Development of a Novel Approach for Cell Surface Protein Analysis

by

Mehmet Akdağ

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This is to certify that I have examined this copy of a master's dissertation by

Mehmet Akdağ

and have found that is complete and satisfactory in all respects, and

that any and all revisions required by

examining committee have been made.

Committee Members:

_____

Assoc. Prof. Dr. Nurhan Özlü (Advisor, Koç University)

_____

Assoc. Prof. Dr. Ceren Çıracı (Istanbul Technical University)

_____

Asst. Prof. Dr. Serkan Kır (Koç University)

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Abstract

Plasma membrane is an evolutionary conserved cellular compartment for all cells. It directly or indirectly participates in vital pathways in the cell such as nutrition uptake, signal transduction, cell to cell communication, migration, adhesion, and cell division. Therefore, to elucidate molecular mechanisms of the cell or regulation of these pathways, we need to understand how plasma membrane components interact with inner part of the cell and their specific roles in these pathways. One unbiased way to address these questions is to take proteomics approach. Yet, unique structure of the plasma membrane and low abundancy of the cell membrane proteins make it challenging to study plasma membrane proteins with traditional proteomics approach. Therefore, there is a need for more effective approaches for identification of plasma membrane proteins.

In the first part of my thesis, I developed a novel approach for plasma membrane protein enrichment. In this method, I used centrifugation and BioID enrichment methods subsequently. To that extent, I modified biotin ligase (BirA) enzyme to target and biotinylate cell membrane proteins. Then, by using subsequent physical and affinity-based enrichment methods, plasma membrane protein levels increased in the sample. Proteins then identified by using mass spectrometry.

In the second part of my thesis, I focused on two members of Chloride Intracellular Channel (CLIC) proteins, CLIC1 and CLIC4. In a recent quantitative proteomics study from our laboratory showed that CLIC1 and CLIC4 enriched at the plasma membrane during cell division Currently, CLICs are not associated with any cell cycle dependent function. In the second part of my thesis, I focused on the characterization of the CLIC1 and CLIC4 proteins in cell cycle dependent manner. To that extent, I generated CRISPR-Cas9 mediated knockout cell lines and investigated their effects on cytokinesis fidelity. CLIC1 and CLIC4 knockout cells increased cytokinesis abnormalities indicating their role at the plasma membrane during cell division.

Özet

Plazma membran tüm hücrelerde evrimsel süreçte korunmuş bir hücresel yapıdır. Hücresel madde alış verişi, sinyal iletimi, hücreler arası iletişim, hücre göçü ve hücre bölünmesi gibi birçok hayati metabolik yolakta direk veya dolaylı olarak görev alır. Bu yüzden bu gibi mekanizmaların anlaşılması, bu yolakların kontrollerinin aydınlatılması için plazma membranını oluşturan bileşenlerin görevlerini ve bunların hücrenin iç bölgeleriyle nasıl etkileşimde olduklarını bilmemiz gerekmektedir. Proteomik metotları kullanmak bu gibi soruları cevaplamak için kullanılabilecek tarafsız yollardan biridir. Fakat plazma membran proteinlerinin kendilerine özgü yapıları ve az miktarda bulunmaları normal proteomik metotların plazma membranı üzerinde kullanımını engeller. Bu yüzden daha etkili plazma membran protein analiz yöntemlerine ihtiyaç duymaktayız.

Tezimin ilk bölümünde, plazma membranı proteinlerini zenginleştirmek için yeni bir metot geliştirdim. Bu metotta santrifüj ve BioID protein zenginleştirme metotlarını birlikte kullandım. Bunun için bir Biotin ligaz enzimi olan BirA enzimini hücre zarını hedefleyecek ve biotinleyecek şekilde modifiye ettim. Sonrasında peş peşe fiziksel ve affinite temelli protein zenginleştirme metotlarını kullanarak plazma zarı proteinlerinin eldesini hedefledim. Sonrasında proteinleri hücre spektrometrisini kullanarak proteinleri analiz ettim.

Tezimin ikinci bölümünde, Chloride Intracelluar Channel (CLIC) protein ailesinin iki üyesi olan CLIC1 ve CLIC4 proteinlerinin karakterizasyonu üzerine çalıştım. Yakın zamanlarda laboratuvarımızdan yayınlanan çalışmada CLIC1 ve CLIC4 proteinlerinin mitoz sırasında hücre zarında zenginleştiğini gösterdik. Literatürde henüz CLIC proteinlerinin hücre döngüsüyle ilgili bir fonksiyonu rapor edilmedi. Bu sebeple, CRISPR-Cas9 metodunu kullanarak CLIC1 ve CLIC4 nakavt memeli hücre hattı ürettik ve bu hücrelerde sitokinez analizi yaptım. CLIC1 ve CLIC4 nakavt hücrelerde sitokinez hatalarının daha fazla olduğunu ve bu sebeple daha yüksek oranda çok çekirdekli hücre oluşturduklarını bulduk.

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ABBREVIATIONS

ALIX	Apoptosis-linked gene 2-interacting protein X
ATP	Adenosine triphosphate
BioID	Proximity-dependent biotin identification
BirA*	Promiscuous Escherichia coli biotin ligase enzyme
BSA	Bovine Serum Albumin
C	Cytokinesis phase
CDK	Cyclin Dependent Kinase
CD44	CD44 antigen
CLIC	Chloride intracellular channel Protein
CLIC1	Chloride intracellular channel Protein 1
CLIC4	Chloride intracellular channel Protein 4
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4’,6- diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
FBS	Fetal Bovine Serum
G0	Resting phase
G1	Gap 1 phase
G2	Gap 2 phase
GFP	Green Fluorescence Protein
GSH	Glutathione
GST	Glutathione S-transferase

GO	Gene Ontology
GO-BP	Gene Ontology Biological Process
GO-CC	Gene Ontology Cellular Compartment
GO-MF	Gene Ontology Molecular Function
HEK293T	Human Embryonic Kidney Cell Line
HeLa S3	Human Cervical Cancer Cell Line S3
HRP	Horseradish Peroxidase
IAA	Iodoacetamide
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
LB	Lysogeny Broth medium
M	Mitosis phase
Myr	Myristoyl group
Myrpalm	Myristoyl and palmitoyl lipid groups
Myrpalm-BirA	Myristoylated and palmitoylated biotin ligase enzyme
MS	Mass Spectrometry
NP-40	Nonyl phenoxypolyethoxyethanol
Opti-MEM	Reduced Serum Medium
Palm	Palmitoyl group
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEI	Polyethyleneimine
PFA	Paraformaldehyde
RhoA	Ras homology gene family, member A
RNA	Ribonucleic acid
Rpm	Round per minute
S	Synthesis phase
SAINT	Significance Analysis of Interactome
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sgNT	nontargeting guide RNA

sgRNA	guide RNA
SOSUI	Classification and Secondary Structure Prediction of Membrane Proteins
TBS	Tris Buffered Saline
TBS-Tx	0.1% Triton X containing Tris Buffered Saline
TBS-Tw	0.1% Tween 20 containing Tris Buffered Saline
TEMED	Tetramethylethylenediamine
WCL	Whole Cell Lysate

INTRODUCTION

All cells, whether it is bacteria, archaea or eukaryotes, undergo cell division. Cell division is vital machinery for living cells to survive, grow or reproduce. Therefore, cell division is kept under strict regulation throughout the cell cycle. During cell cycle progression, several sequential events take place. Duplication of genomic materials, segregation of chromosomes and physically separation of cytosol are among those. The cell cycle is comprised of two main phases which are interphase and mitosis. During interphase cells prepare themselves to division, while in mitosis cell division occurs. Mitosis is comprised of different stages as well. These stages are prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis. Compared to other phases of cell cycle, cytokinesis is not well characterized. Throughout cell division, several changes occur in composition of both cytosol and cellular membrane. Despite having several studies on cytosolic changes during cell division, unfortunately we do not have same level of understanding about what is happening on plasma membrane during mitosis.

The plasma membrane usually considered a physical barrier, yet this is simply underestimation of its importance for the cell. The plasma membrane plays crucial roles in many cellular pathways such as signal transduction, nutrient exchange, cellular communication. Despite their importance, their unique properties make it a challenging task to investigate plasma membrane proteins compared to soluble proteins. Therefore, quantitative proteomics analysis has become commonly used approach to reveal dynamic behavior of plasma membrane proteins. Yet traditional protein identification techniques are not quite compatible for plasma membrane proteomics analysis. For that reason, plasma membrane enrichment strategies commonly used in the plasma membrane proteomics studies. However, the efficiency of the plasma membrane enrichment strategies is not at the desired level.

That is why to elucidate the interaction of membrane proteins with other proteins and characterization of the plasma membrane proteins, we developed a novel method. In this method, we combined different membrane enrichment methods and characterize the plasma membrane proteins. To that extent, we used a physical enrichment method, sucrose density gradient fractionation, and an affinity-based enrichment method, BioID, together. BioID method took advantage of the biotinylation ability of a biotin ligase (BirA) enzyme. Mutant

BirA*(R118G) enzyme can biotinylate surrounding proteins promiscuously[1]. To recruit BirA enzyme to cell membrane we used lipid modifications. Lipid modifications commonly used in the cell to target a protein to its destination. These proteins are mostly targeted to cell membrane, Golgi and endoplasmic reticulum[2]. Identification and characterization of biotinylated proteins become straightforward, by subsequent streptavidin pulldown and proteomics analysis.

According to a recent proteomics study done by our research group, Ozlu, N. et al. (2014), the quantity of plasma membrane and cytosolic proteins change depending on cell cycle. Some proteins get enriched on plasma membrane during mitosis compared to interphase. Chloride intracellular channel proteins (CLIC proteins) are among those proteins. Especially amount of CLIC4 and CLIC1 proteins on plasma membrane increases during mitotic phase compared to their levels in interphase[3].

