $\mu$ l Buffer AE was added into the column. The sample was incubated at room temperature for 5 minutes then centrifuged at 6000  $\times$  g for 1 minute. Concentration of the genomic DNA was measured using Thermo Fisher 2000 NanoDrop spectrophotometer. The sample was stored at +4<sup>0</sup>C for further use.

#### 4.3.2. shRNA-specific Barcode PCR Amplification

To determine shRNA barcodes in monoclonal cells, PCR performed with primer sets designed to amplify the barcode sides on genomic DNA of the samples (Table 4.1). Each 25  $\mu$ l reaction included 50 ng gDNA of monoclonal cells, 10  $\mu$ M dNTP, 0.5  $\mu$ M primers, Taq polymerase, Taq polymerase buffer and nuclease free water.

**Table 4.1** PCR reaction Primers

| Primer Name | Sequence                         |
|-------------|----------------------------------|
| FwdHTS      | 5'-TTCTCTGGCAAGCAAAAGACGGCATA-3' |
| FwdU6-1     | 5'-CAAGGCTGTTAGAGAGATAATTGGAA-3' |
| FwdU6-2     | 5'-CCTAGTACAAAATACGTGACGTAGAA-3' |
| RevCPPT-5   | 5'-TGCCATTTGTCTCGAGGTCGAGAA-3'   |
| RevHTS1     | 5'-TAGCCAACGCATCGCACAAAGCCA-3'   |

**Table 4.2** PCR reaction Reagents

| Reaction Reagents         | Amount ( $\mu$ l)       |
|---------------------------|-------------------------|
| 10X Taq polymerase buffer | 2,5                     |
| Forward primer            | 1                       |
| Reverse primer            | 1                       |
| dNTP (10 $\mu$ M)         | 0,5                     |
| gDNA 50ng                 | Determined to each gDNA |
| Taq polymerase            | 0,25                    |
| Nuclease-free Water       | Up to 25 $\mu$ l        |

PCR was performed at 95<sup>0</sup>C for 1 minute followed by 30 cycles of 95<sup>0</sup>C 30 minutes, 55<sup>0</sup>C 30 minutes, 72<sup>0</sup>C 30 minutes. Final polymerase step was performed at 72<sup>0</sup>C 3 minutes. PCR products were stored at +4<sup>0</sup>C for further use.

**Table 4.3** PCR reaction for Barcode amplification

|                      | Cycle | Time       | Temperature $^{\circ}$ C |
|----------------------|-------|------------|--------------------------|
| Initial Denaturation | 1     | 1 minute   | 95                       |
| Amplification        | 30    | 30 seconds | 95                       |
|                      |       | 30 seconds | 55                       |
|                      |       | 30 seconds | 72                       |
| Final polymerase     | 1     | 3 minutes  | 72                       |

### 4.3.3. Agarose Gel Electrophoresis

1 gram of agarose powder was mixed with 50 ml 1X TAE buffer in a 100 ml microwavable flask for the preparation of 2% agarose gel. The flask was then placed into

the microwave until the agarose powder dissolved completely in the buffer. The solution then left at room temperature for cooling. Mixture was poured carefully without making bubbles into a clean gel tray and comb was placed on the tray. Gel was stayed to dry for 20-30 minutes at room temperature. After the gel began to polymerize, it solidified. Dried gel was placed in the electrophoresis tank filled with 1X TAE buffer. 20 µl samples were prepared by mixing 4 µl 6X DNA loading dye. In first well, molecular weight DNA ladder was loaded and each sample were loaded. The gel was run at 100 Volt constant voltage for 30 minutes. After running, the gel was placed and incubated for 20 minutes into 100 ml 1X TAE buffer containing 5 µl of ethidium bromide. After EtBr incubation, the gel was rinsed with water. DNA bands were visualized under UV-light.

#### **4.3.4. Purification of PCR Products**

PCR products were run on 2% agarose gel and the fragments were visualized under low exposed UV-light. DNA fragments were isolated from the gel by using bistoury. The isolated fragment was weighted in 1.5 ml microcentrifuge tubes. DNA fragments were purified by using QIAquick PCR purification kit according to the manufacturer's *instructions*. Briefly, 200 µl Buffer NT1 was added in the tube containing DNA fragment and incubated at 50 °C for 10 minutes. The mixture was vortexed each 3 minutes. QIAquick PCR purification kit column was placed in 2 ml collection tubes. Then melted gel sample was transferred into columns and centrifuged at 11000 × g for 30 seconds for DNA binding on silica membrane. 700 µl Buffer NT3 was added to the column for cleaning of the membrane. The liquid collected in the tube was discarded and column was centrifuged one more time at 11000 × g for 1 minute in order to eliminate remaining buffer. Then column was placed on clean 1.5 ml microcentrifuge tube. 20 µl Buffer NE was added and the column was incubated 1 minute at room temperature. Then the column was centrifuged at 11000 × g for 1 minutes. Concentration of the purified DNA was measured by using ThermoFisher 2000 NanoDrop spectrophotometer. Samples were sent to BMLabosis (Turkey) for Sanger sequence analysis.

#### **4.3.5. Target Gene Identification**

DECIPHER<sup>TM</sup> barcoded shRNA library, Mouse Module 1 (Collecta): Pathway Targets, includes 27,500 shRNAs with 4,625 genes. Manufacturer provides a document including

target mRNA RefSeq# and HUGO gene symbol. Barcode sequences were searched at the document included HUGO Gene Symbol and RefSeq.

| gene# | gi        | RefSeq         | HUGO Gene Symbol | CDS/UTR | sense                | sense + mismatches   | 18-nt barcode        |
|-------|-----------|----------------|------------------|---------|----------------------|----------------------|----------------------|
| 2032  | 62909982  | NM_001013391.1 | Cpsf6            | CDS     | CTGGTGATTATGGGAGTGC  | CTGGTGATTgTGGGAGTg   | CAGTGTGTGTGTGTGTC    |
| 2032  | 62909982  | NM_001013391.1 | Cpsf6            | CDS     | GAAATCTAACATGGTGGAC  | GAAATCTAACATGGTGGAt  | TGGTGTGTGTGTGTGTAC   |
| 2967  | 118130112 | NM_011580.3    | Thbs1            | CDS     | CTGTAGGTTATGATGAGTI  | CTGTAGGTTATGATGAGTI  | ACGTGTGTGTGTGTGTG    |
| 1398  | 31982067  | NM_009015.2    | Rad541           | CDS     | GTCCATTAAAGAAGCGAGCC | GTCCATTAAAGAAGtGAGCt | CAGTGTGTGTGTGTGTCAGT |
| 3309  | 121583921 | NM_031156.2    | Ide              | CDS     | CCTGGTCATTATCTTGGTC  | CCTGGTCATTATCTTGGTt  | ACGTGTGTGTGTGTGCACA  |
| 4313  | 145966832 | NM_007891.3    | E2f1             | CDS     | CATCCAGCTCATTGCCAAG  | CATCCAGCTtATTGctAAG  | GTGTGTGTGTGTGTCAAC   |
| 1637  | 34996508  | NM_194350.1    | Maf              | CDS     | CAAGGAGAAATACGAGAAG  | CAAGGAGAAATtGAGAAG   | TGGTGTGTGTGTGTGCATG  |
| 1637  | 34996508  | NM_194350.1    | Maf              | CDS     | ACAAGGAGAAATACGAGAA  | ACAAGGAGAAATtGAGAA   | ACGTGTGTGTGTGTACGT   |

**Figure 4.1.** Collecta Decipher shRNA Library document. Barcode sequence site was highlighted and indicated with an arrow.

#### 4.4. Measurement of Targets’ mRNA level by RT-q-PCR

##### 4.4.1. RNA Isolation

In order to determine the amount of TRPV6 and RAB23 mRNA level in monoclonal cells, RNA isolation was applied as following. MEF GFP-LC3 monoclonal cells were seeded in 6 well culture plates as  $1 \times 10^6$  cell/well. Next day cells were treated with 0.006% DMSO as vehicle control and 100nm PMA for 30 minutes, EBSS and EBSS with 100nm PMA for 2 hours. After the treatment procedure, cells were washed with 2 ml 1X PBS. RNA extraction was conducted by using Genezol RNA isolation reagent. 1 ml Genezol RNA reagent was put on the cells for lyses and incubated for 5 minutes at room temperature. Cell lysate was collected into 1.5 ml microcentrifuge tube and 200  $\mu$ l chloroform was added. The mixture was up&down for 15 seconds to mix homogenously. The color change was observed from pink to pale pinkish after mixing. The solution was incubated 15 minutes at room temperature. After incubation, the tube was centrifuged at  $12000 \times g$  for 10 minutes at room temperature. The phase separation was observed respectively

upper colorless, middle white line and bottom pink. The upper phase was carefully transferred into clean 1,5 ml microcentrifuge tube with avoiding touch to white phase. 500  $\mu$ l ice-cold 2-isopropanol was added on the colorless phase and incubated on ice for 15 minutes. After incubation, the tube was centrifuged at  $12000 \times g$  for 10 minutes at  $+4^{\circ}\text{C}$ . Then supernatant was discarded, and 1 ml 75% ethanol was added onto the pellet. The mixture was centrifuged at  $12000 \times g$  for 10 minutes at  $+4^{\circ}\text{C}$ . Supernatant was discarded, and the pellet was incubated at room temperature for 20-30 minutes in order to eliminate ethanol. RNA pellets were dissolved in RNase free water by gently pipettage. Concentration of the RNA samples was measured using Thermo Fisher 2000 NanoDrop spectrophotometer and stored at  $-80^{\circ}\text{C}$  for further use.

#### 4.4.2. cDNA Synthesis

cDNA was synthesized from 1000 ng RNA samples by reverse transcription PCR. First Dnase cycle was applied at  $37^{\circ}\text{C}$  for 30 minutes. Then, EDTA cycle was applied for  $75^{\circ}\text{C}$  for 10 minutes. Then, 10  $\mu$ l PCR master mix including 5X reaction buffer, 10 mM dNTP, random hexamer (10 ng/ $\mu$ l), ribolock (40U/  $\mu$ l), RT enzyme (200U/  $\mu$ l) , nuclease-free water were added to EDTA cycle product. The cycles at  $25^{\circ}\text{C}$  10 minutes,  $37^{\circ}\text{C}$  60 minutes and  $70^{\circ}\text{C}$  10 minutes were applied 3 times. At the end of cycles, PCR reaction was stoped by incubating PCR products on ice for 1-2 minutes. The samples were stored at  $-20^{\circ}\text{C}$  for further analysis. The reagents and the process for cDNA synythesis are stated as tables at the below.

**Table 4.4** DNase Cycle Reagents

| Reagents           | Amount ( $\mu$ l) |
|--------------------|-------------------|
| 1000 ng RNA        | 7                 |
| 10X DNase I Buffer | 1                 |
| DNase I enzyme     | 1                 |

**Table 4.5** EDTA Cycle Reagents

| Reagents            | Amount ( $\mu$ l) |
|---------------------|-------------------|
| DNase cycle product | 9                 |
| 25 mM EDTA          | 1                 |

**Table 4.6** Reverse transcription PCR (RT-PCR) Reagents

| Reagents                        | Amount ( $\mu$ l) |
|---------------------------------|-------------------|
| EDTA cycle product              | 10                |
| Nuclease-free Water             | 3,2               |
| 5X reaction buffer              | 4                 |
| 10 mM dNTP                      | 0,5               |
| Random hexamer (10 ng/ $\mu$ l) | 0,2               |
| Ribolock (40U/ $\mu$ l)         | 0,2               |
| RT enzyme                       | 0,1               |

#### 4.4.3. RT-q-PCR

mRNA expression level of target genes upon PMA and starvation treatment was analyzed with Quantitative Real-Time PCR reaction. cDNA samples were diluted 1:5 ratio with PCR grade water and 5  $\mu$ l of the dilution was used as template in RT-qPCT analysis. 0,5  $\mu$ l 10  $\mu$ M forward and 0.5  $\mu$ l 10  $\mu$ M reverse primers, 4  $\mu$ l water and 10  $\mu$ l 2x Sybr Green was added in a well of white 96 well plate on ice with dark condition. The 96 well plate was sealed with colorles sealer and centrifuged at 1500  $\times$  g for 2 minutes.  $\beta$ -glyceraldehyde 3-fosfat dehydrogenase (GAPDH) was houskeeping gene used as control. The reaction began with

1 cycle preincubation at 95 °C for 10 minutes and followed 50 cycle amplification at 95 °C for 10 secs, 60 °C for 1 minute and 72°C for 10 seconds, 1 cycle melting curve at 95 °C for 5 seconds, 55 °C for 1 minute and 97 °C continuous and 1 cycle cooling at 40 °C for 30 seconds. Results were analysed by  $\Delta\Delta$ CT method.

**Table 4.7** List of RT-qPCR Primers

| <b>Primer Name</b>  | <b>Sequence</b>              |
|---------------------|------------------------------|
| Mouse Forward TRPV6 | 5'- TTGGGCTGGTGTCAATGTCA-3'  |
| Mouse Reverse TRPV6 | 5'-AGCCAGCAGAATCGCATCAAG-3'  |
| Mouse Forward RAB23 | 5'-AAAATGAGGAAGCGGAGGGA-3'   |
| Mouse Reverse RAB23 | 5'-AGTGACTTCTGACCGATGCA-3'   |
| Mouse Forward GAPDH | 5'-TCACCACCATGGAGAAGGC-3'    |
| Mouse Reverse GAPDH | 5'- GCTAAGCAGTTGGTGGTGTCA-3' |

**Table 4.8** RT-q-PCR Reagents

| <b>Reagents</b>          | <b>Amount (µl)</b> |
|--------------------------|--------------------|
| 1:5 diluted cDNA         | 5                  |
| 10 µM Forward Primer     | 0,5                |
| 10 µM Reverse Primer     | 0,5                |
| PCR Grade Water          | 4                  |
| 2x Sybr Green Master Mix | 10                 |

**Table 4.9** RT-q-PCR reaction

| <b>Cycle</b>   | <b>Time</b> | <b>Temperature °C</b> |
|----------------|-------------|-----------------------|
| Pre-incubation | 1           | 95                    |
| Amplification  | 50          | 95                    |
|                |             | 60                    |
|                |             | 72                    |
| Melting Curve  | 1           | 95                    |
|                |             | 55                    |
|                |             | 97                    |
| Cooling        | 1           | 40                    |

#### **4.5. LC3 Dot Analysis**

HeLa LC3 stable cells were seeded on coverslips in the 6 well-plates as  $1,25 \times 10^5$  cell/well and MEF GFP-LC3 transgenic cells were seeded in 12 well-plates as  $2 \times 10^4$  cell/well. After 36 hours, corresponding wells were treated with EBSS for 2 hours. In the last 30 minutes of EBSS, 100 nM PMA and the necessary amount of DMSO were added to the media. Cells were fixed with 4% paraformaldehyde for 20 minutes on ice and mounted on the slides. Minimum 100 cells were counted for each sample under 60x magnification on Olympus BX60 fluorescence microscope and thresholds were determined as > 20 dots for HeLa LC3 stable cells and > 8 dots for MEF GFP-LC3 transgenic cells, respectively. Threshold number of LC3 puncta were determined in vehicle control group. Cells having LC3 puncta more than threshold number were counted as positive autophagic cells and cells having LC3 puncta less than threshold number were counted as negative autophagic cells.

#### 4.6. Cell Lysate Preparation and Immunoblotting

Cells were lysed with RIPA buffer containing 1% complete protease inhibitors and 1 mM PMSF. The lysed was incubated on ice for 20 minutes and every 5 minutes it was mixed by vortexing. Then the lysed was centrifuged for 15 minutes at 13200 rpm, 4°C. After centrifugation, the supernatant was taken into new tube and protein concentration was determined by Bradford Assay. For immunoblotting, 40 µg proteins were denatured in 3X protein loading dye for 10 minutes at 95°C. Denatured proteins were loaded to the SDS-Polyacrylamide (PAGE) gels (Table 4.11 and 4.12) and separated using 15% gel for LC3 and 10% gel for other proteins. Proteins were transferred to nitrocellulose membranes at 250 mA for 1 hour for LC3 and 1 hours 15 minutes for other proteins. Membranes were blocked in 5% non-fat milk in PBS-T for 1 hour at room temperature and washed with PBS. Then membranes were incubated with primary antibodies diluted in red solution with 1:1000 ratio for 1 hour at room temperature or overnight at 4°C then washed with PBS-T three times at each 5 minutes. After washing, the membranes were incubated with secondary antibodies prepared in 5% non-fat milk solution with 1:10000 ratio for 1 hour at room temperature and rinsed with PBS-T three times at each 5 minutes. Then membrane was placed on cassettes and signals were displayed by chemiluminescence reagent or homemade ECL solution. After observing signals, films were placed on membranes and the cassettes were closed and stored in dark for at least 20 minutes. Then the films were developed and fixed by using developer and fixer solutions until the bands arise. The band intensities were analyzed with ImageJ software.