CLIC protein family contains six-member (CLIC1-6) and these members shares evolutionarily conserved domains in their structure. CLIC proteins are ubiquitously expressed proteins that exist as both soluble cytosolic form and membrane-bound form. Although they are known as ion channel proteins their functions have not been fully understood. CLIC proteins are involved in several important biological function such as membrane remodeling, intracellular trafficking, angiogenesis. CLIC proteins and omega-class of Glutathione S-transferase (GST) enzymes share structurally similar domains yet, it is still unknown whether CLIC proteins show the same enzymatic activity under physiological conditions.

CLIC4 is one of the best studied CLIC family proteins along with CLIC1. They are both ubiquitously expressed in cell. Although they are mostly in soluble form, membrane-bound forms are also present in the cell. It is known that it plays important roles in apoptosis, cell differentiation, endosomal trafficking, actin-cytoskeleton dependent membrane remodeling, and cell migration. Yet cell cycle dependent role of CLIC4 is not known. Therefore, in this study I investigated the roles of CLIC4 and CLIC1 in cell cycle dependent manner in mammalian cells. Chapter 2 contains a detailed review related to plasma membrane proteins and the current state of membrane proteomics. In Chapter 3, materials and methods used during the study are explained. Chapter 4 consists of the results of the experiments. In Chapter 5, detailed interpretation and discussion of the results are presented together with future directions of the study.

Chapter 6 contains a detailed literature review related to the CLIC study. In Chapter 7, the materials and methods used during the study are explained. Chapter 8 consists of the results of the experiments. In Chapter 9, detailed interpretation and discussion of the results are presented together with future directions of the study.

1. DEVELOPMENT OF A NOVEL APPROACH FOR PLASMA MEMBRANE PROTEINS

1.1. Literature Review

1.1.1. Membrane Structure and Properties

All cells share three common structures genomic material, cytosol, and cell membrane. The cell membrane also called the plasma membrane is the barrier that separates inner part of the cell from external environment and it plays important roles in many vital pathways. Aside being a barrier some of the main responsibilities of the plasma membrane are providing mechanical strength, preserving cellular and genomic integrity, compartmentalization of organelles, protecting the cell against extreme environmental conditions, defending the cell against dangerous organisms or agents, initiating signal cascades and regulation of material exchange. Most of these tasks are executed by the activity of membrane proteins[4].

Plasma membrane proteins can be clustered into two subgroups, integral membrane proteins and peripheral membrane proteins (**Figure 1**)**.** Integral membrane proteins are embedded into phospholipid bilayers while peripheral membrane proteins interact with integral membrane proteins or head groups of phospholipids. Several integral membrane proteins pass through the entire lipid bilayer and interact with both cytosolic and extracellular environments. These proteins are also called transmembrane proteins. Peripheral membrane proteins do not penetrate through the lipid bilayer, instead they are linked to integral membrane proteins or phospholipids[5].

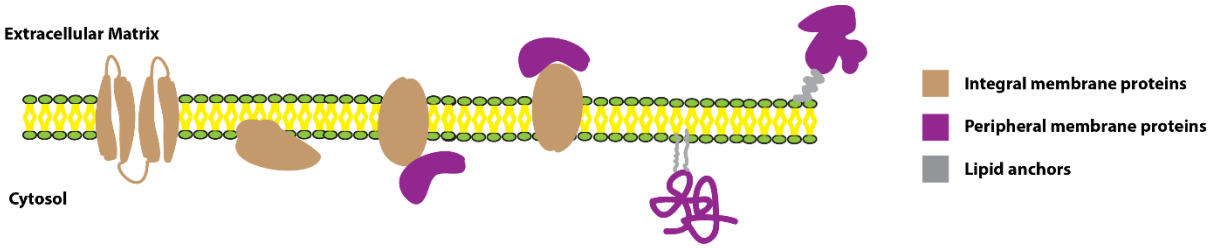

Figure 1 Membrane proteins can be found in various types in the cell.

(Adapted from [5])

Plasma membrane proteins usually carry distinct motifs that provide characteristic properties to them. These motifs are attached to proteins as post-translational modifications (PTMs). While some proteins bear single modification, others contain various PTMs. Most abundant groups of post-translational modifications are phosphorylation, glycosylation, ubiquitylation, methylation, acetylation, and lipidation[6].

Protein lipidation usually results in translocation of the protein to the membrane, organelles or vesicles. Several lipidation modifications present in the cell yet myristoylation and palmitoylations are the most studied ones (**Figure 2**)[7, 8].

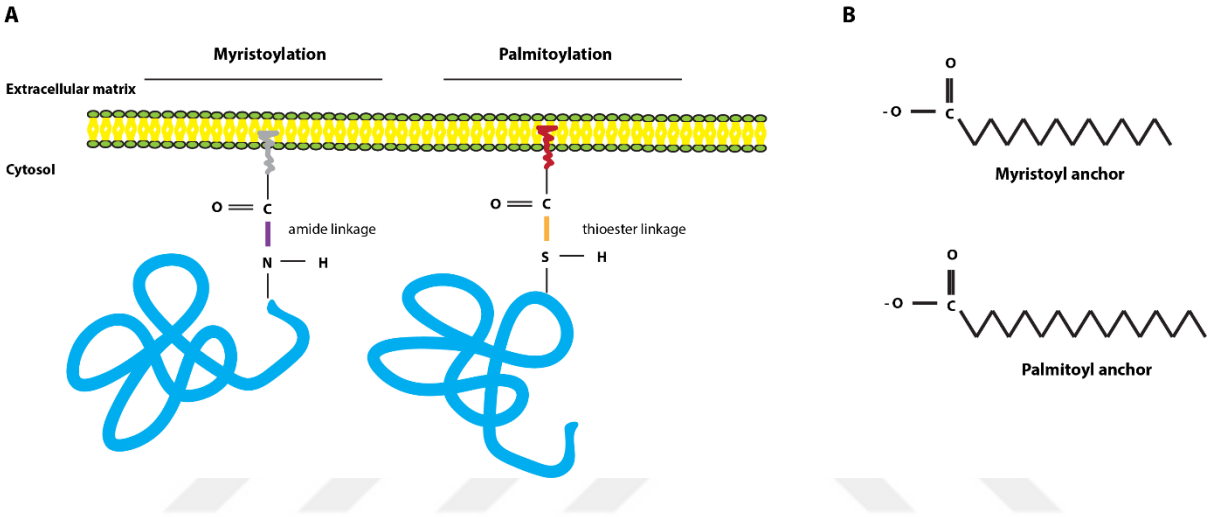

Figure 2 Membrane proteins anchoring by myristoylation and palmitoylation.

A- Proteins anchored to the membrane by amide or thioester bonds by myristoylation and palmitoylation respectively.

B- Schematic representations of myristoyl and palmitoyl groups. [5]

1.1.1.1. N-Myristoylation

Myristoylation is the addition of myristate group, 14 carbon saturated fatty acid group, to the nascent proteins. In eukaryotes about 0.5-0.8% of the proteins are N-myristoylated. Myristoylation is catalyzed by the enzymatic activity of N-myristoyl transferase enzyme (NMT). The enzyme recognizes the sequence of Met-Gly-X-X-X-Ser/Thr and adds myristoyl group to the amino terminus of the Glycine residue after removing initial Methionine amino acid[2].

Myristoylation supply a hydrophobic region to a protein that targets the protein to the membrane. Myristate group also plays roles in signaling pathways. The cell regulates translocation of peripheral membrane proteins between membrane and cytosol through

myristate group. However, myristoylation alone does not provide enough hydrophobicity to membrane proteins to anchor them to phospholipids inside the membrane. Therefore, to obtain stable interaction secondary signals required. These signals can be a basic amino acid residue (lysine, arginine, histidine) or addition of palmitate group[9, 10].

1.1.1.2. Palmitoylation

Palmitoylation is a covalent lipid modification of a protein that results in attachment of 16 carbon saturated acyl groups (palmitate) by esterification reaction. A diverse range of membrane proteins gets palmitoylated in the cell. The palmitate group binds to protein through cysteine residue. Palmitoylation can occur with or without accompanying other modifications. Palmitoylation reaction is catalyzed by palmitoyl acyltransferase enzyme (PATs) family and depalmitoylation is regulated by palmitoyl protein thioesterase enzymes (PTEs). Cellular localization of palmitoylated proteins are regulated by palmitoylation and depalmitoylation cycles[11].

S-palmitoylation of protein results in different outcomes depending on the combinatorial effect of the post-translational modifications of the protein. Palmitoylation is an important signal sequence responsible for diverse functions such as membrane localization of proteins, anterograde protein trafficking between ER and Golgi, protein recycling from lysosomal/endosomal vesicles, and secretion of signaling molecules[10, 12].

1.1.1.3. Importance of Membrane Proteins

Their roles in important pathways make them interesting targets for protein research, especially in the pharmaceutics field. Currently more than 50% of FDA approved therapeutics targets membrane proteins, yet their potential as drug target is not fully exploited[13, 14]. Therefore, several studies focus on identification and characterization of plasma membrane proteins.

Quantitative proteomics approaches promise a bright future for large scale protein analysis especially after recent advances in high throughput data analysis and development in mass spectrometry (MS). Despite all the efforts and investments, MS-based plasma membrane proteomics analysis still struggles to tackle many difficulties.