**Table 4.10** SDS- PAGE Separating gel preparation

| Lower Gel               | 15%     |         |        | 10%     |         |        |
|-------------------------|---------|---------|--------|---------|---------|--------|
|                         | 5 ml    | 10 ml   | 20 ml  | 5 ml    | 10 ml   | 20 ml  |
| <b>ddH<sub>2</sub>O</b> | 850 µl  | 1.75 ml | 3.5 ml | 1.7 ml  | 3.4 ml  | 6.8 ml |
| <b>50 % Glycerol</b>    | 400 µl  | 750 µl  | 1.5 ml | 400 µl  | 750 µl  | 1.5 ml |
| <b>Lower Buffer</b>     | 1.25 ml | 2.5 ml  | 5ml    | 1.25 ml | 2.5 ml  | 5ml    |
| <b>Bis/Acrylamide</b>   | 2.5 ml  | 5 ml    | 10 ml  | 1.65 ml | 3.35 ml | 6.7 ml |

|                |            |             |             |            |             |             |
|----------------|------------|-------------|-------------|------------|-------------|-------------|
| <b>10% APS</b> | 50 $\mu$ l | 100 $\mu$ l | 200 $\mu$ l | 50 $\mu$ l | 100 $\mu$ l | 200 $\mu$ l |
| <b>Temed</b>   | 5 $\mu$ l  | 10 $\mu$ l  | 20 $\mu$ l  | 5 $\mu$ l  | 10 $\mu$ l  | 20 $\mu$ l  |

**Table 4.11** SDS- PAGE Stacking gel preparation

| <b>Upper Gel</b>        | <b>2.5 ml</b> | <b>5 ml</b> | <b>7.5 ml</b> | <b>12.5 ml</b> |
|-------------------------|---------------|-------------|---------------|----------------|
| <b>ddH<sub>2</sub>O</b> | 1.62 ml       | 3.25 ml     | 4.88 ml       | 8.13 ml        |
| <b>Upper Buffer</b>     | 625 $\mu$ l   | 1.25 ml     | 1.87 ml       | 3.12 ml        |
| <b>Bis/Acrylamide</b>   | 250 $\mu$ l   | 500 $\mu$ l | 750 $\mu$ l   | 1.25 ml        |
| <b>10% APS</b>          | 20 $\mu$ l    | 40 $\mu$ l  | 60 $\mu$ l    | 100 $\mu$ l    |
| <b>Temed</b>            | 5 $\mu$ l     | 10 $\mu$ l  | 15 $\mu$ l    | 25 $\mu$ l     |

#### **4.7. Prediction of Protein-Protein Interaction of Target Protein and PKC by Bioinformatics-Based Software**

Based on the findings indicating the presence of interaction between TRPV6 or RAB23 with PKCs, a preliminary analysis based entirely on amino acid sequence information was performed to give an idea of the regions through which this interaction could be achieved. For this purpose, bioinformatics tools, GPS 2.0, Phosphomotif, DISPHOS, KinasePhos2.0, NetPhos 3.1, NetPhorest, PKIS, PhosphoPick, and Muside, were used.

#### **4.8. HeLa Cell Line Transfection Protocols**

##### **4.8.1. Transfection with Calcium-Phosphate**

HeLa cells were seeded to 6-well plates as 150 000 cell/well. Next day, cells were transfected with 4 µg of pcDNA3.1 for control, pMAX GFP for transfection control by using Calcium-Phosphate Transfection. The total volume of plasmid mixture was completed to 54 µl with Hyclone water and 6 µl 2.5 M CaCl<sub>2</sub> was added. This mixture was added onto 60 µl of 2xHBS drop by drop with continuous vortex. After 20 minutes of incubation, the solution was distributed to well as 120 µl. 8 hours after post transfection, cells were washed with 1x PBS twice and 2 ml complete medium added.

#### **4.8.2. Amaxa Nucleofactor Transfection**

HeLa cells are homogenized and counted with Trypan blue. 225 000 cell/well were transferred to eppendorfs and centrifuged at 3000 × g for 5 minutes. Nucleofactor reagents were mixed with 4.5:1 nucleofactor to supplement ratio. Supernatant was discarded and cells were resuspended in 90 µL nucleofactor mixture including 4 ug pMAX GFP. Mixture is taken to cuvette and electroporated with AMAXA machine. After incubation for 10 minutes, cells were mixed with medium. Then they were seeded to 6 well plates. 8 hours after post transfection, cells were washed with 1x PBS twice and 2 ml complete medium added.

#### **4.8.3. Lipofectamine Transfection**

HeLa cells were seeded to 6-well plates as 125 000 cell/well. Next day, cells were transfected with 6 µg of pcDNA3.1 for control or pMAX GFP for transfection control by using Lipofectamine 2000. 6 µg of plasmids were mixed with 100 µL OptiMEM separately. The optimum ratio of DNA to Lipofectamine is ½ for HeLa cells. In another eppendorf tube, 12 µL Lipofectamine was mixed with 200 µL OptiMEM. The solutions were allowed for incubation for 5 min at RT. Then mixture with DNA were added on top of tube including Lipofectamine-OptiMEM. The solution was vortexed and incubated for 15 minutes at RT and vortexed each 5 minutes. During incubation process, cells were washed with 1X PBS once. After washing, 800 µL OptiMEM were given to each well. After incubation, Lipofectamine-plasmid solution was added to wells. After 4 hours from

post transfection, cells were washed with 1X PBS twice then 2 mL complete medium added.

#### **4.8.4. Polyethyleneimine Transfection**

HeLa cells were seeded to 6-well plates as 125 000 cell/well. Next day, cells were transfected with 4 µg of pcDNA3.1 for control or pMAX GFP for transfection control by using PEI. In one tube, 100 µL only DMEM mixed with 9 µL PEI. In another tube, 100 µL only DMEM mixed with 4 µg plasmid DNA. Then PEI-DMEM mixture was added drop by drop into plasmid-DMEM mixture on vortex. The solution was incubated for 15 minutes at RT. Cells were washed with 1X PBS ones and 2 ml complete medium added. After incubation solution was given to cells and 8 hours later the cells were washed with 1X PBS twice and complete medium added.

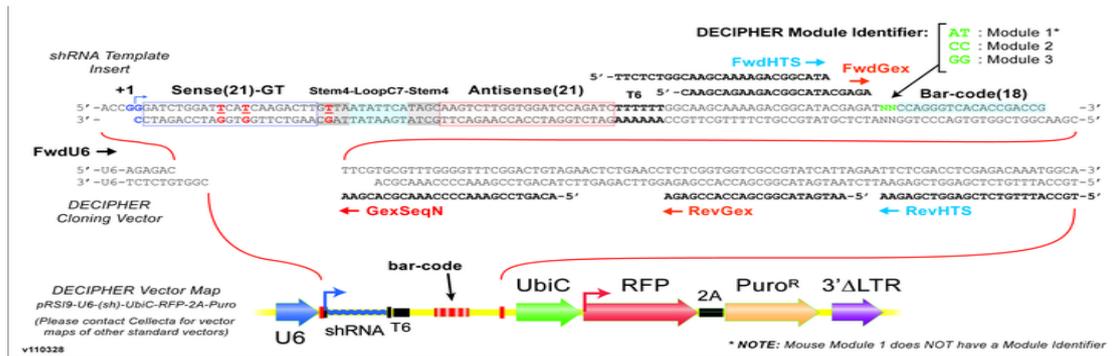
#### **4.8.5. FuGENE Transfection**

FuGENE 6 Transfection Reagent was used according to the manufacturer's *instructions*. Briefly, HeLa cells were split in 6-well plates as 150000 cells/well. Next day, FuGENE transfection was performed with 4 µg of pcDNA3.1 as control and pMAX-GFP for transfection control. In microcentrifuge tubes, pcDNA3.1 or pMAX-GFP was mixed with 90 µl OptiMEM then FuGENE 6 reagent was added to DNA-OptiMEM mixture as 3:1 ratio (FuGENE 6 Reagent (µl): DNA (µg)). The transfection reagent with DNA mixture was vortexed and incubated for 15 minutes at room temperature. Media of the cells was replaced with OptiMEM. After incubation, the mixture was given to cells drop by drop. 8 hours later, the cells were washed with 1X PBS and fresh media was added.

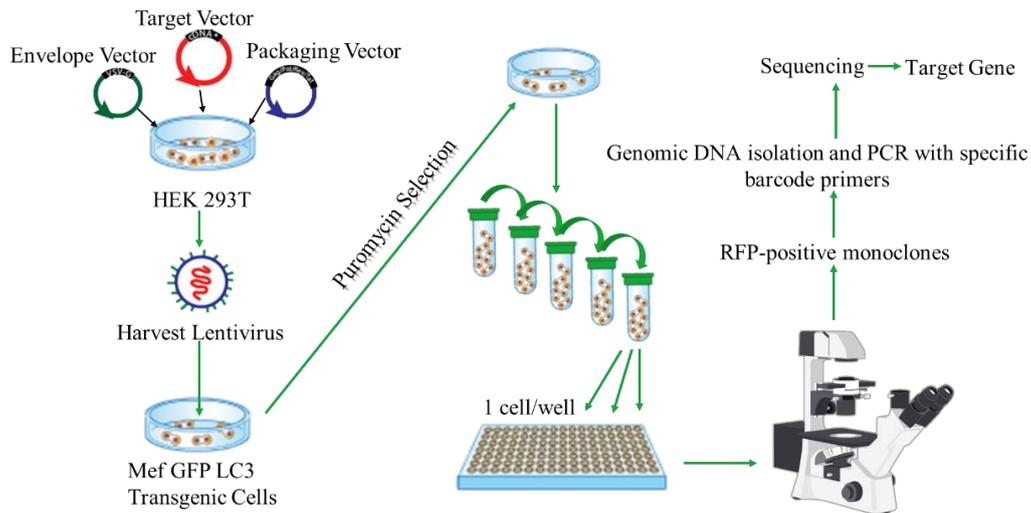
### **4.9. Flow Cytometry**

For transfection efficiency check, flow cytometry protocol was applied. The cells were washed with 1X PBS and detached with trypsin. Detached cells were resuspended in 1 ml PBS including 10% FBS and centrifuged at 300 g for 5 minutes. Then pellets were resuspended in 200 µl PBS including 10% FBS and transferred into FACS tubes.

Cells were analysed with BD FACSCanto II flow cytometry system with green laser. Fluorescence measurements were blank corrected with the fluorescence signals of non-transfected cells (20000 cells/ flow) and normalized with the same number of cells.



**Figure 4.2.** Representation of target gene including barcode sequence and shRNA region



**Figure 4.3.** Lentivirus transduction and single cell monoclonal production

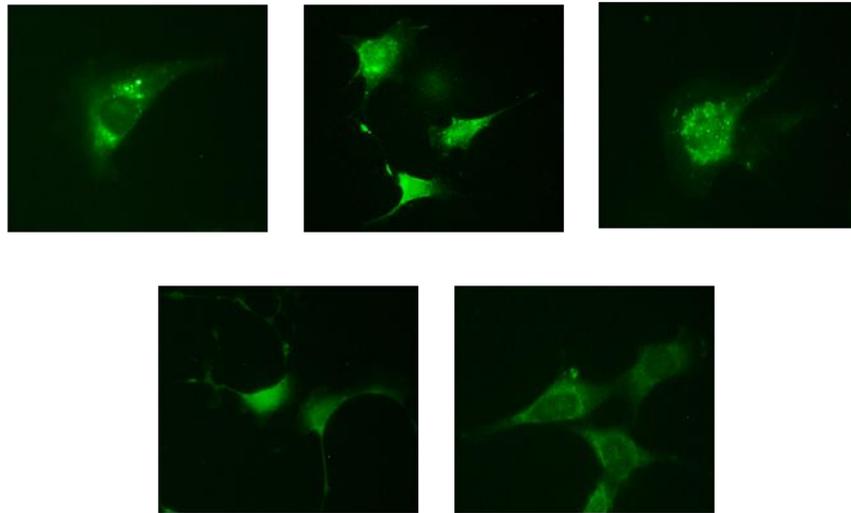
## 5. RESULTS

### 5.1. Autophagy is inhibited by PMA treatment in MEF GFP-LC3 cells

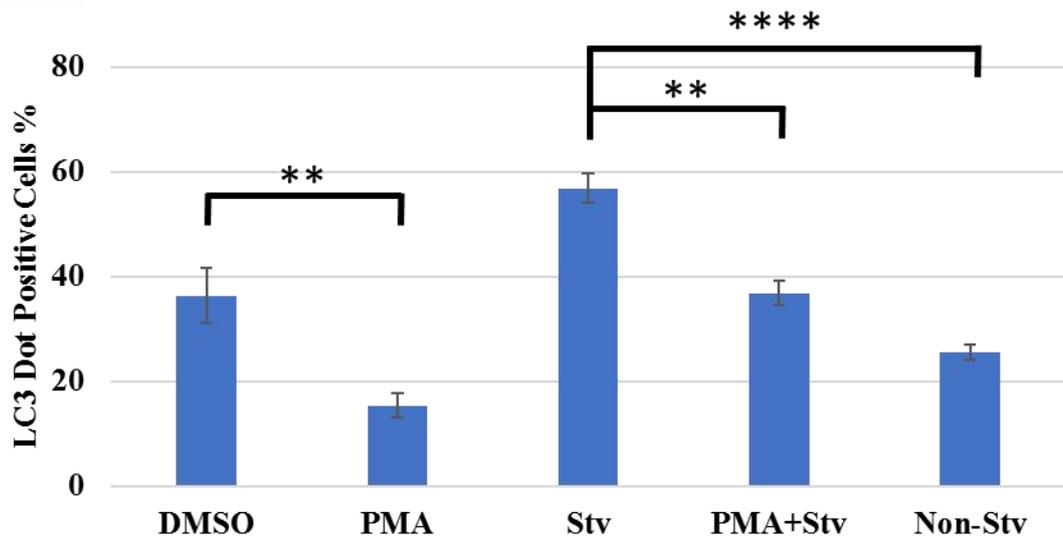
PMA is a synthetic drug that has structural and functional similarities with diacyl glycerol. Because of these characteristics, it is generally used as an activator of novel PKC isozymes in molecular biology. Previously, we showed that the stimulation of PKC isozymes by PMA treatment causes an alteration in autophagy regulation compared to basal condition. In order to confirm the effect of PMA in autophagy, we performed GFP-LC3 analysis.

GFP-LC3 dot counting is common technique used for visualization of autophagy activation under different conditions. Green dots in the cytosol represent the autophagosome-associated LC3-II formation. When MEF GFP-LC3 transgenic cells were treated with PMA, the number of GFP-LC3 dot formation was decreased compared to control group. Under starvation condition, autophagy is upregulated in order to maintain cellular homeostasis. The number of LC3-II dots was higher in starved cells, while lower in PMA group compared to control. The effect of PMA was confirmed by combined treatment with starvation media. PMA with starvation showed significant decrease in the number of LC3 puncta compared to starvation. Accordingly, autophagy activation is suppressed under PMA exposure in MEF GFP-LC3 cells, both in basal and starvation-induced conditions.

A.



B.



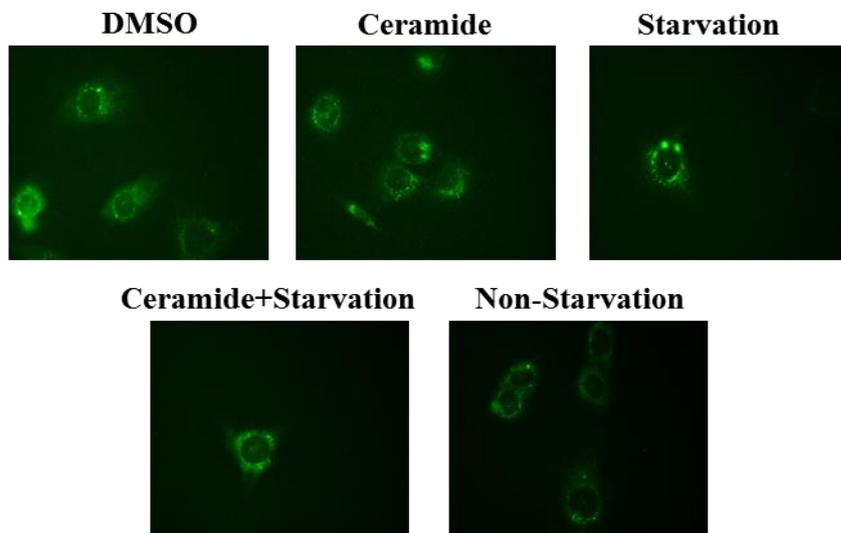
**Figure 5.1.** GFP-LC3 dot formation upon PMA and starvation treatment. (A.) Cells were treated with DMSO or PMA (100 nM) for 30 minutes and EBSS for 2 hours. Microscopic images were visualized under fluorescence microscopy with 60x objective (B.) Quantification of GFP-LC3 positive cells. Percentage of positive cells in total counted cells (minimum 100 cells) were compared in the graph. DMSO was used as a vehicle control of PMA. Data were shown as mean  $\pm$  SD of independent experiments, n=3. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

## 5.2. Autophagy is induced by PKC activation with Ceramide treatment in MEF

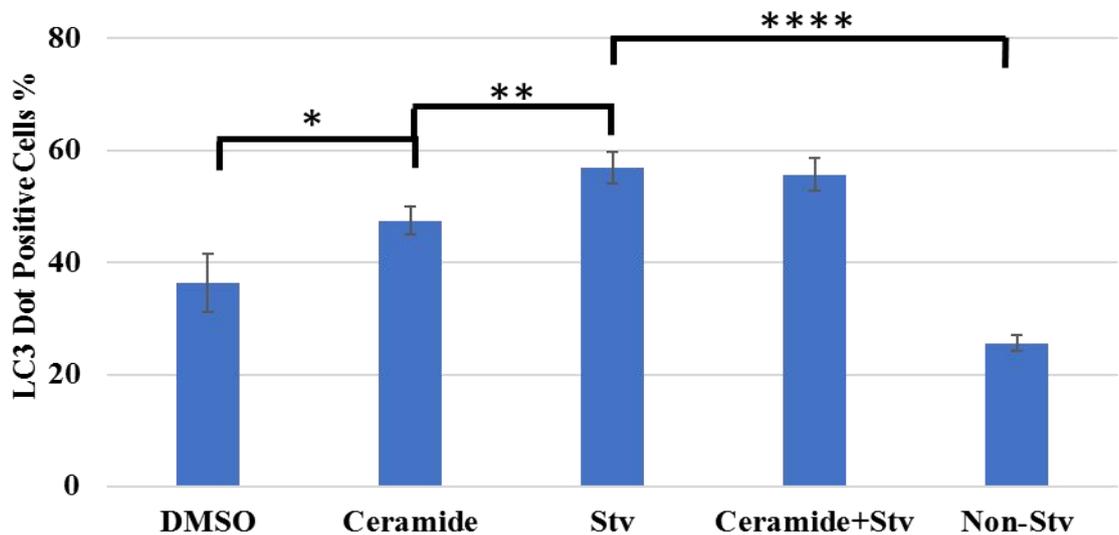
### GFP-LC3 cells

Ceramide is commonly used as a secondary messenger in order to activate atypical PKC $\zeta$ . Previously, we found that the atypical PKC $\zeta$  promotes autophagy. In order to confirm previous data we performed autophagy analysis. Upon 1  $\mu$ M Ceramide treatment for 30 minutes, the number of LC3 dots were increased in basal condition.