1.1.2. Membrane Proteomics Challenges

The main challenges of membrane proteomics arise from their hydrophobic nature and their low abundance. The unique structure of the membrane and characteristic properties of

membrane proteins makes it difficult to investigate plasma membrane proteins. Membrane proteins interact with high levels of lipid molecules which interferes with several methods to study proteins. Plasma membrane proteins show split characteristic properties with hydrophobic and hydrophilic regions. Hydrophobic regions of proteins are covered with lipid molecules, while hydrophilic regions can freely interact with water molecules. Therefore, plasma membrane proteins show tendency to form aggregates when exposed to aquatic environments. Additionally, their compact structure interferes with tryptic digestion by covering trypsin targets in hydrophobic regions. Therefore, the analysis lacks hydrophobic regions of proteins. Besides hydrophobicity, low abundances of many plasma membrane proteins result in underrepresentation of plasma membrane proteins in proteomics analysis. Their low expression levels lead to masking of these proteins by abundant proteins in proteomics analysis[15-18].

1.1.3. Membrane Enrichment Methods

The quality of plasma membrane proteomics study is tightly related to how efficient plasma membrane proteins enriched in a sample. For enrichment, fractionation and labeling methods are heavily used in proteomics analysis. Isoelectric focusing (IEF), 2D gel electrophoresis (2DGE), sucrose density gradient fractionation are fractionation techniques used in proteomics studies during sample preparation. Since plasma membrane proteins contain hydrophobic regions, they could form aggregates in the 2DGE fractionation method. Therefore, sucrose density gradient fractionation is the most commonly used fractionation method for membrane proteins[19].

1.1.3.1. Sucrose Density Gradient Fractionation

Sucrose density gradient fractionation is a centrifuge based physical separation technique. This method depends on separation of molecules according to their size and density. Various sucrose gradients fractionation methods are available for fractionation of different cellular compartments.

Sucrose density gradient fractionation forms various layers each having different density so that membrane protein separated from the sample according to their densities. However, due to similarities of plasma membrane structure with other subcellular compartments such as endoplasmic reticulum and Golgi, membrane fraction obtained from centrifugation contains cytoplasmic organelle and non-membrane protein contaminations[20, 21].

In many studies, centrifuge-based methods are accompanied by additional enrichment methods to eliminate cytosolic proteins. One of the methods is using chloroform/methanol in a basic solution to extract plasma membrane proteins from lipid bilayers[22]. Another approach is called on-membrane digest. In this method, as its name refers, membrane proteins are exposed to trypsin for a short time and aquatic regions of proteins. Despite successfully enriching plasma membrane proteins, the overall representation of membrane proteins is not on the desired level. Using specific antibodies against target protein is one of the methods. Manipulation of antibodies together with density gradient centrifugation can enrich the desired protein levels in analysis up to three-fold compared to centrifugation-based methods [23]. However, antibody-based methods are not suitable for omics science since they are expensive and not available for all proteins. Colloidal silica beads can enrich membrane proteins by using electrostatic interactions. Since these beads are cationic, they interact with anionic membrane proteins, when they are presented to intact cells. By crosslinking the beads with an anionic polymer, membrane protein levels can be efficiently enriched. This method depends on the homogenization of tissues and disruption of cell membrane, therefore, it is applicable to soft membrane having tissues[22].

1.1.3.2. Chemical Labeling Techniques

Cell surface labeling is another commonly used method in membrane proteomics studies. Despite having several chemically labeling approaches present, the most commonly used one is biotinylation. In this method, mostly posttranslational modifications of plasma membrane proteins are exploited. Membrane impermeable biotin derivatives target plasma membrane proteins and interact with their targets. These biotinylated proteins can be enriched from the proteome by taking advantage of the biotin affinity towards avidin/streptavidin. Despite increased cell surface protein ratio, the efficiency of the chemical biotinylation does not meet the expectation with low coverage numbers of membrane proteins[22, 24].

1.1.3.3. Proximity Dependent Biotin Identification

Various biotinylation dependent identification method is proposed in the literature, one of them is called BioID. This method depends on the proximity-dependent biotinylation activity of a mutant biotin ligase enzyme, BirA(R118G). BirA enzyme originally presents in Escherichia coli and its mutated version promiscuously biotinylates its vicinity. This method requires a fusion protein expression of BirA(R118G) enzyme tagged bait proteins[1]. The enzyme

catalyzes the labeling reaction of molecules in 10 nm proximity [25]. Subsequently, biotinylated proteins isolated by streptavidin resins and further analyzed by using mass spectrometry. BioID proposed a promising approach for identification of interaction partners of proteins. Although there are several successful studies present in the literature, high background in the analysis is the limiting factor for this method. Another limitation is the difference between wild type and fusion protein localization due to additional 35 kDa BirA* enzyme.

1.1.4. The motivation of the study

Although we have many techniques to investigate plasma membrane proteins, each method brings different limitations. Using only one method leads to underrepresentation of membrane proteins or enrichment of only small fraction of the membrane proteins. A combination of methods increases the efficiency, yet additional purification steps cause losing important portion of membrane proteins.

That is why in this study, I aimed to develop an alternative strategy to physiologically label the cell membrane proteins and isolate them to acquire more comprehensive plasma membrane proteomics analysis. To that extent, I generated a stable mammalian cell line expressing myristoylated and palmitoylated BirA enzyme. Since these two lipid modifications can translocate the BirA enzyme to the plasma membrane, activation of BirA results in the biotinylation of cell membrane proteins. I isolated biotinylated proteins from membrane fractions by using streptavidin beads to enrich the membrane proteins. Additionally, I also used sucrose density gradient fractionation to further enrich the membrane proteins. Then I analyzed isolated proteins by using mass spectrometry-based proteomics tools.

1.2. Materials & Methods

1.2.1. Generation of lentiviral vectors

Myrpalm and CD44 isoform 12 sequences were cloned into pcDNA3.1 MCS-BioID(R118G)-HA (Addgene, Plasmid #36047) by Gürkan Mollaoğlu (former M.Sc. student of Cell Biology and Proteomics Laboratory, Koç University). pcDNA3.1 MCS-BirA(R118G)-HA was a gift from Kyle Roux (Addgene plasmid # 36047 ; http://n2t.net/addgene:36047 ; RRID:Addgene_36047) [1].

1.2.1.1. Cloning of BioID, Myrpalm-BioID, and CD44-BioID into pLenti CMV Hygro DEST vector

To generate stable cell lines, I cloned these inserts into pLenti-CMV-Hygro-DEST vector by using Gateway Cloning (Invitrogen) system. pLenti CMV Hygro DEST (w117-1) was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 17454 ; http://n2t.net/addgene:17454 ; RRID:Addgene_17454)[26]. These inserts do not have an appropriate restriction site. Therefore, to insert *attB* and *attP* restriction sites which are necessary for Gateway Cloning, I amplified BioID, Myrpalm-BioID, and CD44-BioID plasmids by using PCR.

Following reaction mixture was prepared for this PCR:

4.0 µL 5X GC Buffer, 0.4 µL 10mM dNTP, 10 µL forward primer, 10 µL reverse primer, 0.6 µL 100 % DMSO, 100 ng template DNA, 0.2 µL Phusion HF DNA Polymerase (NEB, M0530S) and 12.7 µL ddH_2O.

Primers used in this study is given in **Table 1:**

Table 1 Primers used during the study

Primers	Primer Sequence	Plasmids
144-B1-frw	5-GGGGACAAGTTTGTACAAAAAAGCAGGC TTTATGAGCAGAGCCGCCATCAACAAGC-3	BioID
146-B1-frw	5-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATG GGATGTATAAAATCAAAAGGGAAAGACAGCG-3	Myrpalm-BioID
148-B1-frw	5-GGGGACAAGTTTGTACAAAAAAGCAGGC TTTATGGACAAGTTTTGGTGGCACGCAGC-3	CD44-BioID
144-B2-rev	5-GGGGACCACTTTGTACAAGAAAGCTGGGT CTCAGCGGTTTAAACTTAAGGCCGCC(TA)-3	BioID, CD44-BioID Myrpalm-BioID

Following PCR conditions were used for these amplifications:

Initial denaturation at 98°C for 30 seconds; denaturation at 98°C for 10 seconds, annealing at 75°C for 30 seconds, extension at 72°C for 45 seconds, final extension at 72°C for 10 minutes; 30 cycles. To confirm product sizes PCR products were run in 1% agarose gel. Confirmed PCR products were purified from the gel by using the Clean-up Gel Extraction kit (Macherey Nagel, 740609.50).

Then by using *BP Clonase II* enzyme activity PCR products were ligated into *pDONR221* vector (3 µl of PCR product, 1 µl of 75ng/µl pDONR221 vector, 1 µl of BP Clonase II enzyme). Reaction occurred overnight at room temperature (25°C for 16 hours), then I stopped the reaction by adding 1 µl of *Proteinase K* and incubate it at 37°C for 15 minutes.

After that, I transformed chemical competent *Escherichia coli Stbl3* cells with the ligation product to 50 µg/ml kanamycin containing LB agar plate. Transformation was done according to traditional transformation protocol (Mix and incubate 3 µl of DNA and 50 µl of bacteria on ice for 30 minutes, heat shock at 42°C for 45 seconds, incubate on ice for 2 minutes, add 800 µl LB and incubate the culture for 60 minutes at 37°C with 300 rpm shaking condition, plate the culture to antibiotic-containing LB plate, incubate it for 16 hours at 37°C). Single colonies grown from these plates were randomly picked and inoculated into antibiotic containing liquid LB media. LB cultures were incubated for 16 hours at 37°C with 200 rpm shaking conditions. Plasmid isolation was performed from bacteria cultures by using MN NucleoBond Xtra Midiprep kit (Macherey-Nagel #740410.100), by using manufacturers protocol.

Inserts in pDONR221 vector cloned into pLenti CMV Hygro DEST vector by using LR Clonase II enzyme activity (Mix 1,5 µl of 75 ng/µl pDONR insert, 1,5 µl of 75 ng/µl pLenti-CMV-Hygro-DEST, 1,5 µl LR Clonase II, 3.0 µl TE buffer pH 8 .0 and incubate for 16 hours at 25°C). The reaction was terminated by using Proteinase K and incubate it at 37°C for 15 minutes.

1.2.2. Cell culture

HeLa S3 and Human Embryonic Kidney 293T (HEK293T) cells were grown routinely cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 unit/ml Penicillin and 100 µg/ml Streptomycin (Lonza, DE17-602E) at 37°C and 5% CO_2.

For BioID experiments, cells were grown until they reach 70% confluency and treated with 50mM D-Biotin (BioBasic, BB0078) and 1µM ATP (Sigma, A2383). Cells were harvested after 16 hours of incubation[27].

1.2.2.1. Stable cell line generation

I used HEK293T cells for production of viruses. HEK293T cells were cultured in a 100mm^2 plate until they reach 80-90% confluency. For transfection, I mixed packaging plasmids (psPAX2 (Addgene #12260) (5µg), pMD2.G (Addgene #12259) (2.5µg)) with my plasmids (7.5µg) in OPTI-MEM. psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260 ; http://n2t.net/addgene:12260 ; RRID:Addgene_12260). pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259 ; http://n2t.net/addgene:12259 ; RRID:Addgene_12259).

Then I add PEI to the mixture with a 1:3 ratio (45µl). After incubating mixture for 45 minutes at room temperature, cells were treated with the mixture. Cells were incubated overnight, and the spent media was discarded the next day. After 48 hours, viruses can be harvested by filtering the spent media. For long term storage, viruses were stored at -80°C.

HeLa S3 cells treated with viruses. After three days, the expression of the plasmids reaches the level which I initiated the selection of transfected cells by using hygromycin (300µg/µl) for 10 days.

1.2.2.2. Single Cell Isolation

Cells surviving in the hygromycin selection constitute the pool population. Then I used single-cell isolation technique to the pool population and grow each colony from a single cell.

Heterogenous pool populations were diluted and 10 mL cell suspension with a concentration of 8 cells/mL was obtained. 100 µL of this cell suspension was seeded to each well of the 96 well plates. Cells were grown in the conditions mentioned above and grown colonies transferred into bigger dishes. Colonies were screened by immunoblotting and immunofluorescence to verify Myrpalm-BirA and BirA localization and enzyme activity.

1.2.3. Characterization of Stable Cell Lines

1.2.3.1. Western Blot

Cells were lysed by using EDTA-free protease inhibitor cocktail (Thermo) supplemented NP-40 lysis buffer (1% NP-40, 150mM NaCl, 50mM pH8.0 Tris-Cl). Cell debris was removed by

using centrifugation at 14.000 rpm at 4°C for 10 minutes. To asses protein concentration, BCA kit (Pierce, 23227) was used. Proteins were boiled in 2X Laemmli sample buffer (4% SDS, 0.02% bromophenol blue, 100mM dithiothreitol (DTT), 20% glycerol, 120mM pH6.8 Tris-Cl) for 5 minutes at 95°C.

Western blot separates proteins according to their sizes. I used 10% SDS-PAGE gels and proteins were separated on the gel at 120V for 90-120 minutes.

After that proteins were transferred to the nitrocellulose membrane (GVS, 1215471) from the gel at 45V at 4°C for 16 hours. Transfer efficiencies were controlled by Ponceau staining. Membranes were blocked with 4% non-fat dry milk in TBS-Tween 20 (0.1%) for 60 minutes. Then, I incubated membranes with primary antibodies in 2% BSA containing TBS-Tween 20 (0.1%) for 3 hours at RT or 16 hours at 4°C. Excess antibodies and nonspecific bindings removed by washing with excess TBS-Tween 20 (0.1%) washes for 10 minutes. Then membranes incubated with secondary antibodies which are counterparts of the primary antibodies for 1 hour. Secondary antibodies were prepared in 4% non-fat dry milk in TBS-Tween 20 (0.1%). Then excess bindings were removed by washings again. Then membranes were detected by using horseradish peroxidase - luminol based chemiluminescence method (Pierce, 32106 and Pierce, 32132).

1.2.3.2. Immunofluorescence

Myrpalm-BirA and BirA cell lines were grown on coverslips until they reach 60% confluency. Cells were fixed by using 3.2% PFA solution and permeabilized by PBS-Triton-X (0.1%). Fixed cells were blocked by using 2% BSA containing PBS-Triton X (0.1%) for 1 hour. Then cells incubated with primary antibodies for 3 hours at RT or 16 hours at 4°C. Excess antibodies and nonspecific bindings were removed by PBS-Triton X (0.1%) washes. Then cells treated with appropriate secondary antibodies for 45 - 60 minutes in dark. Images were taken by using confocal microscopy (Eclipse 90i, Nikon). Quantifications for multinucleation experiments were done by using Image J software.

1.2.3.3. Antibodies

Following primary antibodies were used in this study: anti-EGFR (Sc-03, Santa Cruz), anti-β-Actin (ab6276, Abcam), anti-Biotin (noncommercial) and anti-Lamin B1 (PA5-19468, Thermo

Scientific), anti-HLA (ab7855, Abcam), anti-BirA (BID-CP-100, BioFront) and anti-HA-tag (ab16918, Abcam).

Following antibodies were used: HRP conjugated anti-mouse IgG (Cell Signaling, 7076S) and HRP conjugated anti-rabbit IgG (Cell Signaling, 7074S) antibodies used for western blot while Alexa Flour 488- and 555- conjugated anti-chicken, anti-mouse, and anti-rabbit (Cell Signaling) antibodies were used for immunofluorescence. Alex Flour 488- conjugated anti-Streptavidin (S-32354, Invitrogen) antibody is used for microscopic analysis.

1.2.4. Membrane Enrichment

We used two different enrichment methods in this study.

1.2.4.1. Membrane enrichment by sucrose density gradient fractionation

Cells are cultured until they reach 80-90% confluency in the 150mm^2 plate. Then cells were harvested by scraping in ice-cold PBS and snap-frozen in liquid nitrogen. Cell pellet lysed by using Hypotonic Lysis Buffer (10mM HEPES pH 7.4, 1.5mM $MgCl_2$, 10mM NaCl, 1mM EDTA, and protease inhibitor) for 15 minutes on ice. Then cell lysates were homogenized by using Zirconium Oxide beads (0.5mm diameter, Next Advance) in Bullet Blender (BB24-AU, Next Advance) for 90 seconds at 4°C and then beads and pellets were discarded by subsequent centrifugation at 2000 rpm for 1 minute at 4°C.

For fractionation, we used two different concentrations of sucrose solution 50% and 10% sucrose solutions. To get fractions, the first ice-cold 2mL 50% sucrose solution, then 6 mL 10% sucrose solution was added to ultracentrifuge tubes. Then the homogenized sample was added to the tube. We used following conditions for the first centrifugation 25.000 rpm for 18 hours at 4°C.

Then we took the midzone between two gradients and resuspended this fraction in 8mL Extraction Buffer (0.1mM Na_2CO_3 and 1mM EDTA pH11.3). The pellet coming from the first fractionation contains high amount of nuclear protein, so we called the pellet as nuclear fraction. Then resuspended midzone was centrifuged again at 35.000 rpm for 90 minutes at 4°C. This pellet composed of membrane enriched proteins and further analysis was done by using this sample.

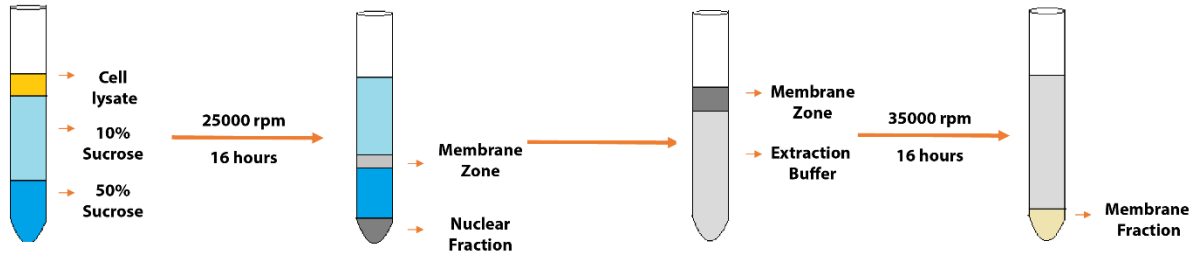

Figure 3 Schematic representation of the sucrose gradient density fractionation for plasma membrane analysis

1.2.4.2. Membrane enrichment by BioID

We used the BioID method to enrich both membrane fractionated samples and whole-cell lysates. The procedure is similar for both cases. Myrpalm BirA and BirA cell lines were biotinylated as explained above and following the BioID procedure was applied [28].