A.



B.



**Figure 5.2.** GFP-LC3 dot formation upon Ceramide and starvation treatment. (A.) Cells were treated with DMSO or ceramide (1  $\mu$ M) for 30 minutes and EBSS for 2 hours. Microscopic images were visualized under fluorescence microscopy with 60x objective (B.) Quantification of GFP-LC3 positive cells. Percentage of positive cells in total counted cells (minimum 100 cells) were compared in the graph. DMSO was used as a vehicle control of ceramide. Data were shown as mean  $\pm$  SD of independent experiments, n=3. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

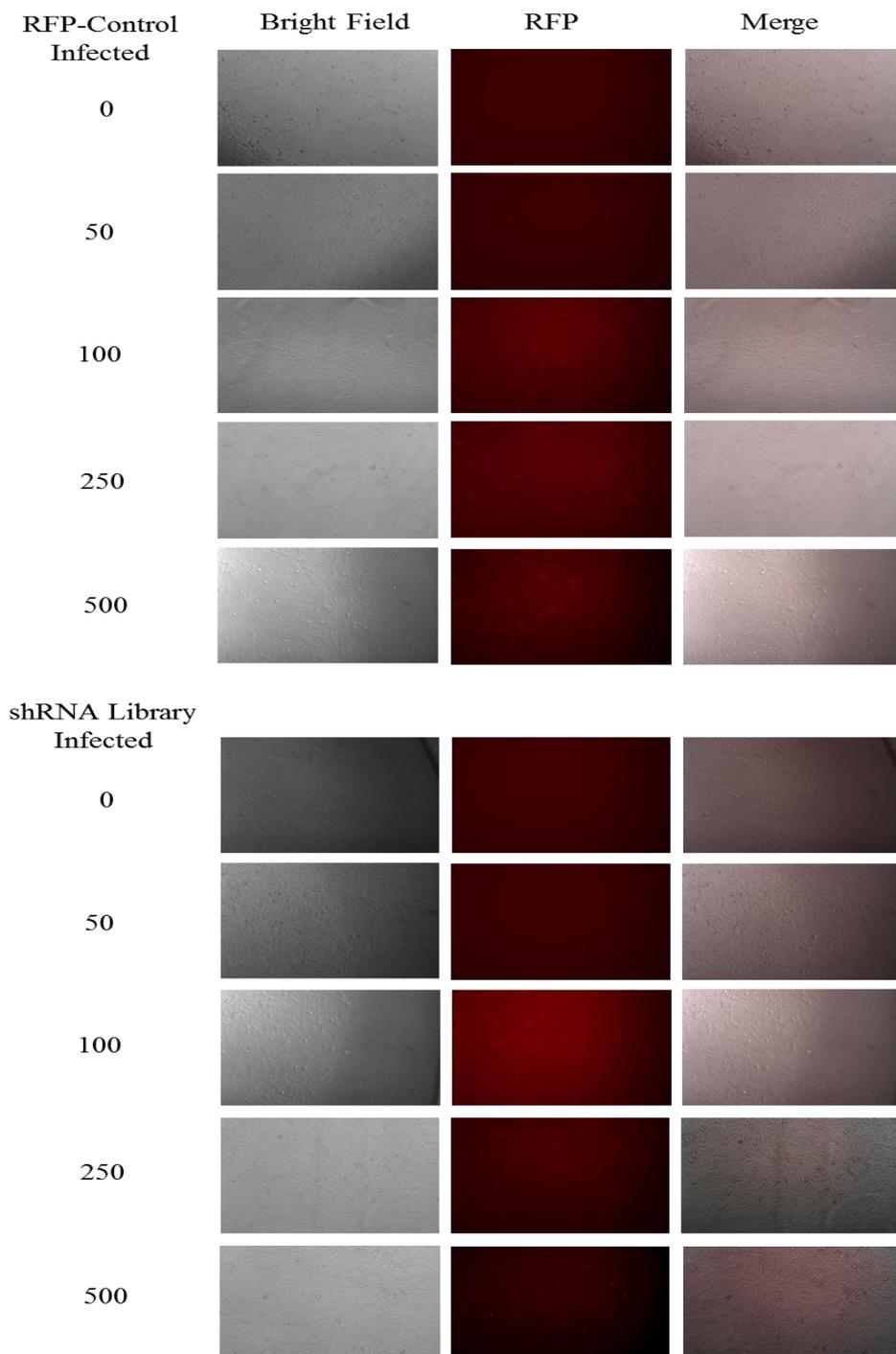
### 5.3. Lentivirus Transduction to MEF GFP-LC3 transgenic cells

In the previous part, the alterations in autophagy mechanism were demonstrated under PMA, ceramide and starvation-treated conditions. In order to investigate the relationship of PKC isozymes and their targets in the signaling pathways related to autophagy mechanism, shRNA lentiviral pool infection was performed in MEF GFP-LC3 transgenic cells. shRNA lentiviral pool is a pathway target specific lentivirus mixture containing more than 27000 vectors targeting 4625 genes. Our first aim was to produce lentiviral vectors in HEK293T cells and followed by infection of MEF GFP-LC3 transgenic cells with produced lentivirus pool. Since the final goal was to identify specific targets related with PKC isozymes and their effect on autophagy mechanism, monoclonal cells were generated.

HEK293T cells were transduced with shRNA lentiviral library with calcium phosphate transfection protocol. RFP shRNA control lentiviral vector was used as an empty control. Both helper and envelope plasmids were used in combination with shRNA lentivirus to increase efficiency of virus production in the cells. The ratio of vectors was 5 lentivirus pool; 1 envelop vector pmD2.G; 4 helper vector psPAX2. After transduction of HEK293T cells, the virus titers were collected at 36 and 48 hours. After filtration, MEF GFP-LC3 transgenic cells were transduced with 0, 50, 100, 250 and 500  $\mu$ l of shRNA lentiviral library or RFP shRNA control vector. 50  $\mu$ l lentivirus group has lower RFP signals so their propagation might be difficult under antibiotic selection. On the other hand, almost each cell in 250 and 500  $\mu$ l lentivirus groups have RFP signal so it was suspected that the

cells might have more than one lentivirus infection. We aimed to give one vector to each cell so 250 and 500  $\mu$ l lentivirus groups were eliminated. The RFP signal ratio in 100  $\mu$ l lentivirus group was moderate compared to others so we choose to continue antibiotic selection by using this titration (Figure 5.3).

Transduced cells including puromycin resistance gene were subjected to puromycin treatment for 10 days in order to produce monoclonal cells. The cells without lentiviral vectors died in 2 days while propagation of transduced cells increased.



**Figure 5.3.** Transduction efficiency with different virus titers.

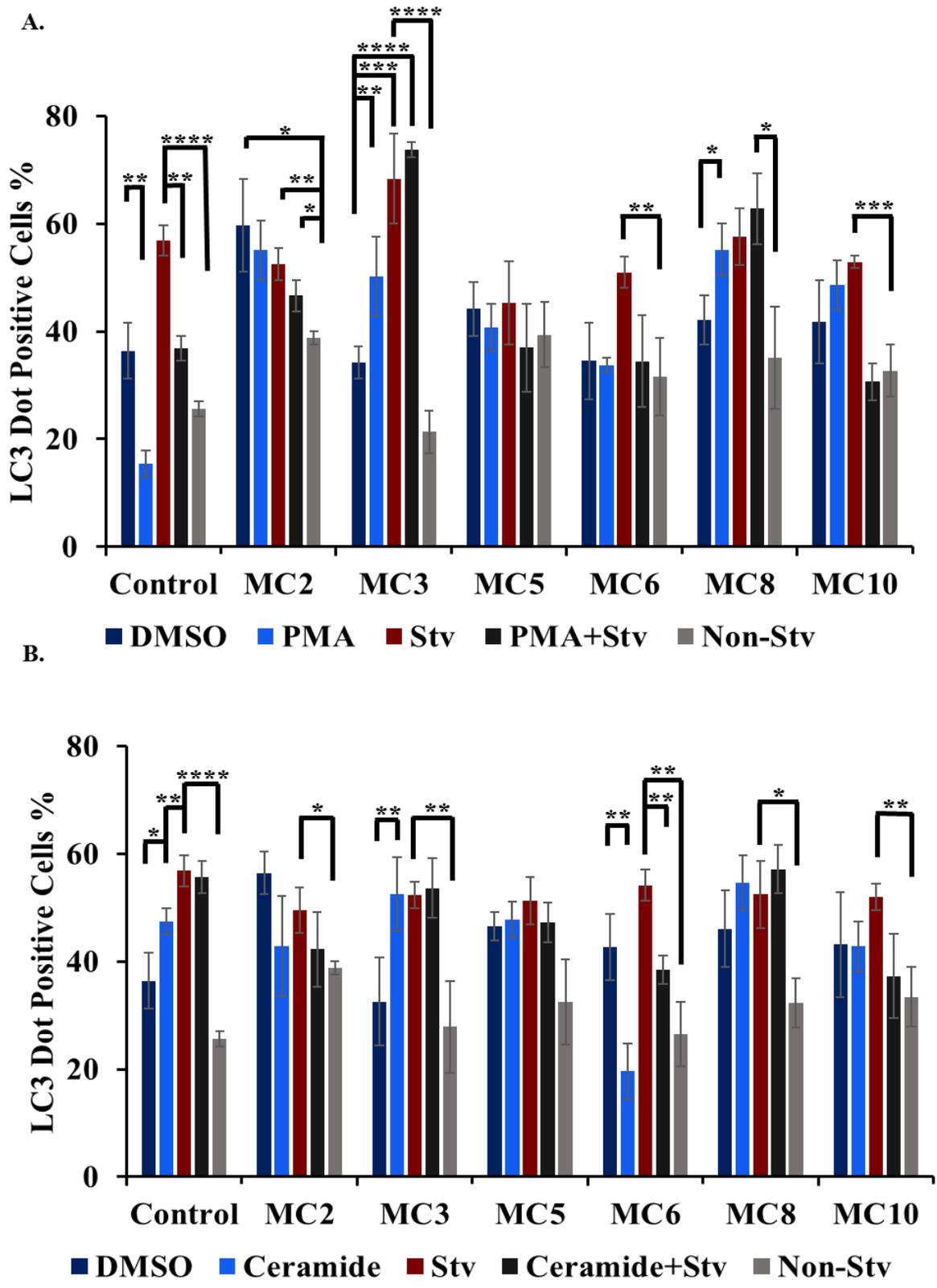
#### **5.4. The effect of PMA and Ceramide treatment on monoclonal cell lines**

After puromycin selection, transduced cells were diluted and seeded in 96 well culture plate. Each well was checked under fluorescence microscopy with green and red channels. The wells including single cell were determined and labelled to follow the further colony growth. 12 monoclonal cell lines including shRNA lentiviral library and 10 monoclonal cell lines including RFP shRNA control vector were labelled in 96 well plates, however, half of them died during propagation processes. When each single colony covered 80% of wells, they were transferred to 24 well plate, 12 well plate, 6 well plate and 10 cm culture plate respectively. After growing in 10 cm plate, they were divided into two as one for propagation in order to use for further analysis; one for barcode sequence analysis. Total 6 monoclonal cell lines having shRNA lentiviral library vector and 3 monoclonal cell lines having RFP shRNA control vector were produced.

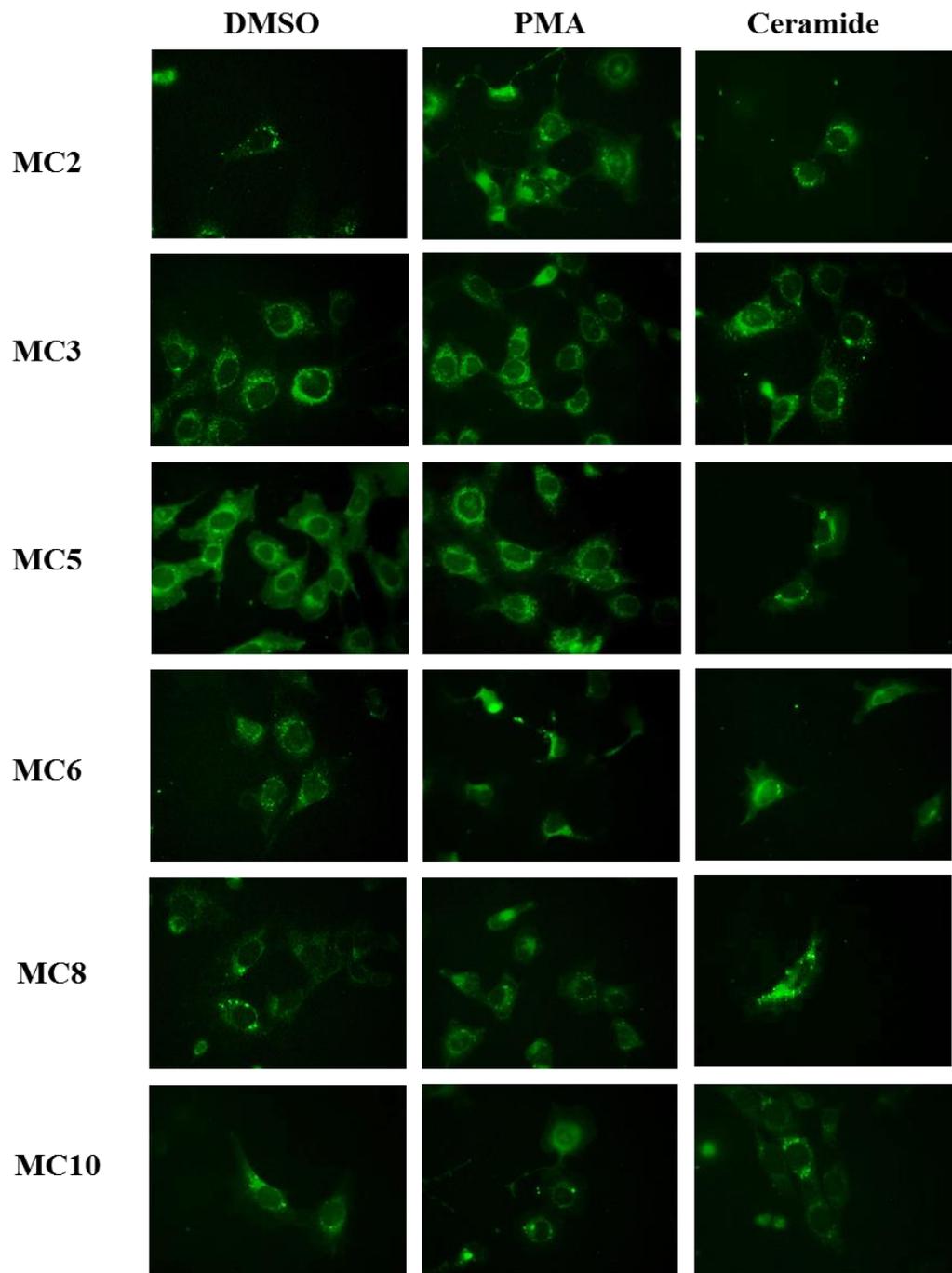
To determine the change in autophagy mechanism after PMA and ceramide treatment, GFP-LC3 puncta count was performed by using each monoclonal cell lines. It was interesting that autophagic activity was upregulated in some monoclonal cell lines upon PMA treatment. As indicated in the first section, PMA had inhibitory effect on autophagy in MEF GFP-LC3 transgenic cells. Reverse result of PMA treatment in single cell monoclonal cell lines supports our hypothesis, that downregulation of specific gene by shRNA causes change in autophagy activation. Especially, MC3 monoclonal cell line showed significant increase in autophagy under PMA treatment compared to basal condition (Figure 5.4 A). The downregulation of specific gene in MC3 clone may cause the impairment of autophagy under PKC activation.

Although ceramide treatment induced autophagy in MEF GFP-LC3 transgenic cells indicated as control in Figure 5.4, MC6 single cell monoclonal cell line suppressed autophagy upon ceramide treatment in both basal and starvation-induced condition (Figure 5.4 B). This result showed that the decrease in LC3 puncta formation may be due to inhibition of target gene in MC6 monoclonal cell line by shRNA.

As expected, starvation condition caused the significant increase in the number of LC3 dots in monoclonal cell lines. GFP-LC3 dot formation in monoclonal cell lines was shown in Figure 5.5.