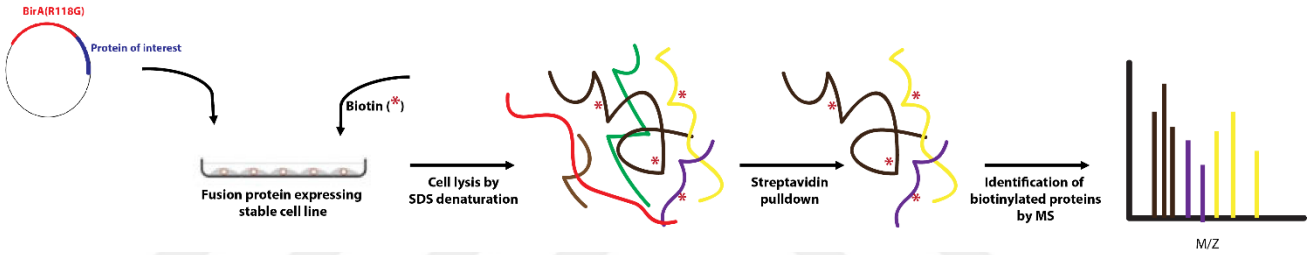

Figure 4 Representative scheme of proximity dependent biotin identification (BioID) protocol

Cell lysates and membrane enriched samples were used for streptavidin pulldown experiments. For that we lysed the sample with Lysis Buffer (0.5% SDS, 2% NP-40, 150mM NaCl, 1mM EDTA, 10mM 2- Chloroacetamide, 10mM Tris-Cl pH7.6 and protease inhibitor). Cell debris was removed by centrifugation at 7.500 rpm for 15 minutes at 4°C. Proteins were incubated with pre-equilibrated Streptavidin Plus UltraLink Resin (Thermo, 53117) for 16 hours at 4°C with rotation. Unbound proteins were removed by centrifugation at 1.000rpm for 5 minutes. Nonspecific bindings were removed by washing and centrifugation steps.

As wash buffers, we used four different buffers: Wash Buffer 1 (2% SDS), Wash Buffer 2 (50mM HEPES pH7.5, 2% sodium deoxycholate, 1% Triton-X, 50mM NaCl, 1mM EDTA), Wash Buffer 3 (10 mM Tris pH8.1, 1 mM EDTA, 500 mM NaCl, 1% Triton X, 0.5% sodium deoxycholate, 0.5% NP-40) and Wash Buffer 4 (50mM TrisCl pH7.4). After each wash beads were collected by 1000 rpm 5 minutes of centrifugation. Each wash step was applied three times.

For immunoblot analysis, after that point I boiled the beads with 100mM DTT containing Laemmli Sample Buffer for 10 minutes at 95°C.

For mass spectrometry analysis, we applied the on-bead digest protocol to the samples.

As control group we used empty BirA stably expressing HeLa S3 cell line. The same procedure was applied control group as well.

1.2.4.3. Membrane enrichment by combining sucrose density gradient fractionation and BioID

For this enrichment, we combined sucrose gradient fractionation and BioID technique. Cells cultured in biotin containing media. Cells were harvested, lysed and fractionated as it described in sucrose density gradient fractionation technique. After fractionation membrane fraction was incubated with streptavidin beads and isolation of biotinylated proteins were done according to BioID protocol described above.

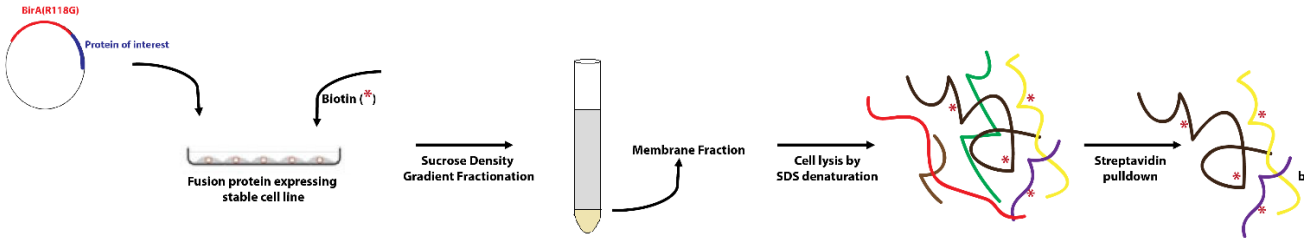

Figure 5 Schematic representation of our plasma membrane enrichment protocol for proteomics analysis.

1.2.5. Proteomics

1.2.5.1. On-bead Tryptic Digest

Streptavidin beads were washed with urea solution (8M urea in 0.1M TrisCl pH8.5). After urea washes beads were collected. After denaturing the proteins by high urea concentration, beads were incubated with 100 mM DTT containing urea solution for reducing the proteins for 30 minutes at 56°C and then the beads were collected by centrifugation. After that, the beads were washed with urea solution and chloroacetamide wash (50mM 2-Chloroacetamide in urea solution) for alkylation for 20 minutes at room temperature. Then the beads were washed with a urea solution again for 15 minutes. Then to remove urea, the beads were washed with Ammonium bicarbonate solution (50mM NH_4CO_3) twice for 15 minutes to

remove urea. Then the beads were incubated with trypsin (0.1µg/µl in 1mM HCl) for 16 hours at 37°C for digestion.

After tryptic digestion, peptides are separated from the beads. Therefore, we took the supernatant after centrifugation the beads at 1000 rpm for 5 minutes. Then the beads were washed with ammonium bicarbonate solution to get the remaining peptides on the beads. After centrifugation, the tryptic digest pool and ABC washing pool were combined. pH of the peptides was adjusted to pH 2-3 by using 10% formic acid. Peptides were concentrated by using Speed-vac (SPD1010, Thermo).

1.2.5.2. Desalting

Concentrated peptides contain lots of salts and ions to remove them we used the StageTip method. In this method, Empore Octadecyl C18 (66883-U, Empore Sigma) columns were placed into tip and conditioned by methanol and Stage Buffer A (0.5% formic acid in 5% acetonitrile). Peptides were dissolved in Stage Buffer A and sonicated at 4°C to be completely dissolved in the buffer. The sample is applied to columns and centrifuged at 2500 rpm for 10 minutes. Peptides bound to the column and washed with Stage Buffer A twice. After each wash we centrifuged the tips to remove flow through at 2500 rpm for 10 minutes. Then peptides were eluted by using Stage Buffer B (0.1% formic acid in 70% acetonitrile) and centrifuging at 3000 rpm for 15 minutes. Then peptides were concentrated by using speed-vac. Dried samples were stored at -20°C for mass spectrometry analysis.

1.2.5.3. Data acquisition

Dried samples were eluted in LC Buffer (5% formic acid in 5% acetonitrile). Peptides were analyzed by online C18 nanoflow reversed-phase nano liquid chromatography (Dionex Ultimate 3000 RS nLC, Thermo Scientific) combined with orbitrap mass spectrometer (Q Exactive Orbitrap, Thermo Scientific. Samples were separated in an in-house packed 75 µm i.d. × 25 cm C18 column (Reprosil-Gold C18, 3 µm, 200 Å, Dr. Maisch) using 70 minute linear gradients from 5 to 40% acetonitrile in 0.1% formic acid with 300 nL/min flow in 90 minutes total run time. The scan sequence began with an MS1 spectrum (Orbitrap analysis; resolution 70,000; mass range 400–1,500 *m*/*z*; automatic gain control (AGC) target 3e6; maximum injection time 60 ms). Up to 15 of the most intense ions per cycle were fragmented and analyzed in the orbitrap with Data Dependent Acquisition (DDA). MS2 analysis consisted of collision-induced dissociation (higher-

energy collisional dissociation (HCD)) (resolution 17,500; AGC 1e6; normalized collision energy (NCE) 26; maximum injection time 100 ms). The isolation window for MS/MS was 2.0 *m*/*z*.

1.2.5.4. Data processing

Raw files were processed with Thermo Proteome Discoverer version 1.4. Carbamidomethylation of cysteine was used as fixed modification and acetylation (protein N-termini) and oxidation of methionine were used as variable modifications. Maximal two missed cleavages were allowed for the tryptic peptides. The precursor mass tolerance was set to 10 ppm and both peptide and protein false discovery rates (FDRs) were set to 0.01. The other parameters were used with default settings. The database search was performed against the human Uniprot database (release 2016)[29]. Protein groups that are reported as contaminant or identified by only one modification site or having more than half of its peptides as reverse hits, were removed from Thermo Proteome Discoverer results.

1.2.5.5. Data analysis

Significantly enriched proteins were detected by using SAINTexpress software 3.6.3. version[30]. Identified proteins were analyzed against their corresponding controls. Proteins with higher than 0.5 SAINT score was taken also Bayesian False Discovery Rate (BFDR) value was lower than 0.05.

Cellular localizations of significant proteins were obtained from Gene Ontology terms of Uniprot database. GO analysis were performed by using g: Profiler Gene Ontology web server and Top 10 hits were presented[31].

1.3. RESULTS

1.3.1. Generation of Stable Cell Line

To check the activity and integrity of BioID constructs, first HEK293T cells were transfected with pcDNA3.1 Myrpalm-BioID (R118G)-HA construct. Then cells were fixed and stained against BirA antibody to examine the location of the Myrpalm-BioID construct by microscopy (**Figure 6**).

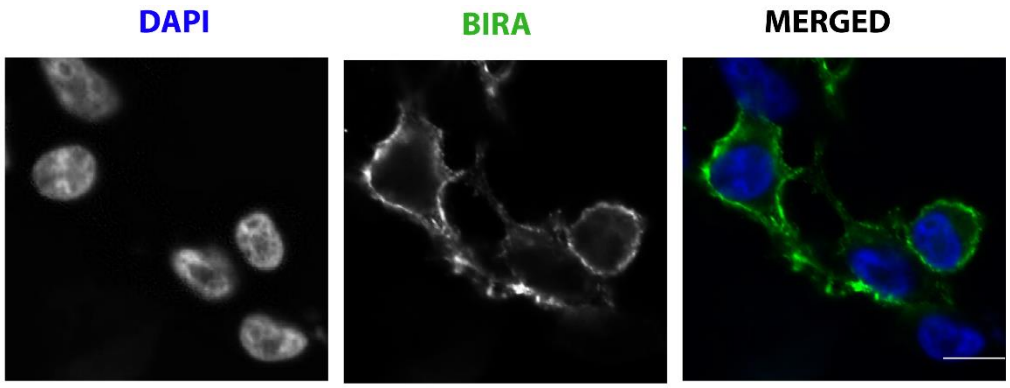

Figure 6 Myrpalm-BioID plasmid translocates into the plasma membrane in HEK293T cells.

HEK293T cells transiently transfected with pcDNA3.1 Myrpalm-BioID construct by using PEI. Cells were stained against BirA antibody (green) and DAPI (blue). (Scale bar 10µm)

Figure 6 shows the localization and enrichment of the Myrpalm-BirA enzyme on the cell membrane. After confirming the membrane localization of the Myrpalm-BioID construct, I cloned BirA-HA and Myrpalm-BirA-HA sequences into lentiviral plasmid backbones (pLenti CMV-hygro DEST Addgene #36047) by using Gateway cloning techniques. Plasmid sequences were verified by sequencing **(Appendix)**.

I generated HeLa S3 cell lines stably expressing BirA and Myrpalm-BirA and verified the BirA enzyme localization in those cells (**Figure 7**) by immunofluorescence. These results verify that the plasmids are intact and BirA enzyme can translocate itself on the plasma membrane in HeLa S3 cells.

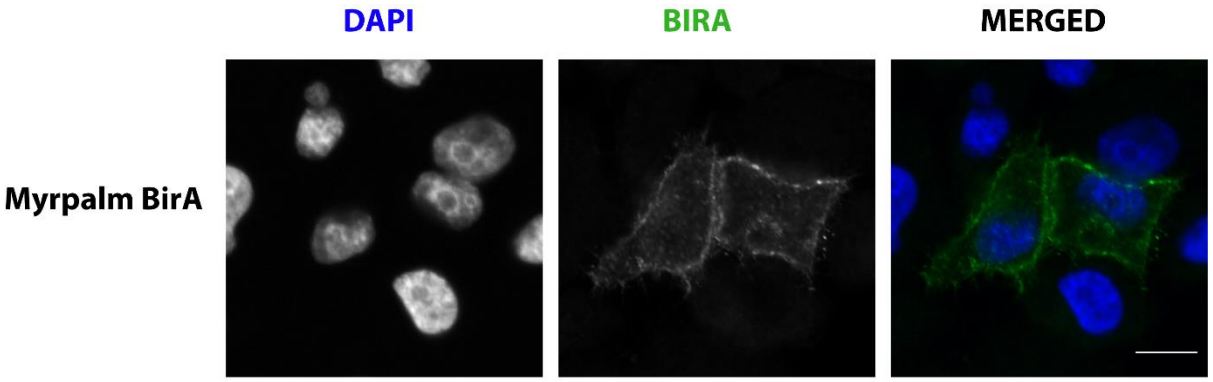

Figure 7 pLenti-Myrpalm-BirA plasmid localization in HeLa S3 cell line.

pLenti-Myrpalm-BirA plasmid expression is verified in HeLa S3 cell line. Transduced cells express the enzyme on their cell membranes. Cells were stained against BirA antibody (green) and DAPI (blue) (Scale bar 10µm).

1.3.1.1. Characterization of Single Colonies

BirA staining verified enzyme localization on the plasma membrane. To asses enzymatic activity, cells were treated with biotin and ATP. **Figure 8** shows the biotinylation activity of the populations. Some mitochondrial proteins biotinylated physiologically biotinylated inside the cell regardless of excess biotin and ATP presence. These proteins were used as a loading control for the experiment. When HeLa S3 cells treated with biotin, biotinylation profile of the cell did not change. Therefore, biotin treatment does not lead to unspecific biotinylation by itself. Biotinylation patterns of BirA and Myrpalm BirA show that BirA enzyme does not cause biotinylation alone as well. Therefore, BirA dependent biotinylation occurs when the enzyme is present with biotin and ATP.

BirA and Myrpalm BirA enzymes showed increased biotinylation bands compared to the control group. However, the enzyme was showing decreased activity than our expectations due to low transduction efficiency and heterogeneity of the population. Therefore, I generated single-cell clones and expand them to obtain a homogenous population. Single colonies were screened by immunofluorescence and we decided to continue with BirA-colony1 and Myrpalm-BirA-colony2 for further experiments. Single colonies were analyzed under microscopy to see cellular localization of the biotinylated proteins inside the cell (**Figure 9)**.

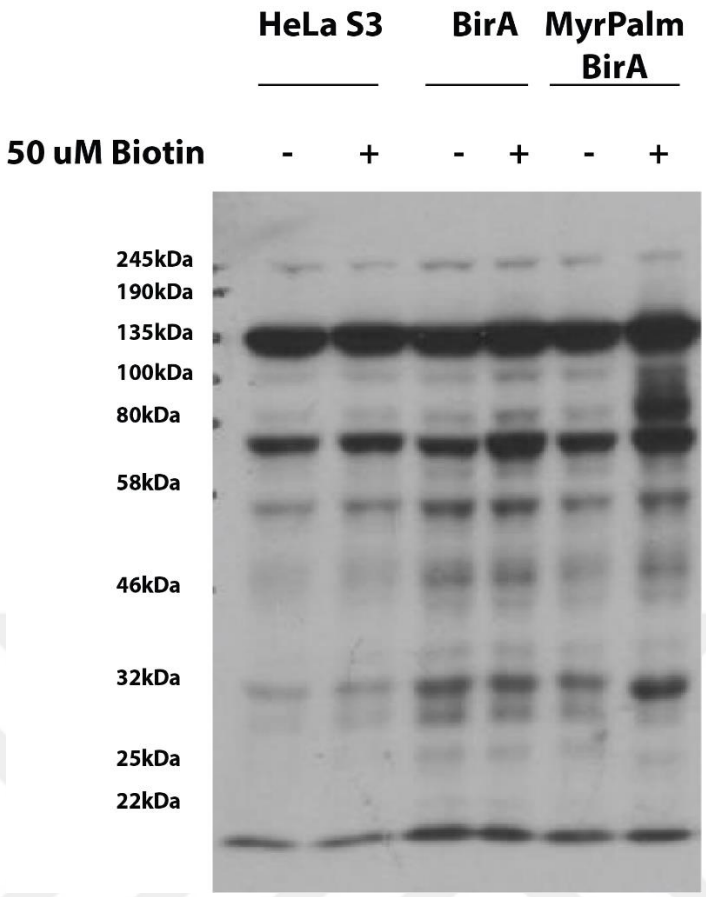

Figure 8 Western Blot analysis of heterogenous HeLa S3 populations.

Biotinylation efficiencies of heterogenous HeLa S3 cells stably expressing BirA or Myrpalm-BirA is analyzed by Western Blot analysis. Nontreated HeLa S3 cells were used as the control group. Increased enzymatic activity is observed in the presence of Biotin and ATP.

Internally biotinylated cells were visualized in control HeLa S3 cells. Biotinylated proteins are mostly cytosolic and mitochondrial proteins. In cytosolic BirA expressing HeLa S3 cells (BirA-c1), levels of biotinylated proteins increased, and they are distributed inside cells without a specific enrichment. On the other hand, Myrpalm-BirA-c2 cells showed a distinct biotinylation profile compared to others. Biotinylation reaction occurred specifically on cell membrane and plasma membrane proteins are selectively biotinylated. Cytosolic biotinylation is significantly lower than control group and BirA-c1 cells.

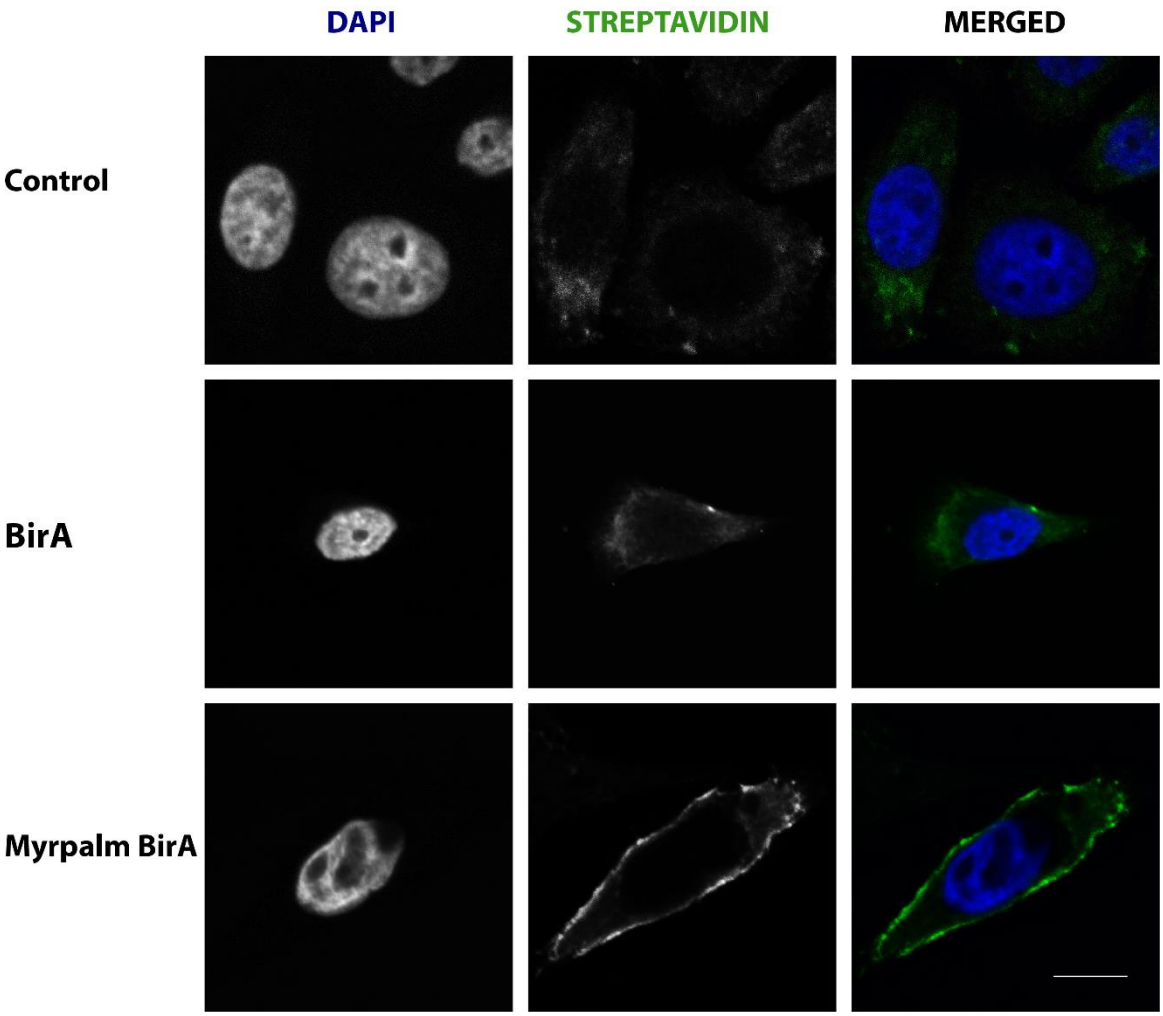

Figure 9 Enzymatic activity of the BirA and Myrpalm-BirA expressing HeLa S3 cells were analyzed under fluorescence microscopy.