**Figure 5.4.** GFP-LC3 puncta count upon (A.) PMA and (B.) ceramide treatment in monoclonal cells. DMSO was used as a vehicle control of PMA and ceramide. Control group was results of non-transfected MEF GFP-LC3 cells. Data were shown as mean  $\pm$  SD of independent experiments, n=3. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

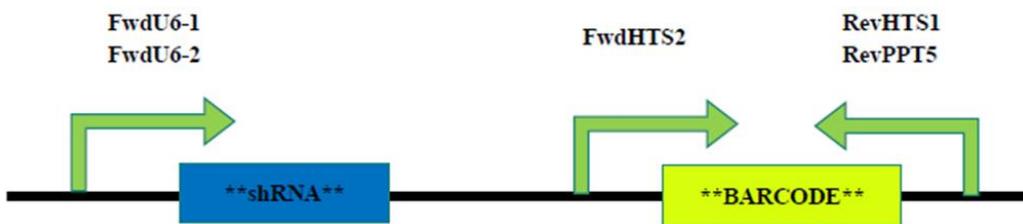


**Figure 5.5.** GFP-LC3 puncta images upon PMA and ceramide treatment of single cell monoclonal cells under fluorescence microscopy with 60x objective. DMSO was used as a vehicle control of PMA and ceramide.

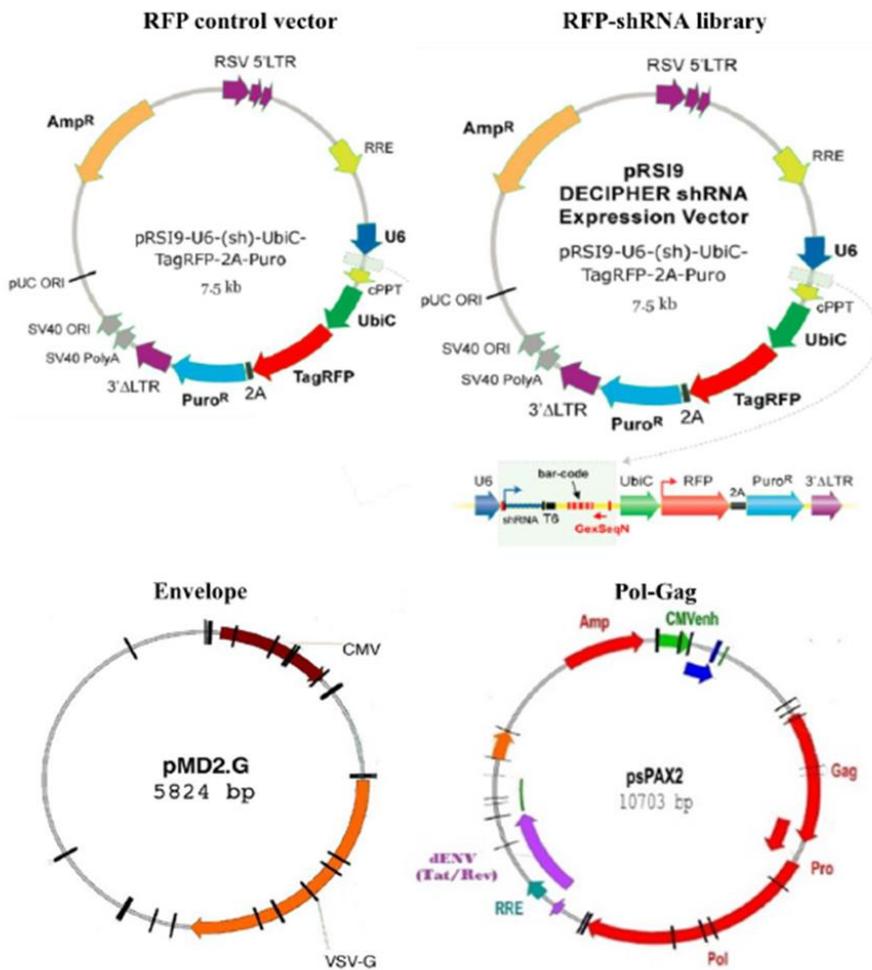
## 5.5. Monoclones's Sequence Result

Six different single clones were propagated and their barcode sequence in genomic DNA were amplified with PCR. Four different primer pairs were used in order to amplify barcode sequence or barcode with shRNA sequence. Barcode sequence amplification was represented at Figure 5.6 part A.

A.



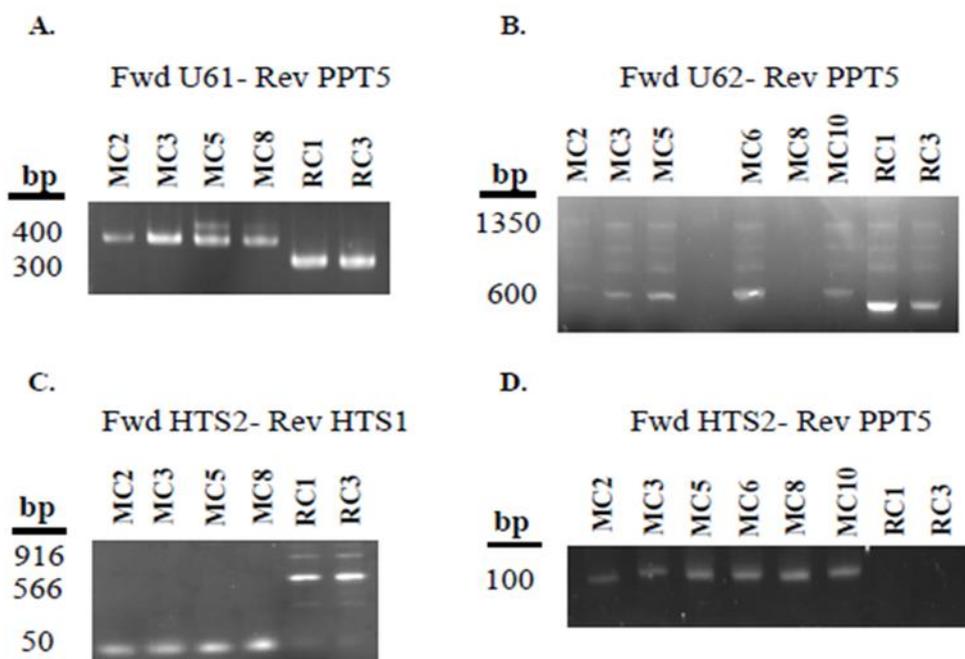
B.



**Figure 5.6.** Barcode sequence amplification (A.) Representation of primer, barcode and shRNA sites of vector. (B.) Maps of lentivirus plasmids.

PCR products were visualized on agarose gel in order to check the fragment of interest. Forward U61-Reverse PPT5 primer pairs and Forward U62-Reverse PPT5 primer pair amplified both shRNA and barcode sequence together. However, it was observed that this primer pairs also amplified non-specific sequences in genomic DNA of monoclonal cells including RFP shRNA control vector (RC) (Figure 5.7 A-B). The primer pairs were not appropriate for barcode amplification because they were not site specific for shRNA and barcode regions. Forward HTS2-Reverse HTS1 and Forward HTS2-Reverse PPT5 primer pairs were used for only barcode sequence amplification. However, as previous primer pairs, non-specific sequences in genomic DNA of monoclonal cells including RFP shRNA control vector were amplified with Forward HTS2-Reverse HTS1 primer pair (Figure 5.7 C). Meaning that, the primer pair was not specific for barcode sequence amplification. Forward HTS2-Reverse PPT5 primer pair amplified only barcode sequence in monoclonal cells having shRNA and they did not amplify any region in genomic DNA of monoclonal cells including RFP shRNA control vector (Figure 5.7 D).

Agarose gel images showed that only PCR reaction with Forward HTS2 and Reverse PPT5 primer pair gave the correct fragments in monoclonal cells including lentivirus library, but not monoclonal cells including RFP shRNA control vector. PCR products were purified from agarose gel and sent to sequence analysis in order to determine corresponding gene of interest.



**Figure 5.7.** Agarose gel images of PCR amplification in monoclonal cell lines. (A.) PCR product of monoclonal cell lines amplified with Forward U61-Reverse PPT5 primer pairs. (B.) Forward U62-Reverse PPT5 primer pair. (C.) Forward HTS2-Reverse HTS1 primer pair. (D.) Forward HTS2-Reverse PPT5 primer pair. RC clones were used as negative control, which include only RFP shRNA control vector and did not contain any barcode sequence.

According to the DNA sequence data (Figure 5.8), MC3 monoclonal cell line has barcode sequence specific for TRPV6 gene and MC10 monoclonal cell line has barcode sequence specific for Rab 23 gene (Table 5.1.).

| gene# | gi        | RefSeq      | HUGO Gene Symbol | CDS/UTR | sense                 | sense + mismatches    | 18-nt barcode      | barcode ID |
|-------|-----------|-------------|------------------|---------|-----------------------|-----------------------|--------------------|------------|
| 4096  | 142276464 | NM_008143.3 | Gnb2l1           | CDS     | GCAAGATCATTGTAGATGAAT | GCAAGATCATTGTAGATGAAT | TGGTGTCAAGTGTGGTCA | 1072       |
| 4096  | 142276464 | NM_008143.3 | Gnb2l1           | CDS     | CCGAGACAAGCATAAAGTT   | CCGAGACAAGCATAAAGTT   | CAGTGTCAAGTGTGGTCA | 1074       |
| 4096  | 142276464 | NM_008143.3 | Gnb2l1           | CDS     | GATGTTGTTATCTCTCTGAT  | GATGTTGTTATCTCTCTGAT  | GTTGTTCAAGTGTGGTCA | 1075       |
| 4096  | 142276464 | NM_008143.3 | Gnb2l1           | CDS     | TCTGGCTAACTGCAAGCTAAA | TCTGGCTAACTGCAAGCTAAA | TGGTGTCAAGTGTGGTCA | 1076       |
| 4080  | 142274273 | NM_022413.3 | Trpv6            | CDS     | CCCTACTGGAACTTATTGTTA | CCCTACTGGAACTTATTGTTA | CAGTACGTCAACACGTAC | 8610       |
| 2339  | 86262150  | NM_009706.2 | Arhgap5          | CDS     | CCCACAAAGTAAGGAGATA   | CCCACAAAGTAAGGAGATA   | ACGTGTCAAGTGTGGTCA | 1078       |
| 2339  | 86262150  | NM_009706.2 | Arhgap5          | CDS     | CCCTATAACAACCTACCTGAT | CCCTATAACAACCTACCTGAT | CAGTGTCAAGTGTGGTCA | 1079       |
| 4098  | 142277153 | NM_008999.3 | Rab23            | CDS     | ACACTCAAGTAGTAACAGAT  | ACACTCAAGTAGTAACAGAT  | CAGTTGACTGGTACTGAC | 15150      |
| 1507  | 22813438  | NM_177262.3 | Fknl             | CDS     | GCACATTATAGCGAGTGT    | GCACATTATAGCGAGTGT    | CAGTGTCAAGTGTGGTCA | 1081       |
| 1507  | 22813438  | NM_177262.3 | Fknl             | CDS     | GCCAGACAGATGAACATAGAT | GCCAGACAGATGAACATAGAT | TGGTGTCAAGTGTGGTCA | 1082       |
| 1507  | 22813438  | NM_177262.3 | Fknl             | CDS     | GCAGGACAGTAAGACATAGAT | GCAGGACAGTAAGACATAGAT | ACGTGTCAAGTGTGGTCA | 1083       |
| 1507  | 22813438  | NM_177262.3 | Fknl             | CDS     | GCCATCAAAGCCTTGAAGAAA | GCCATCAAAGCCTTGAAGAAA | CAGTGTCAAGTGTGGTCA | 1084       |
| 2642  | 142261805 | NM_178654.3 | Fknl             | CDS     | CGGGAAAGTGTAGTAACTTT  | CGGGAAAGTGTAGTAACTTT  | ACGTGTCAAGTGTGGTCA | 1085       |

**Figure 5.8.** Barcode Sites of monoclonal cell lines was highlighted at Collecta shRNA Library document

**Table 5.1.** Sequence Result

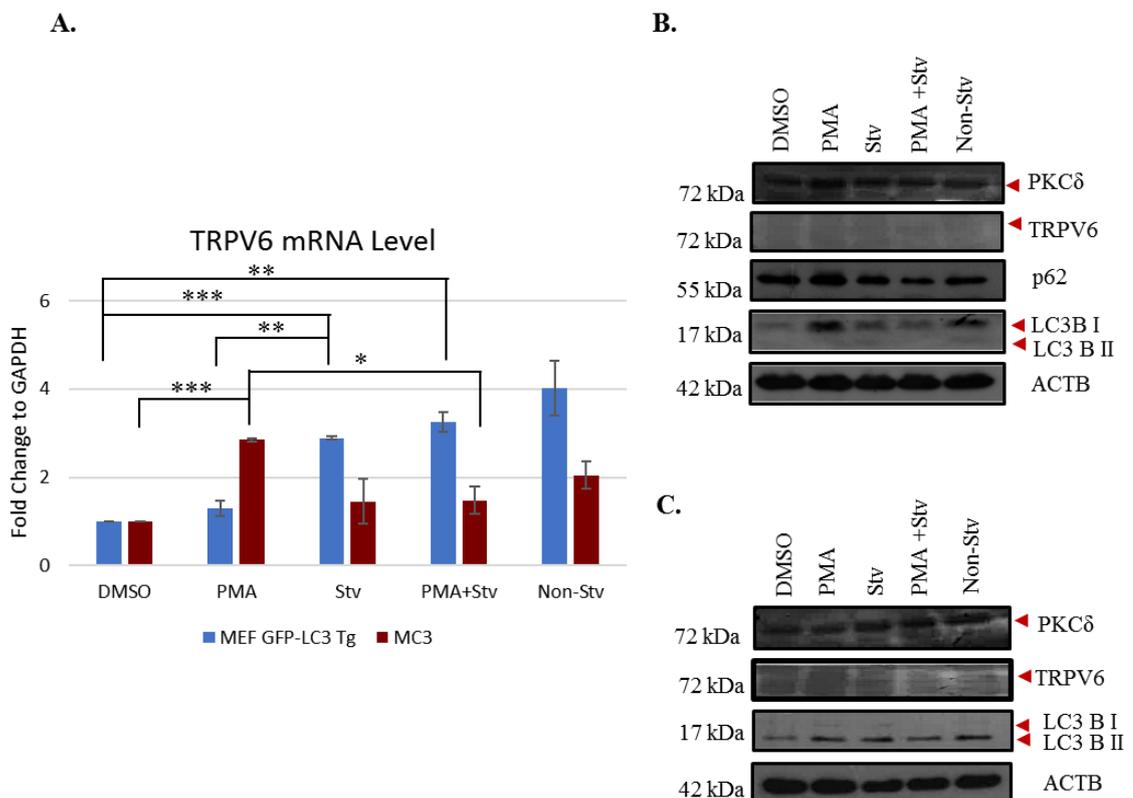
| Monoclonal cell line | NM code     | Hugo gene symbol | 18-nt- Barcode     |
|----------------------|-------------|------------------|--------------------|
| MC 10                | NM_008999.3 | Rab23            | CAGTTGACTGGTACTGAC |
| MC 3                 | NM_022413.3 | Trpv6            | CAGTACGTCAACACGTAC |

## 5.6 TRPV6 gene expression and protein level were examined in MC3 monoclonal cell line

In order to confirm gene silencing, mRNA expression of TRPV6 under PMA and starvation treatments in MEF GFP-LC3 transgenic cells and MC3 monoclonal cell line was examined by RT-q-PCR analysis. Under basal condition mRNA level of TRPV6 in MC3 is significantly lower than MEF GFP-LC3 cells (Figure 5.9 A).

p62 is a receptor for ubiquitinated proteins and directed them to the autophagic degradation. p62 is degraded by autophagy so the decrease in p62 indicates autophagic activity. Increased level of autophagosome-associated LC3 I and increased level of p62 in MEF GFP-LC3 transgenic cells confirmed inhibitory effect of PMA on autophagy (Figure 5.9 B)

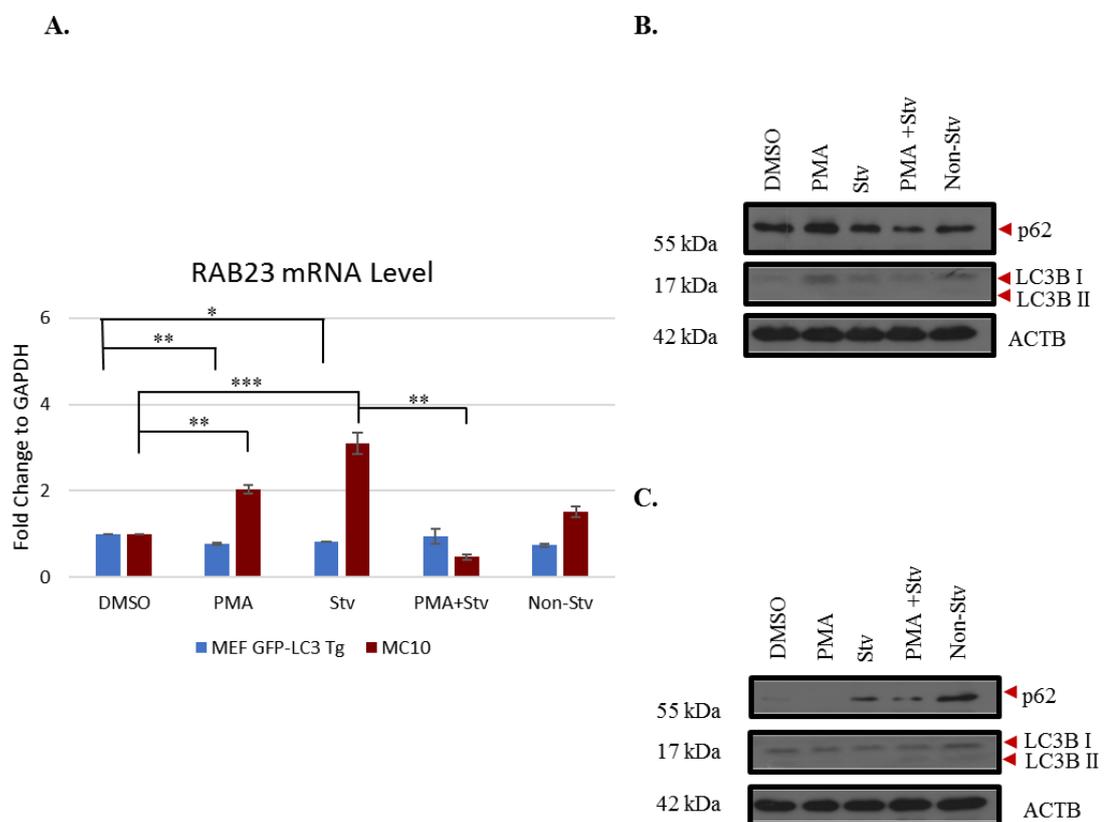
While mRNA level of target protein was significantly lower, there was not prominent change in the target protein level in MEF GFP-LC3 transgenic and MC3 monoclonal cell lines (Figure 5.9 B-C). Under PMA treatment PKC $\delta$  expression increased in both basal and starvation-induced condition in MEF GFP-LC3 transgenic and MC3 monoclonal cell lines (Figure 5.9 B-C). These results indicated that downregulation of TRPV6 gene could be a target of PKC $\delta$  that regulate autophagy.