Cells were fixed and stained against streptavidin (green) and DAPI (blue). Scale bar (10µm)

Biotinylation profile of the Myrpalm-BirA-c2 is assessed by western blotting analysis (**Figure 10**). When compared to the heterogeneous Myrpalm-BirA population and HeLa S3 control group, Myrpalm-BirA-c2 showed significantly higher biotinylation activity. As expected, biotinylation activity is seen only in the presence of biotin and ATP.

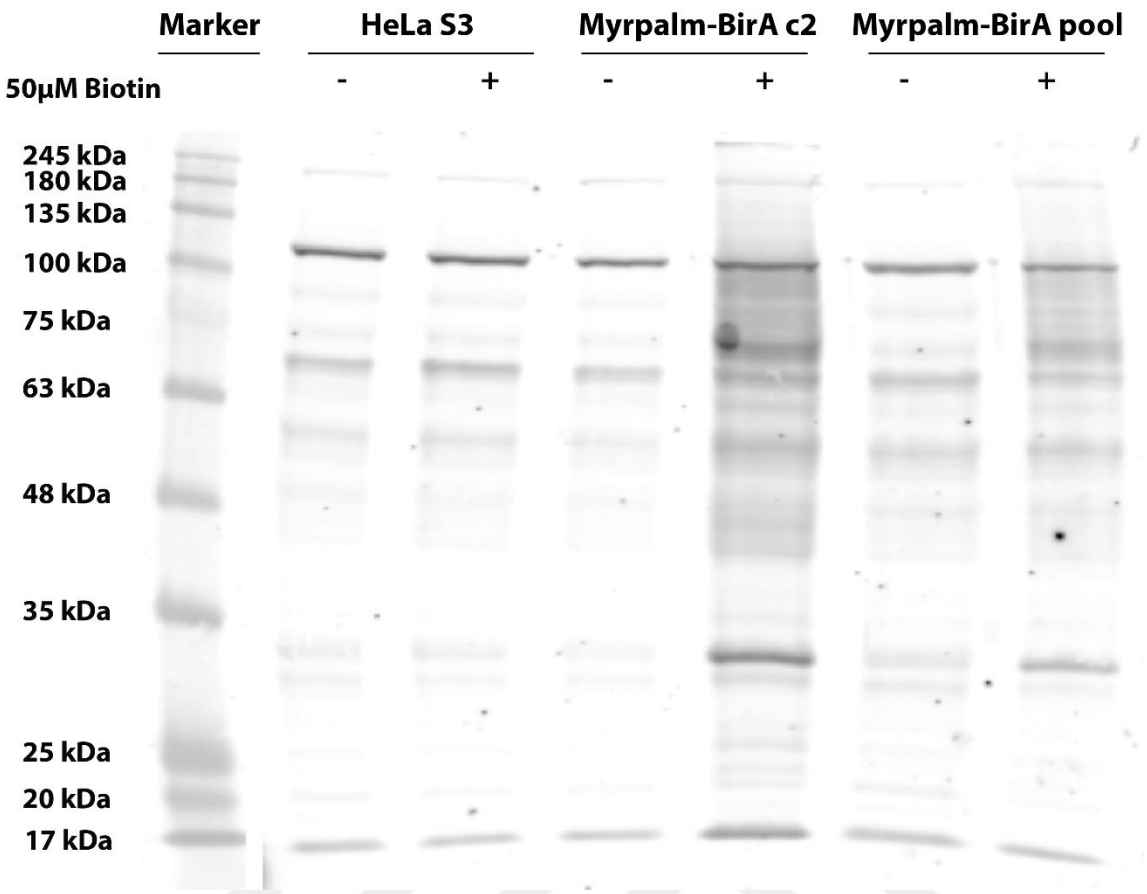

Figure 10 Western Blot analysis showing the biotinylation efficiency of Myrpalm-BirA-colony-2.

Single colonies were analyzed by using biotin antibody. Immunoblotting analysis against biotin antibody to show the enzymatic activity of Myrpalm-BirA cell lines.

1.3.2. Membrane Enrichment by Sucrose Density Gradient Centrifugation

To increase membrane fraction, I used a centrifugation-based membrane enrichment method optimized by Dr. Zeynep Sabahat Yunt. According to this method, we used 50% and 10% sucrose solution to create a density gradient, then cell lysates were centrifuged at 25,000 rpm for 16 hours at 4°C. After centrifugation cell lysate separated into two fractions one of them is the pellet which contains high amount of nuclear proteins which we called **Nuclear Fraction (NF)** and the other one is the soluble fraction. The latter is precipitated by second centrifugation at 35,000 rpm for 90 minutes at 4°C. We will refer to this second pellet as **Membrane Fraction (MF)**.

To show the membrane enrichment efficiency of the procedure, I did an immunoblotting analysis against markers from different fractions (**Figure 11**). We used a membrane receptor

protein, EGFR, as a membrane marker; and a nuclear lamina protein, Lamin B1, as a nuclear marker.

Band intensities are normalized to control groups and then I did quantification by comparing these intensities to their actin bands. This analysis showed that our centrifugation-based method increases the membrane protein levels 375% while nuclear protein levels decrease by up to 90%. On the other hand, in the nuclear fraction, we get a two-fold increase in membrane proteins, yet there is around 50% increase in nuclear proteins as well. Since using this fraction would bring nuclear protein contamination, further experiments were performed by using Membrane Fraction.

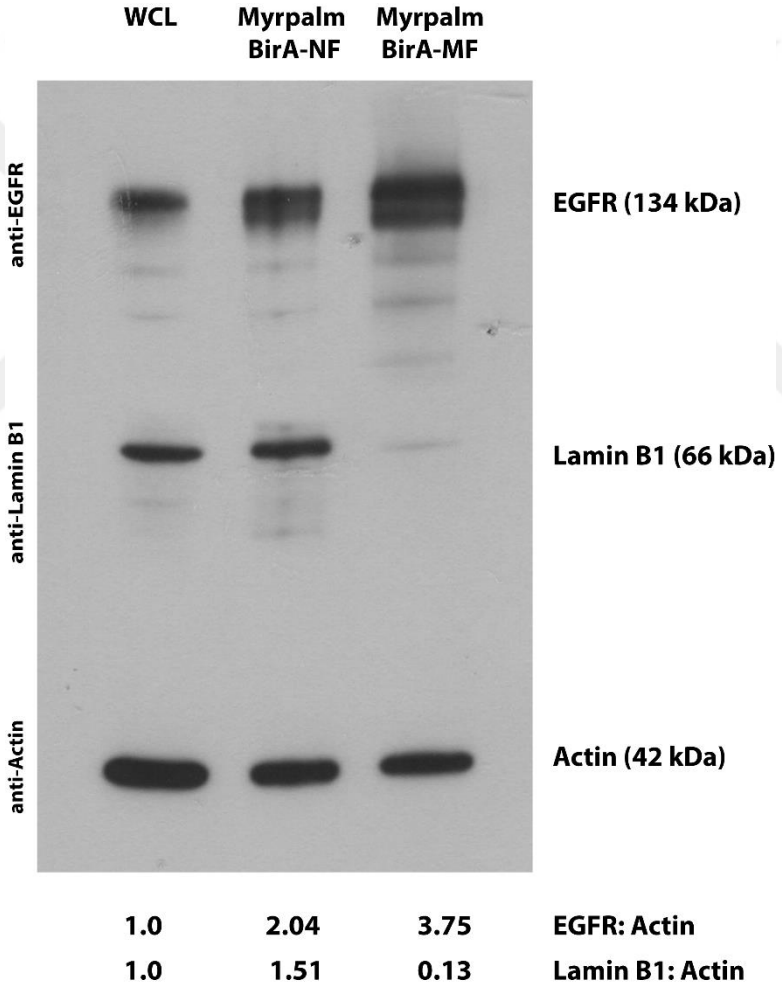

Figure 11 Western Blot analysis showing the membrane enrichment efficiency by centrifugation.

HeLa S3 fractionation by density gradient ultracentrifugation to show fractionation efficiency. (WCL: whole cell lysate, NF: Nuclear Fraction, MF: Membrane Fraction)

1.3.3. Membrane enrichment by BioID

Several biological and chemical reactions take place simultaneously on the cell periphery. This leads to cell membrane proteins to build dynamic and transient interactions with other cell periphery proteins. As we see in **Figure 9**, BirA and Myrpalm-BirA enzymes biotinylate proteins in the cell and **Figure 10** shows that Myrpalm-BirA enzymatic activity. We aimed to isolate these biotinylated proteins by using their affinity towards streptavidin resin.