**Figure 5.9.** Confirmation of gene silencing of TRPV6 and its effect on autophagy in MEF GFP-LC3 transgenic cells (A and B) and MC3 monoclonal (A and C) under PMA and starvation-induced conditions. (B and C) Immunoblots showing protein expression of PKC $\delta$ , TRPV6, p62 and LC3. Cells were treated with DMSO or PMA (100nM) for 30 minutes and EBSS for 2 hours. ACTB was used as a loading control. Data were shown as mean  $\pm$  SD of independent experiments, n=3, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

### 5.7 RAB23 gene expression and protein level were examined in single cell clone

RAB23 gene was the silenced gene according to barcode sequence analysis in MC10. However, mRNA expression of RAB23 in MC10 was not lower than MEF GFP-LC3 cells (Figure 5.10 A). Maybe the sequence result or primer pairs in RT-q-PCR were not completely specific for RAB23. However, it is interesting that under PMA treatment, p62 level was decreased compared to non-starvation and starvation conditions in MC10 cells (Figure 5.10 C.).



**Figure 5.10.** Confirmation of gene silencing of RAB23 and its effect on autophagy in MEF GFP-LC3 transgenic cells (A and B) and MC10 monoclonal (A and C) under PMA and starvation-induced conditions. (B and C) Immunoblots showing protein expression of p62 and LC3. Cells were treated with DMSO or PMA (100nM) for 30 minutes and EBSS for 2 hours. ACTB was used as a loading control. Data were shown as mean  $\pm$  SD of independent experiments, n=3, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

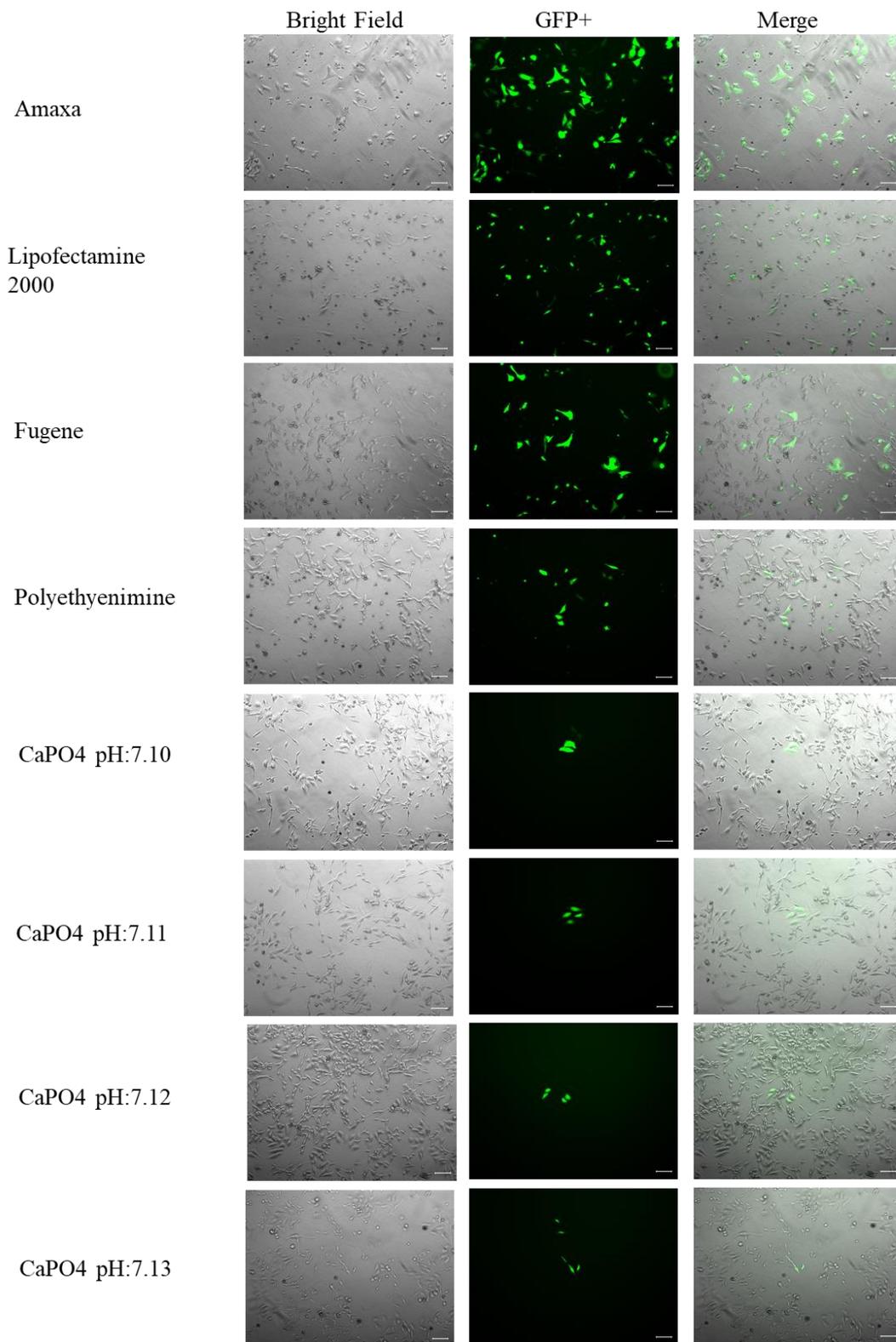
### **5.8 Confirmation of the effect of PMA and expression of TRPV6 in HeLa GFP-LC3 Stable Cells**

In the literature, the alteration of TRPV6 expression was detected in development and progression of prostate cancer, lung cancer, ovarian cancer and breast cancer (Haustrate, Hantute-Ghesquier, Prevarskaya, & Lehen'kyi, 2019; Kaemmerer, Turner, Peters, Roberts-Thomson, & Monteith, 2018; Lutes et al., 2018; Song et al., 2018). In the light of that, the interaction of TRPV6 with PKC $\delta$  and its effect on autophagy mechanism was aimed to investigate in HeLa GFP-LC3 stable cell line. However, HeLa GFP-LC3 cells could not be transfected by using conventional calcium phosphate (CaPO<sub>4</sub>) protocol. Therefore, first, transfection efficiency of several methods was analyzed at 48 h post transfection.

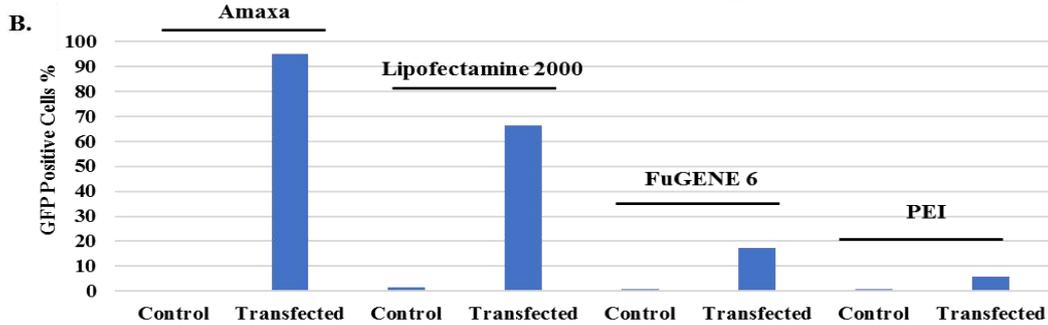
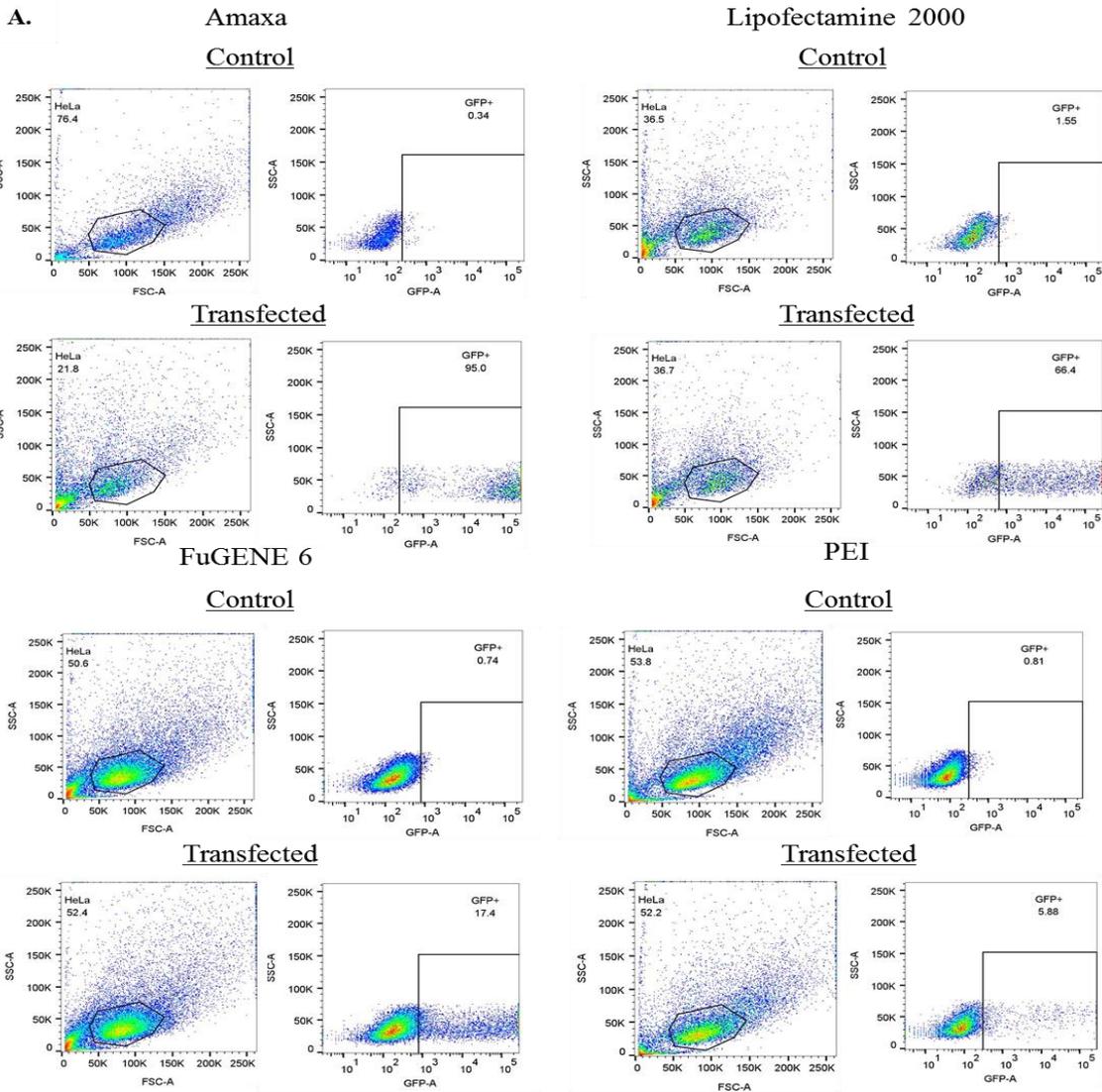
Previously, calcium phosphate (CaPO<sub>4</sub>) transfection protocol was used for HeLa cell line transfection yet, pH of CaPO<sub>4</sub> could not be stabilized. Transfection solutions with different pH groups were tested range from 7.00 to 7.20 and GFP signals were only detected at 7.10 and 7.13 pH value but with very low efficiency.

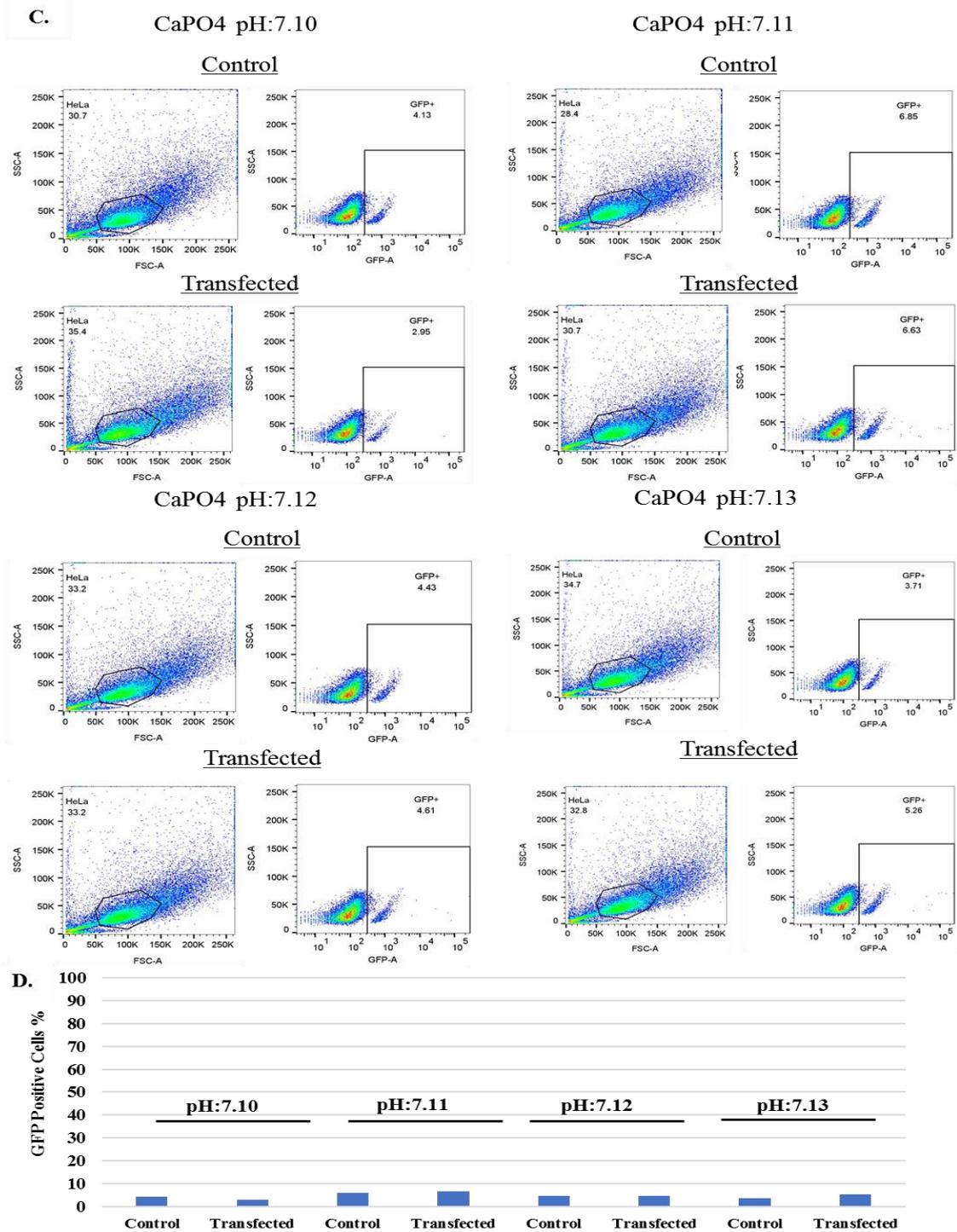
In the literature, lipid or polymer-based transfection reagents such as Lipofectamine and FuGENE were mostly used for HeLa cell line transfection. Instead of CaPO<sub>4</sub> transfection, we decided to check the transfection efficiency of electroporation, Lipofectamine 2000, FuGENE 6 or PEI reagents. The flow cytometry result showed that CaPO<sub>4</sub> transfection had the lowest efficiency with 5%, while Amaxa Nucleofector transfection had the most efficient transfection with 96%, Lipofectamine 2000 had relatively good efficiency as 70%, FuGENE 6 only transfected 17 % of cells and PEI transfected 5% of cells (Figure 5.12). The cytotoxicity of transfection was also important because higher transfection

efficiency resulted in the decrease of viability. Although the initial number of cells were equal for each transfection group, most of cells died after Amaxa Nucleofector transfection compared to other groups. The rate of dead cells was the least in PEI and CaPO<sub>4</sub> transfection groups however changes in cell shape were observed in CaPO<sub>4</sub> transfection groups. The cell death was moderate in Lipofectamine 2000 transfection, so we decided to continue further transfection experiments by using Lipofectamine 2000 reagent.



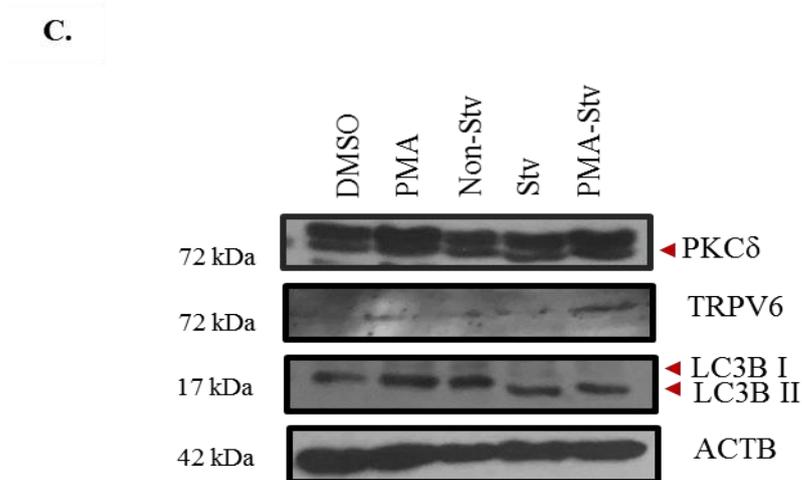
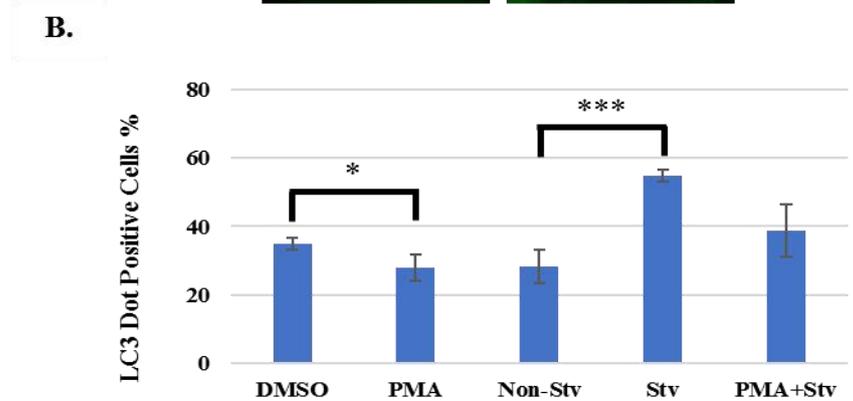
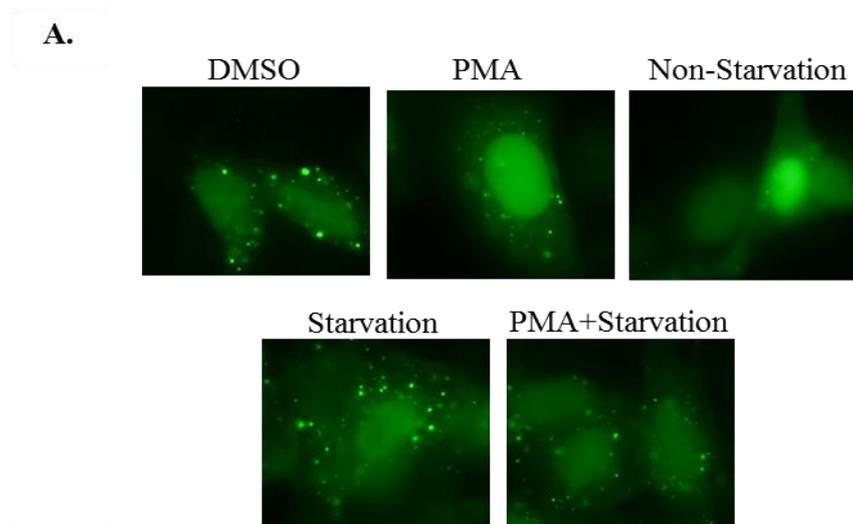
**Figure 5.11.** Fluorescent microscope images of different transfection methods in HeLa cells at 48 h post transfection.





**Figure 5.12.** Evaluation of the transfection efficiencies of Amaxa Nucleofector, Lipofectamine 2000, FuGENE 6, PEI and CaPO4 protocols in HeLa cells. (A and C) Representative flow cytometry dot plots with percentage of GFP expressing cells. (B and D) Bar chart indicating the percentage of GFP-positive cells in HeLa cells transfected by Amaxa Nucleofector, Lipofectamine 2000, FuGENE 6, PEI and CaPO4. pcDNA3 empty vector was used as control.

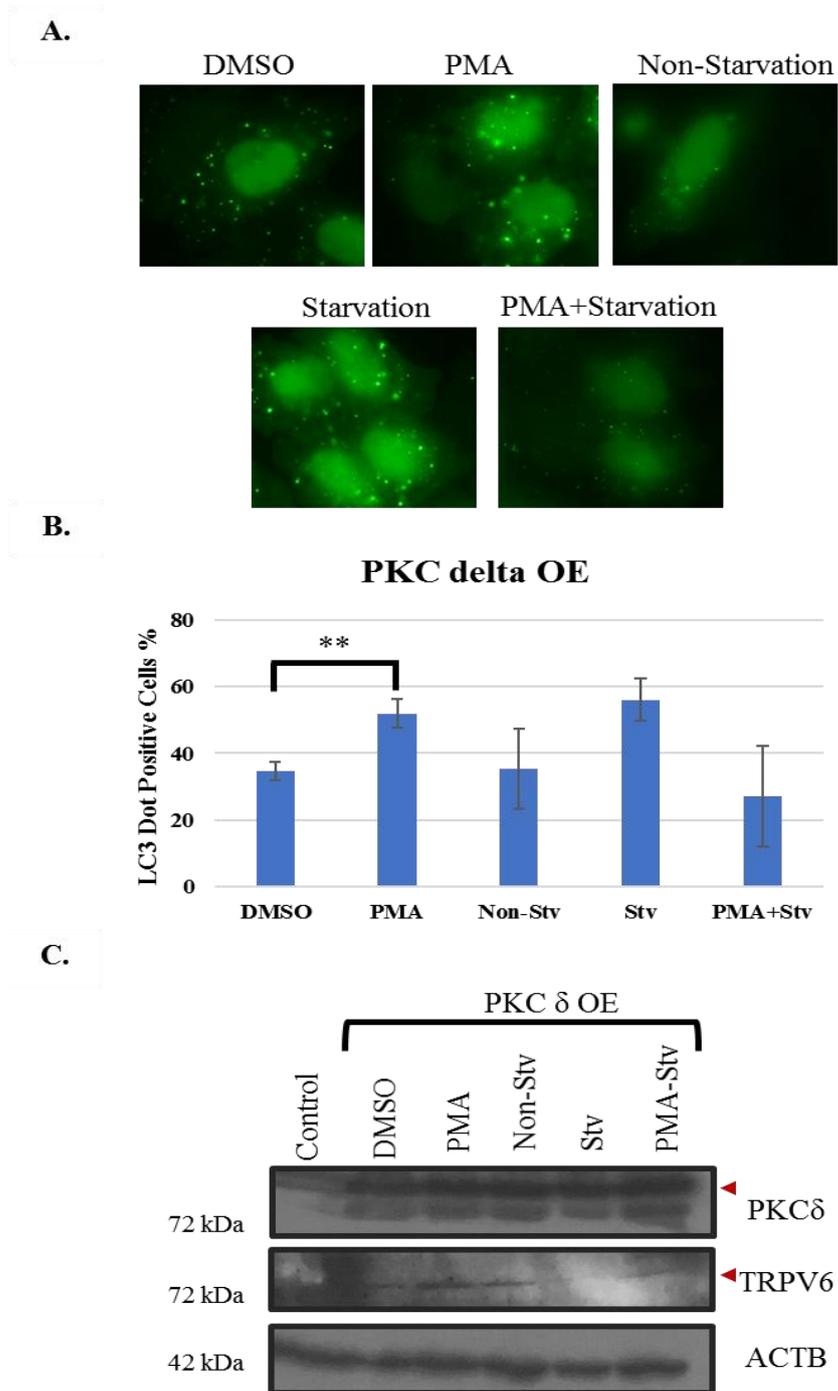
Autophagy activity under PMA and starvation conditions was verified with GFP-LC3 dot counting and LC3 II formation (Figure 5.13 A-B) As shown previously, PMA induced PKC $\delta$  expression compared to vehicle control DMSO. TRPV6 expression was shown in HeLa GFP-LC3 cells and there was an increase in the TRPV6 expression upon PMA with starvation treatment (Figure 5.13 C).



**Figure 5.13.** The effect of PMA on autophagy in HeLa GFP-LC3 stable cells. (A.) GFP-LC3 dot formation upon PMA and starvation treatment was observed under fluorescence microscopy with 60x objective (B.) Number of GFP-LC3 positive cells in each treatment groups were determined. (C.) Immunoblots showing protein expression of PKC $\delta$ , TRPV6 and LC3. Cells were treated with DMSO or PMA (100nM) for 30 minutes and EBSS for 2 hours. ACTB was used as a loading control. Data were shown as mean  $\pm$  SD of independent experiments, n=3, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

### **5.9. The effect of overexpression of PKC $\delta$ on TRPV6 expression and Autophagy**

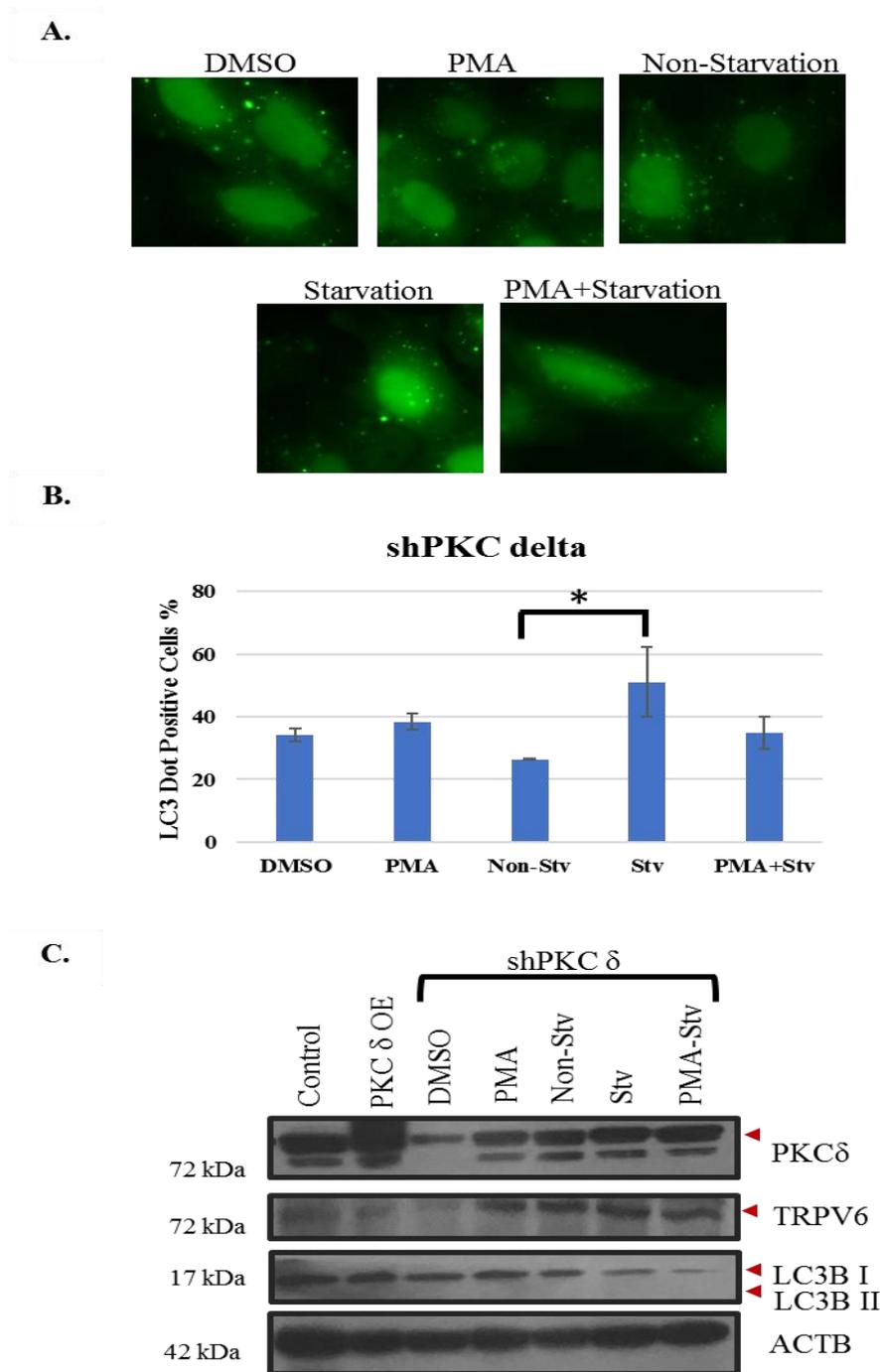
In order to check the relationship of PKC $\delta$ , TRPV6 and autophagy, the effect of PMA and starvation on TRPV6 expression as well as autophagy was investigated by PKC $\delta$  overexpression. PKC $\delta$  overexpression and PMA treatment caused the increase in the number of LC3 dots (Figure 5.14 A-B). Under PMA treatment, TRPV6 expression was increased compared to control, meanwhile the increase in the degradation of TRPV6 under starvation was reversed with PMA with starvation treatment (Figure 5.14 C).



**Figure 5.14.** The effect of PKC $\delta$  overexpression on autophagy in HeLa GFP-LC3 stable cells. (A.) GFP-LC3 dot formation upon PMA and starvation treatment was observed under fluorescence microscopy with 60x objective (B.) Number of GFP-LC3 positive cells in each treatment groups were determined. (C.) Immunoblots showing protein expression of PKC $\delta$ , TRPV6 and LC3. Cells were treated with DMSO or PMA (100nM) for 30 minutes and EBSS for 2 hours. ACTB was used as a loading control. Data were shown as mean  $\pm$  SD of independent experiments, n=3, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

### **5.10 The effect of PKC $\delta$ inhibition on TRPV6 and autophagy**

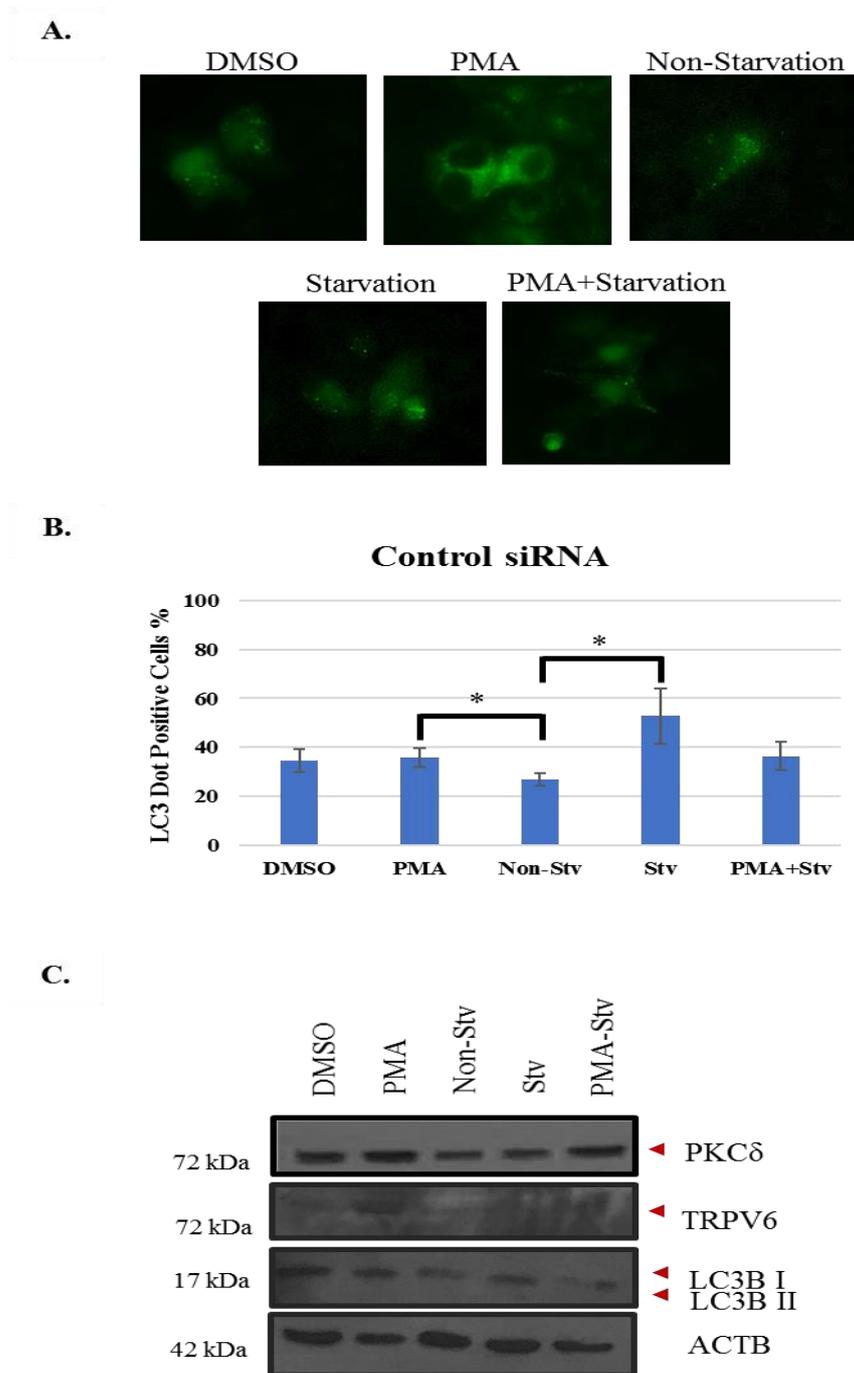
In previous part, it was shown that, the upregulation of PKC $\delta$  caused the increase in TRPV6 expression. We wondered whether knockdown of PKC $\delta$  by shRNA leads to any alteration in TRPV6 expression. Therefore, PKC delta expression was downregulated by shPKC $\delta$  transfection and both autophagy activation and TRPV6 expression were analyzed. While overexpression of PKC $\delta$  caused the increase in autophagy activation, shPKC $\delta$  knockdown led slight decrease in autophagy (Figure 5.15 A-B). It's remarkable that, the level of TRPV6 was increased with under PKC $\delta$  downregulation compared to control and PKC $\delta$  overexpression.



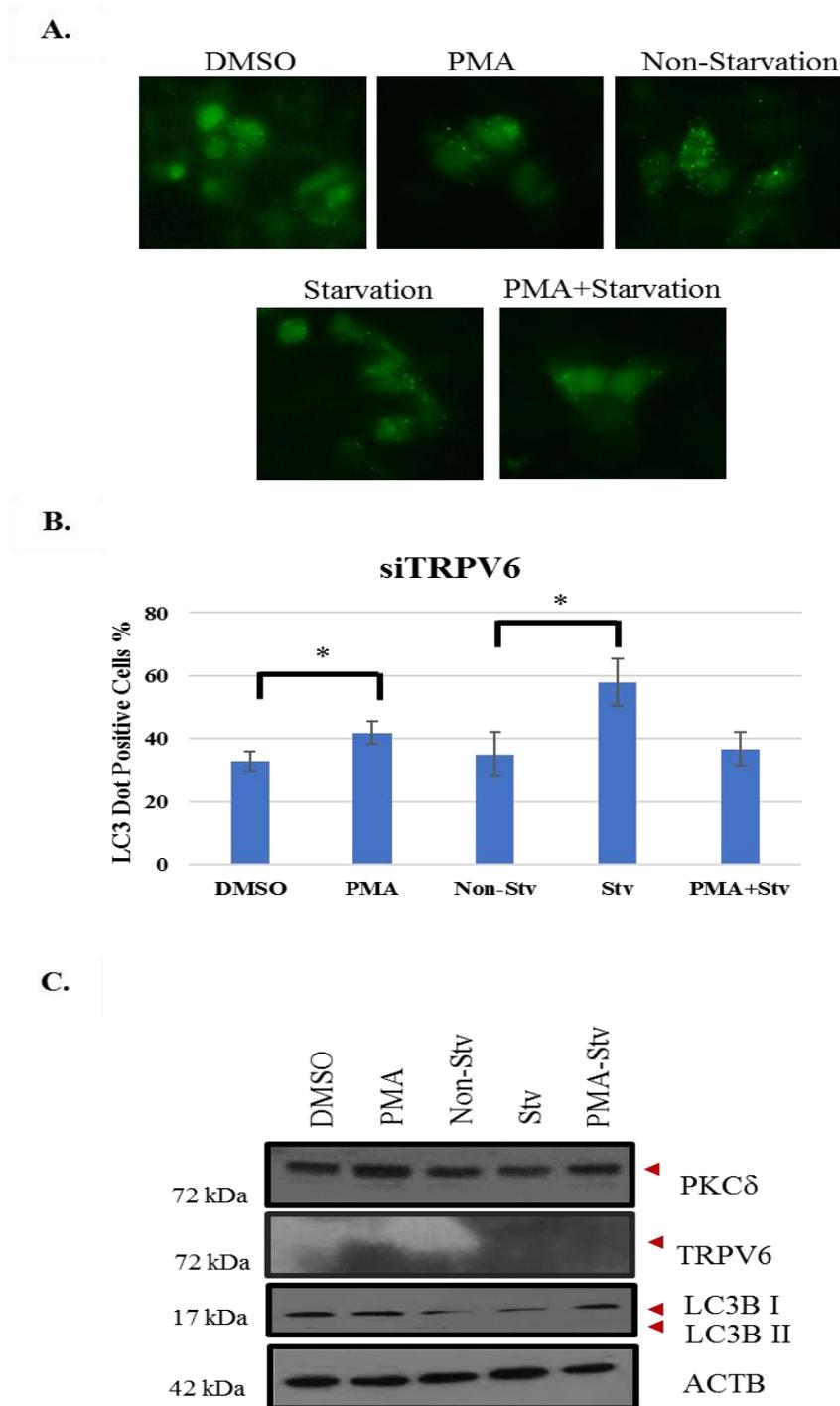
**Figure 5.15.** The effect of shPKC $\delta$  on autophagy and TRPV6 expression in HeLa GFP-LC3 stable cells. (A.) GFP-LC3 dot formation upon PMA and starvation treatment was observed under fluorescence microscopy with 60x objective (B.) Number of GFP-LC3 positive cells in each treatment groups were determined. (C.) Immunoblots showing protein expression of PKC $\delta$ , TRPV6 and LC3. Cells were treated with DMSO or PMA (100nM) for 30 minutes and EBSS for 2 hours. ACTB was used as a loading control. Data were shown as mean  $\pm$  SD of independent experiments, n=3, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.

### **5. 11 Change in PKC $\delta$ and autophagy regulation under TRPV6 knockdown**

So far, we observed an interaction between TRPV6 and PKC $\delta$  and its effect on autophagy mechanism. To understand this interaction, PKC $\delta$  expression and autophagy regulation were analyzed under the suppression of TRPV6. It was shown that PKC $\delta$  expression could not change upon TRPV6 downregulation (Figure 5. 17 C). As in MC3 monoclonal, PMA induced autophagy was observed in TRPV6 suppressed HeLa GFP-LC3 stable cells (Figure 5. 17 A-B). So that downregulation of TRPV6 caused the increase in autophagy activation.



**Figure 5.16.** The effect of control siRNA on autophagy in HeLa GFP-LC3 stable cells. (A.) GFP-LC3 dot formation upon PMA and starvation treatment was observed under fluorescence microscopy with 60x objective (B.) Number of GFP-LC3 positive cells in each treatment groups were determined. (C.) Immunoblots showing protein expression of PKC $\delta$ , TRPV6 and LC3. Cells were treated with DMSO or PMA (100nM) for 30 minutes and EBSS for 2 hours. ACTB was used as a loading control. Data were shown as mean  $\pm$  SD of independent experiments, n=3, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.



**Figure 5.17.** The effect of siTRPV6 on autophagy in HeLa GFP-LC3 stable cells. (A.) GFP-LC3 dot formation upon PMA and starvation treatment was observed under fluorescence microscopy with 60x objective (B.) Number of GFP-LC3 positive cells in each treatment groups were determined. (C.) Immunoblots showing protein expression of PKC $\delta$ , TRPV6 and LC3. Cells were treated with DMSO or PMA (100nM) for 30 minutes and EBSS for 2 hours. ACTB was used as a loading control. Data were shown as mean  $\pm$  SD of independent experiments, n=3, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001

## 5.12 Possible Phosphorylation Sites on TRPV6 and RAB23 by PKC $\delta$

In the literature it was reported that TRPV6 has PKC isozymes mediated phosphorylation sites [86]. In the light of these data, possible phosphorylation sites were predicted by using nine different bioinformatics tools. Accordingly, hit regions that may play a role in the interaction between TRPV6 and PKC $\delta$  were shown in Table 5.1. Also, the possible phosphorylation sites on RAB23 by PKC $\delta$  were indicated in Table 5.2. Consequently, it was observed that the middle and carboxyl end domains of targets protein may be important for this interaction.

**Table 5.2** Predicted Phosphorylation sites of TRPV6 by PKC $\delta$

| Residue | GPS 0.5  | Phosphomotif | DISPHOS  | KinasePhos 2.0 | NetPhos 3.1 | NetPhorest | PKIS     | PhosphoPick | Musid    |
|---------|----------|--------------|----------|----------------|-------------|------------|----------|-------------|----------|
| S-21    |          |              |          |                |             | <i>x</i>   | <i>x</i> |             |          |
| S-22    | <i>x</i> | <i>x</i>     | <i>x</i> |                |             |            | <i>x</i> | <i>x</i>    | <i>x</i> |
| S-44    |          |              |          |                | <i>x</i>    | <i>x</i>   | <i>x</i> |             |          |
| S-57    | <i>x</i> |              |          | <i>x</i>       |             |            |          |             |          |
| S-68    | <i>x</i> | <i>x</i>     | <i>x</i> | <i>x</i>       |             |            | <i>x</i> | <i>x</i>    | <i>x</i> |
| S-72    |          |              |          |                |             |            | <i>x</i> |             |          |
| S-87    |          |              |          |                |             |            | <i>x</i> |             |          |
| T-121   |          |              |          | <i>x</i>       |             | <i>x</i>   | <i>x</i> |             |          |
| T-151   |          |              |          |                |             |            | <i>x</i> |             |          |
| S-152   |          |              |          | <i>x</i>       |             | <i>x</i>   | <i>x</i> |             |          |
| T-159   |          |              |          |                |             |            | <i>x</i> |             |          |
| S-182   | <i>x</i> | <i>x</i>     | <i>x</i> |                |             |            | <i>x</i> | <i>x</i>    | <i>x</i> |
| S-184   | <i>x</i> | <i>x</i>     |          |                | <i>x</i>    | <i>x</i>   | <i>x</i> | <i>x</i>    |          |
| T-188   |          |              |          |                | <i>x</i>    |            | <i>x</i> | <i>x</i>    |          |
| S-190   |          |              |          |                | <i>x</i>    | <i>x</i>   | <i>x</i> | <i>x</i>    |          |
| S-195   | <i>x</i> | <i>x</i>     |          |                |             |            |          | <i>x</i>    |          |
| S-208   | <i>x</i> |              |          | <i>x</i>       |             | <i>x</i>   | <i>x</i> | <i>x</i>    |          |

|       |          |          |  |          |          |          |          |          |  |
|-------|----------|----------|--|----------|----------|----------|----------|----------|--|
| S-215 |          |          |  | <i>x</i> |          | <i>x</i> | <i>x</i> |          |  |
| S-234 |          |          |  |          |          | <i>x</i> | <i>x</i> | <i>x</i> |  |
| T-238 |          |          |  | <i>x</i> |          |          | <i>x</i> |          |  |
| T-250 | <i>x</i> |          |  |          |          |          | <i>x</i> | <i>x</i> |  |
| S-261 | <i>x</i> |          |  |          | <i>x</i> | <i>x</i> | <i>x</i> |          |  |
| T-282 |          |          |  |          |          |          |          |          |  |
| T-293 |          |          |  |          |          |          |          |          |  |
| T-306 | <i>x</i> | <i>x</i> |  | <i>x</i> |          |          |          | <i>x</i> |  |
| T-309 |          |          |  | <i>x</i> | <i>x</i> |          |          |          |  |
| T-314 |          |          |  |          |          | <i>x</i> | <i>x</i> |          |  |
| S-315 |          |          |  |          |          |          | <i>x</i> |          |  |
| T-316 |          |          |  |          |          |          |          |          |  |
| T-321 |          |          |  |          |          |          |          |          |  |
| S-325 |          |          |  |          |          |          | <i>x</i> |          |  |
| S-326 | <i>x</i> |          |  |          |          |          |          |          |  |
| S-331 |          |          |  |          |          |          |          |          |  |
| T-337 |          |          |  |          |          | <i>x</i> | <i>x</i> |          |  |
| T-338 |          | <i>x</i> |  |          | <i>x</i> | <i>x</i> | <i>x</i> | <i>x</i> |  |
| T-339 | <i>x</i> |          |  |          | <i>x</i> |          |          | <i>x</i> |  |
| T-351 |          |          |  | <i>x</i> |          |          |          | <i>x</i> |  |
| S-357 |          |          |  |          |          | <i>x</i> | <i>x</i> |          |  |
| S-358 |          | <i>x</i> |  |          | <i>x</i> |          |          |          |  |
| T-384 |          |          |  | <i>x</i> |          |          |          |          |  |
| T-396 | <i>x</i> | <i>x</i> |  |          |          | <i>x</i> | <i>x</i> | <i>x</i> |  |

|       |          |          |  |          |  |          |          |          |          |
|-------|----------|----------|--|----------|--|----------|----------|----------|----------|
| T-400 |          |          |  |          |  |          |          |          |          |
| S-401 | <i>x</i> | <i>x</i> |  | <i>x</i> |  |          |          |          | <i>x</i> |
| T-406 |          | <i>x</i> |  |          |  |          |          |          |          |
| T-419 | <i>x</i> | <i>x</i> |  |          |  |          |          |          |          |
| S-431 |          |          |  |          |  | <i>x</i> | <i>x</i> | <i>x</i> |          |
| T-452 |          |          |  |          |  | <i>x</i> | <i>x</i> | <i>x</i> |          |
| T-459 |          |          |  | <i>x</i> |  |          |          | <i>x</i> |          |
| T-471 | <i>x</i> |          |  |          |  | <i>x</i> |          |          |          |
| T-479 |          |          |  |          |  | <i>x</i> |          | <i>x</i> |          |
| S-486 | <i>x</i> | <i>x</i> |  |          |  |          | <i>x</i> | <i>x</i> |          |
| S-488 |          |          |  | <i>x</i> |  |          |          |          |          |
| S-495 |          |          |  |          |  | <i>x</i> |          | <i>x</i> |          |
| T-519 |          |          |  | <i>x</i> |  | <i>x</i> |          |          |          |
| S-546 |          |          |  |          |  | <i>x</i> |          | <i>x</i> |          |
| T-554 |          |          |  | <i>x</i> |  |          |          |          |          |
| S-572 |          |          |  |          |  |          | <i>x</i> |          |          |
| T-573 | <i>x</i> |          |  | <i>x</i> |  | <i>x</i> |          |          |          |
| S-579 |          |          |  | <i>x</i> |  |          |          |          |          |
| S-596 | <i>x</i> |          |  | <i>x</i> |  | <i>x</i> |          |          |          |
| T-598 |          |          |  |          |  | <i>x</i> |          |          |          |
| T-607 | <i>x</i> |          |  |          |  |          |          |          |          |
| T-621 |          |          |  |          |  | <i>x</i> |          | <i>x</i> |          |
| T-640 | <i>x</i> |          |  | <i>x</i> |  |          | <i>x</i> |          |          |
| T-641 | <i>x</i> |          |  | <i>x</i> |  |          |          | <i>x</i> |          |

|       |          |          |          |          |          |          |          |          |          |
|-------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| S-656 |          |          |          |          |          |          |          |          |          |
| T-693 |          |          |          |          |          |          |          |          |          |
| S-696 | <i>x</i> | <i>x</i> |          |          | <i>x</i> |          |          |          |          |
| S-703 |          | <i>x</i> |          |          |          |          |          |          |          |
| S-714 |          |          |          | <i>x</i> |          |          |          |          |          |
| S-718 | <i>x</i> |          |          |          |          |          |          |          |          |
| S-723 |          |          | <i>x</i> |          |          | <i>x</i> | <i>x</i> |          |          |
| S-725 |          |          | <i>x</i> |          |          |          |          |          |          |
| S-727 |          |          | <i>x</i> |          | <i>x</i> | <i>x</i> | <i>x</i> | <i>x</i> |          |
| T-728 | <i>x</i> | <i>x</i> | <i>x</i> |          | <i>x</i> | <i>x</i> |          | <i>x</i> |          |
| S-729 | <i>x</i> | <i>x</i> | <i>x</i> |          |          |          | <i>x</i> |          |          |
| S-731 |          |          | <i>x</i> |          |          |          | <i>x</i> |          |          |
| S-732 | <i>x</i> | <i>x</i> | <i>x</i> |          |          | <i>x</i> |          |          |          |
| T-742 | <i>x</i> | <i>x</i> |          | <i>x</i> | <i>x</i> |          |          | <i>x</i> | <i>x</i> |
| S-760 | <i>x</i> |          |          |          |          |          |          |          |          |

**Table 5.3** Predicted Phosphorylation sites of RAB23 by PKC $\delta$

| Residue | GPS 0.5  | Phosphomotif | DISPHOS  | KinasePhos2.0 | NetPhos 3.1 | Muside |
|---------|----------|--------------|----------|---------------|-------------|--------|
| S-24    | <i>x</i> | <i>x</i>     |          |               | <i>x</i>    |        |
| S-23    |          |              |          |               |             |        |
| T-35    |          |              |          |               | <i>x</i>    |        |
| T-41    |          | <i>x</i>     |          |               |             |        |
| T-65    |          |              |          |               |             |        |
| T-75    |          |              |          |               |             |        |
| S-90    |          |              |          | <i>x</i>      |             |        |
| T-91    |          |              |          |               |             |        |
| T-92    | <i>x</i> | <i>x</i>     |          |               |             |        |
| S-96    | <i>x</i> | <i>x</i>     | <i>x</i> |               |             |        |
| S-101   |          |              | <i>x</i> |               |             |        |

|       |          |          |          |          |          |          |
|-------|----------|----------|----------|----------|----------|----------|
| S-102 | <i>x</i> | <i>x</i> |          |          | <i>x</i> |          |
| T-116 |          |          |          | <i>x</i> |          |          |
| S-129 |          |          |          | <i>x</i> |          |          |
| S-150 |          |          |          |          |          |          |
| S-151 | <i>x</i> | <i>x</i> | <i>x</i> |          | <i>x</i> | <i>x</i> |
| T-184 |          |          |          |          |          |          |
| S-186 | <i>x</i> |          |          | <i>x</i> |          |          |
| S-187 |          |          |          |          |          |          |
| S-188 | <i>x</i> | <i>x</i> |          | <i>x</i> |          |          |
| T-196 |          |          |          |          |          |          |
| S-197 |          |          |          |          |          |          |
| S-200 |          |          | <i>x</i> |          |          |          |
| S-202 |          |          | <i>x</i> |          |          |          |
| S-206 |          |          |          |          |          |          |
| T-208 |          |          |          |          |          |          |
| T-224 | <i>x</i> | <i>x</i> | <i>x</i> | <i>x</i> | <i>x</i> | <i>x</i> |
| S-232 | <i>x</i> |          |          |          |          |          |
| S-233 | <i>x</i> |          | <i>x</i> |          |          |          |
| S-235 |          |          | <i>x</i> |          |          |          |

## 6. DISCUSSION

Protein kinase C (PKC) isozymes are members of the Serine/Threonine kinase family regulating cellular events through the activation of various signaling pathways. These pathways involve in cell proliferation, development, cellular survival and death mechanisms. It was shown that some PKC isozymes affect autophagy; yet, the exact mechanism of the relationship between PKC isozymes and autophagy is not clearly identified. In this regard, our former group members worked on the identification of PKC isozymes that take place in the autophagy regulation under PKC activators and starvation induced conditions in HeLa cells. Protein level of each PKC isozymes and autophagy activation were examined under PMA, ionomycin and ceramide conditions. According to their data, under PMA treatment which is novel PKC activator, autophagy regulation was suppressed. When they checked the protein level and phosphorylation of PKC isozymes on activation sites, they found specifically increased protein expression and phosphorylation of PKC $\delta$ . Also, under ceramide treatment they showed the increase in the autophagy compared to basal condition. Thus, in this thesis, we aimed to identify a novel protein associated with PKC isozymes in the fate of autophagy regulation by shRNA-based gene silencing.

Phosphorylation of LC3 by PKC was found, however the role of phosphorylation on autophagy could not be identified after mutagenesis on phosphorylation sites. This is because the endogenous expression of LC3 was enough to regulate autophagy albeit of mutant LC3 proteins (Jiang, Cheng, Liu, Peng, & Feng, 2010). Also, ULK1 phosphorylation by PKC influenced autophagosome formation negatively because of increasing CMA activity in order to recycle phosphorylated ULK1 proteins (C. Wang et al., 2018). PKC $\delta$  activity resulted in the attenuation of autophagy while increasing apoptosis in order to protect kidney cells from cisplatin induced nephrotoxicity. The mechanism occurs due to phosphorylation of AKT and activation of mTOR by PKC $\delta$

(Dongshan Zhang et al., 2017a). Interaction of AKT/mTOR/ULK1 complex with PKC was found important for initiation of autophagy whereas LC3 phosphorylation crucial for autophagosome formation and elongation. Under hypoxic stress, calcium storages in ER were released and caused  $\text{Ca}^{+2}$  dependent PKC $\theta$  activation. PKC $\theta$  signaling resulted in the increase in autophagy regulation in hepatic stellate cells (Jin et al., 2016). In another study, critical role of PKC $\delta$  in autophagy and apoptosis under hypoxic stress was demonstrated. While under short term stress condition, PKC $\delta$  caused the increase in autophagy to protect cells from death, under long term stress PKC $\delta$  initiated apoptosis in order to get rid of nonfunctional damaged cells to protect environment (J.-L. Chen et al., 2009; Liu et al., 2019; So & Oh, 2016). Noticeably, PKC signaling has pivotal role in the regulation of autophagy to decide cells' destiny under stress conditions. Thus, investigation of PKC signaling pathways is important to understand the cell death and survival mechanisms in a broader perspective.

In relation to the data, we have previously obtained, in this thesis, it was crucial to show the effect of PKC $\delta$  activation on autophagy mechanism in MEF GFP-LC3 transgenic cells upon PMA treatment. PMA showed an inhibitory effect on autophagy consistent with our previous results (Figure 5.1). Ceramide is a specific drug used for the activation of PKC $\zeta$ . PKC $\zeta$  is another PKC isozyme that could possibly has function in autophagy regulation, since we showed the change in autophagy activation under ceramide treatment (Figure 5.2).

For further analysis, MEF GFP-LC3 transgenic cells were infected with lentiviral shRNA library, that includes 4625 shRNA targeting genes having role in different signaling pathways. After successful selection with puromycin treatment, monoclonal cells were produced. During monoclonal propagation and maintenance, half of them were died. Probably in those monoclonal cells, crucial cell survival genes were silenced so they could not be propagated. Also, the growth rate and cell size were changed after infection in most of monoclonal cells. They grew slower and had smaller surface area compared to non-transduced cells. To determine the change in PMA or ceramide-dependent autophagy, GFP-LC3 puncta count was performed in each monoclonal cells. MC3 monoclonal cell had significant autophagy activation upon PMA treatment under both basal and starvation-induced conditions. This result is significant because PMA had an inhibitory effect on autophagy regulation in non-transduced cells. According to the sequence analysis, the suppressed gene in MC3 monoclonal cell was TRPV6 which is one of the transmembrane calcium

channels. mRNA expression of TRPV6 showed the successful inhibition of target gene by lentiviral shRNA in MC3 monoclonal cell line. Previously, PMA treatment showed the inhibition of autophagy in non-transduced MEF GFP-LC3 transgenic cells. However, it was reverse in MC3 monoclonal cell line, therefore PMA-induced autophagy might be blocked due to suppression of TRPV6 (Figure 5.9).

TRPV6 is a member of transient receptor potential superfamily and located on both endoplasmic reticulum (ER) and cell membrane. TRPV6 channels are highly sensitive to intracellular  $Ca^{+2}$  concentration and acts either homotetrameric or heterotetrameric structure with TRPV5. An increase in  $Ca^{+2}$  level results in the inactivation of TRPV6 function via the PLC-PIP2 signaling pathway (Haustrate et al., 2019). The change in expression of TRPV6 was demonstrated in several disease progressions such as carcinogenesis, kidney and cardiac failure due to the disruption of  $Ca^{+2}$  uptake (Humeau et al., 2018). Calcium is an essential secondary messenger in signal transduction and has a vital role in cellular homeostasis. Any defect in its turnover may cause blockage of proliferation and initiation of apoptosis since calcium metabolism is important for the cell cycle regulation (Humeau et al., 2018). Excess calcium in the cell is stored in ER and its intracellular signaling initiates from ER to other cellular compartments. Therefore, any fault in intracellular  $Ca^{+2}$  balance results in ER stress (Krebs, Agellon, & Michalak, 2015). Recent studies showed that upregulation of TRPV6 protein results in the increase of  $Ca^{+2}$  level and causes ER stress mediated apoptosis (J. K. Lau et al., 2014). In contrast, TRPV6 knockdown led to initiation of apoptosis since TRPV6 promotes cell survival by providing apoptosis resistance in cancer (Raphaël et al., 2014). It was reported that ER stress caused by elevated  $Ca^{+2}$  level is closely associated with autophagy regulation apart from apoptosis (X. Chen, Li, & Zhao, 2018; Høyer-Hansen & Jäättelä, 2007).

Since the expression of TRPV6 and PKC $\delta$  were reported in different type of cancer cells, we decided to perform further experiments in HeLa cell line. In order to apply gene transfer in HeLa cells stably expressing GFP-LC3, we carried out different transfection methods. While, Amaxa transfection demonstrated the highest efficiency, CaPO4 transfection gave the lowest efficiency (Figure 5.11-12). However, the transfection efficiency was proportional to cell death. Therefore, we preferred to use Lipofectamine 2000 which has second efficient transfection results in order to avoid excessive cell death during transfection process. At first, TRPV6 expression and the effect of PMA on autophagy regulation were confirmed in HeLa GFP-LC3 stable cells under basal

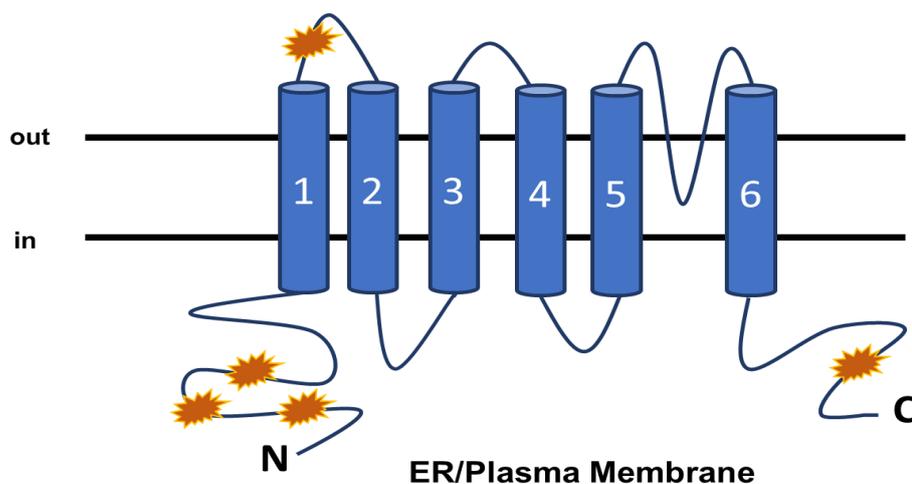
condition. In MEF GFP-LC3 transgenic cells, PMA treatment caused an increase in PKC $\delta$  protein expression while autophagy was attenuated under this condition. Surprisingly, PMA increased both PKC $\delta$  and TRPV6 protein expression compared to both vehicle control and starvation induced condition in HeLa cells (Figure 5.13). It was reported that the change in Ca<sup>+2</sup> level due to ER stimulation caused autophagy activation however there is not any data regarding to TRPV6 dependent autophagy regulation. Thus, it was important to check the interaction of TRPV6 and PKC $\delta$  by establishing overexpression and knockdown experiments.

Our findings demonstrated that upon PMA exposure, autophagy was inhibited due to PKC $\delta$  activation. However, PKC $\delta$  overexpression caused an increase in the autophagy mechanism upon PMA treatment. This increase might be due to the contrary effect of cellular mechanisms in order to keep cellular homeostasis under excessive amount of PKC $\delta$  protein. Meanwhile, in these cells, PMA treatment caused the increase in TRPV6 protein expression in both basal and starvation-induced condition (Figure 5.14). One possibility is that upregulation of PKC $\delta$  caused the change in TRPV6 activity and led to Ca<sup>+2</sup> dependent ER stress and eventually contributed the increase in autophagy under PMA treatment. The another one is that other PKC isozymes activated by PMA may take place in the signaling mechanism and caused the increase in ER stress independent autophagy regardless of TRPV6 activation.

The next step was confirmation of autophagy activity and TRPV6 expression under PKC $\delta$  silenced conditions. We expected that under the suppression of PKC $\delta$ , TRPV6 protein level could be decreased because both PMA treatment and PKC $\delta$  overexpression caused increase in the TRPV6 protein expression. However, it was observed that the expression level of TRPV6 was higher in PKC $\delta$  silenced cells under both PMA and starvation induced condition (Figure 5.15). PKC $\delta$  downregulation affected TRPV6 expression significantly because TRPV6 could interact with other PKC isozymes in order to stabilize channel activity and to normalize intracellular Ca<sup>+2</sup> concentration in the absence of enough PKC $\delta$  activity. In the literature PKC $\alpha$  dependent phosphorylation of TRPV6 resulted in the change of channel function (Niemeyer, Bergs, Wissenbach, Flockerzi, & Trost, 2001). However, PKC $\alpha$  activation depends on Ca<sup>+2</sup> so other signaling pathways may ensue to regulate TRPV6 stimulation under PKC $\delta$  silenced condition.

These results showed that there is a momentous interaction between PKC $\delta$  and TRPV6. The regulation of TRPV6 depends on PKC $\delta$  however we wonder whether PKC $\delta$  expression relates to the change in TRPV6 expression. In order to examine this hypothesis, we downregulated TRPV6 expression by siRNA silencing to determine both autophagic activity and PKC $\delta$  expression. Consistent with MC3 cells, suppression of TRPV6 increased the autophagy upon PMA treatment compared to control in HeLa cells. However, TRPV6 downregulation did not change the PKC $\delta$  expression (Figure 5.17). Therefore, the relation between PKC $\delta$  and TRPV6 is not mutual. In the literature, TRPV6 works both alone or dual with TRPV5 and they have a similar role in Ca<sup>+2</sup> regulation. Thus, TRPV5 can substitute TRPV6 role in Ca<sup>+2</sup> metabolism under the absence of TRPV6.

The change in TRPV6 expression could not cause differences in PKC $\delta$  level so we hypothesized that the phosphorylation of TRPV6 by PKC $\delta$  may happen. Before experimental process, we aimed to determine possible phosphorylation sites on TRPV6 by PKC $\delta$  by using different bioinformatics tools. Interestingly, there were several phosphorylation sites detected and the most common sites located on the N terminus of TRPV6 which is crucial for function of channel activity (Table 5.1, Figure 6.1). Obviously to be determine which sites is important for PKC $\delta$  interaction during autophagy regulation, in-depth search should be applied. Our preliminary findings are important because of introducing novel signaling pathway of PKC on autophagy mechanism. Investigation of this pathway will contribute the understanding of PKC $\delta$  dependent autophagy activation associated with calcium metabolism clearly.



**Figure 6.1.** Possible phosphorylation sites on TRPV6 targeted by PKC $\delta$  were shown in yellow.

## 7. FUTURE PERSPECTIVE

In this M.Sc. thesis, it was described that PKC $\delta$  isozyme interacts with TRPV6, which could have a significant impact on autophagy regulation. The exact mechanism of this interaction could not be identified so far. Our study might be critical, because we investigated possible linker signaling member, TRPV6, to clarify PKC $\delta$  mediated autophagy regulation. There are several phosphorylation sites on TRPV6 targeted by PKC $\delta$  and point mutations on these sites will lighten the site-specific interaction between TRPV6 and PKC $\delta$ . In addition to phosphorylation, the interactions between TRPV6 and PKC $\delta$  should be shown by co-immunoprecipitation assays to understand the relation between PKC $\delta$  and TRPV6 in terms of binding.

Also, intracellular Ca<sup>+2</sup> levels should be determined under each treatment condition in order to predict whether these data are related to ER stress-mediated autophagy. Depending on the Ca<sup>+2</sup> level analysis, Ca<sup>+2</sup> induced PKC $\alpha$  activation may be checked under both PMA and ionomycin treatment. It is already shown that PKC $\alpha$  can phosphorylate TRPV6. It will clarify whether the change in TRPV6 is depending on PMA or Ca<sup>+2</sup> concentration. Flow cytometry analysis after FURA-2AM exposure may give idea about cytoplasmic Ca<sup>+2</sup> concentration related with TRPV6 activity. Also, ER stress inducers such as tunicamycin or thapsigargin may be used as positive control of ER stress during analysis of Ca<sup>+2</sup> level in cells after PMA and starvation treatments. Also, Ca<sup>+2</sup> concentration in ER maybe visualized under fluorescence microscopy after FURA 2AM staining and this will contribute the comparison of TRPV6 role in Ca<sup>+2</sup> concentration both in ER and cytoplasm.

PKC isozymes may have contrary effects on autophagy depending on the cell types. In order to show PKC $\delta$  and TRPV6 relation, different cancer cell lines might be used to confirm this interaction on autophagy mechanism.

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## PUBLICATIONS AND PRESENTATIONS

**Kaleli H.N.**, Ozer E., Kaya V.O., Kutlu O. Protein Kinase C Isozymes and Autophagy During Neurodegenerative Disease Progression (2019) (**under revision**)

Kutlu O., **Kaleli H.N.**, Ozer E., Molecular Pathogenesis of Nonalcoholic Steatohepatitis (NASH) Related Hepatocellular Carcinoma, Canadian Journal of Gastroenterology and Hepatology, doi.org/10.1155/2018/8543763

**Kaleli H.N.**, Kaya V.O., Yedier-Bayram O., Kilic S., Onal G., Yuce A., Dokmeci S., Gozuacik D., Kutlu O., (2019) Reverse Activation of Defective Autophagy Mechanism Might Be an Alternative Approach for Gaucher Disease Therapy, 13th European Working Group on Gaucher Disease Congress (EWGGD 2019), Clermont-Ferrand, France

Ozer O., **Kaleli H.N.**, Taskın E., Kutlu O., (2019) Association Between *PNPLA3* rs738409 polymorphism with Autophagy and Non-alcoholic Steatohepatitis (NASH), 3rd Cukurova Hepatocellular Carcinoma Congress, Turkey

**Kaleli H.N.**, Ozer E., Yedier-Bayram O., Kilic S., Dokmeci S., Gozuacik D., Kutlu O. (2018) Identification of Novel Targets for The Role of PKC Isozymes on The Regulation of Autophagy, 6th International Congress of the Molecular Biology Association of Turkey