To identify cell membrane proteins, BirA and Myrpalm-BirA expressing cell lines were cultured with biotin and ATP. To isolate biotinylated proteins, we used the streptavidin pulldown method. Subsequently, proteins were separated from the beads by on bead tryptic digestion. Then digested proteins were analyzed by LC-MS/MS and the identified proteins were listed.

The number of identified proteins were analyzed and categorized into different groups by using g:Profiler Gene Ontology (GO) analysis[31]. GO analysis categorizes proteins according to their predefined functions, processes, and locations. For this experiment, we used GO Cellular Compartment analysis to group proteins according to their subcellular locations (**Figure 12**).

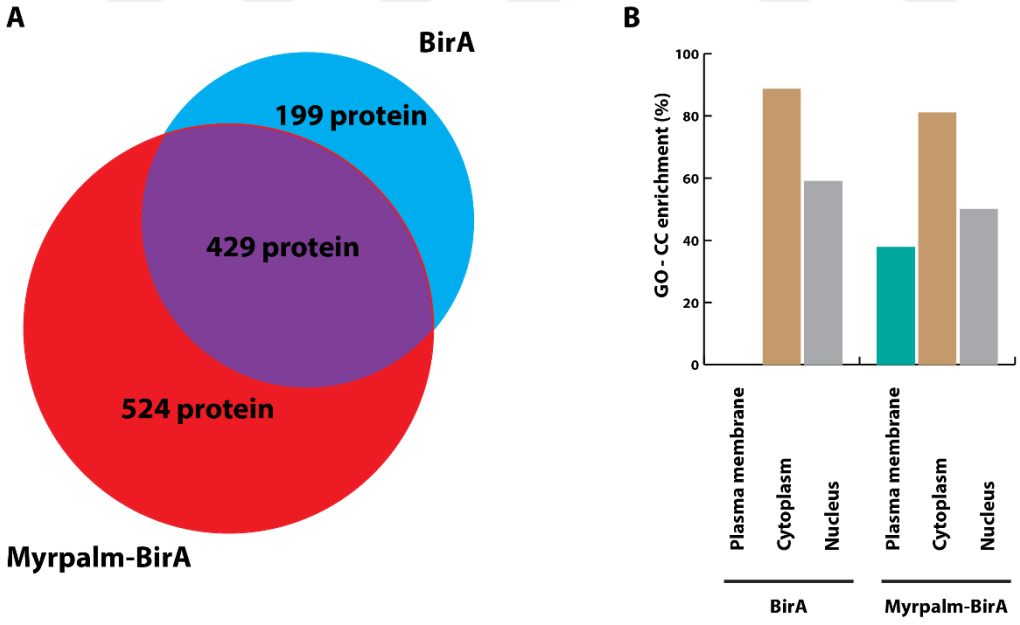

Figure 12 The number of proteins identified and cellular locations of BioID analysis of BirA and Myrpalm-BirA cell line.

A- A comparison of identified proteins in Myrpalm BirA and BirA BioID analysis is shown as a Venn diagram. The number in each zone indicates the number of

identified proteins for that specific zone. Identified plasma membrane proteins are given as percentages. Common proteins are shown in intersection. BirA (green), Myrpalm BirA (purple).

B- Main subcellular localization of identified proteins according to g:Profiler Gene Ontology Cellular Compartment analysis. Plasma membrane (blue), cytoplasm (brown), nucleus (gray).

Cytosolic BirA expressing cell line was used as a control group. 630 proteins were identified in BirA BioID analysis. According to g: Profiler Gene Ontology Cellular Compartment (GO-CC) analysis most of the identified proteins are related to cytoplasm and nucleus. 88.6% (538 protein) were cytoplasm related proteins while 59% (363 protein) of all identified proteins are related to the nucleus. However only 0.8% (5 protein) of all identified proteins are recognized as plasma membrane protein.

The same analysis was applied to the Myrpalm-BirA cell line. MS analysis revealed that 953 proteins were identified. Our BioID results suggested that 37.7% (360 protein) of all identified proteins were categorized as plasma membrane proteins. The same analysis revealed that 81% (774 protein) of the proteins were related to the cytoplasm and 50% (477 protein) of identified proteins were listed as nucleus.

1.3.4. Membrane enrichment by a combinatorial method

To improve membrane protein analysis, we used both sucrose gradient ultracentrifugation and BioID methods sequentially. To apply this method, we first expand BirA and Myrpalm BirA colonies. Then we used centrifugation to fractionate the sample and took the membrane fraction of each sample. Then we used the BioID method to isolate biotinylated proteins from the enriched membrane fractions. Isolated proteins were digested and then analyzed by LC-MS/MS. Identified peptides were categorized by using GO annotation (**Figure 13**).

BirA cell line was used as a control group for the analysis. Only 71 proteins were identified in this analysis. According to GO-CC analysis, 67.1% of the identified proteins are clustered into extracellular exosome compartment with 47 identified proteins. Nucleus and cytosolic proteins comprise 58.6% and 55.7% of the identified proteins respectively. According to GO-CC analysis we did not identify any plasma membrane proteins.

On the other hand, Myrpalm BirA proteomics analysis gave 348 proteins. g:Profiler GO-CC analysis revealed that 60% of the identified proteins were plasma membrane proteins with 173 proteins. Plasma membrane proteins constitute the most abundant group in the analysis. Cytoplasmic proteins form 70% of the total identified proteins.

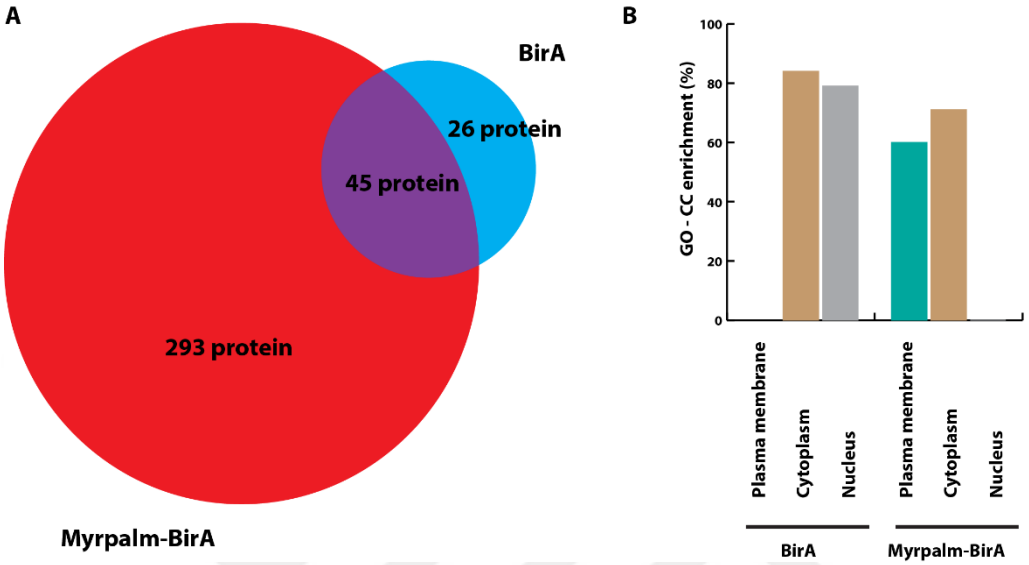

Figure 13 Identified proteins obtained from LC-MS/MS analysis after using density gradient ultracentrifugation and BioID

A- A comparison of identified proteins in BioID analysis of membrane fractions of Myrpalm BirA and BirA cell lines is shown as the Venn diagram. Number in each zone indicates number of identified proteins for that specific zone. Identified plasma membrane proteins are given as percentages. Common proteins are shown in intersection. BirA (green), Myrpalm BirA (purple).

B- Main subcellular localization of identified proteins according to g:Profiler Gene Ontology Cellular Compartment analysis. Plasma membrane (blue), cytoplasm (brown), nucleus (gray).

GO-CC analysis was also applied to the common proteins identified in both Myrpalm BirA and BirA proteomics results. Most of these common proteins were comprised of the extracellular exosome, nuclear and cytosolic proteins respectively.

We identified three palmitoyltransferase enzymes that are required for translocation of Myrpalm-BirA enzyme into plasma membrane when BioID and ultracentrifugation used together. These proteins were not detected when only BioID method is applied.

1.3.5. Comparison of the methods

We compared the enrichment efficiencies of two methods by comparing Myrpalm-BirA proteomics results. A powerful BioID based analysis depends on subtracting background proteins which are unspecific biotinylated proteins, from the protein list. Therefore, to find significantly enriched biotinylated proteins in Myrpalm-BirA cells, we applied SAINT express scoring tool[30]. As a control, we used biotin untreated Myrpalm-BirA cells and applied same experimental procedure. SAINTexpress scores each protein according to PSM numbers and protein size then compares the scores of experimental groups with control group. If a protein has a significantly high SAINTexpress score than this protein is significantly enriched compared to control.

Our BioID analysis constitutes from two biological replicates. To validate the reproducibility of the method, we calculated the Pearson correlation of the biological replicates. The analysis showed high correlation of the protein intensities with 0.8. A high Pearson correlation suggests reproducibility of the data (**Figure 14**).

SAINTexpress significance test revealed that we have 165 and 188 significantly enriched proteins in two biological replicates of Myrpalm BirA BioID analysis. We categorized these proteins according to their subcellular localization. The most abundant subgroup is plasma membrane proteins and they constitute around 75% and 50% of the total proteins in each biological replicate respectively.

We also applied the same significance analysis to Myrpalm-BirA-MF-BioID, and the results revealed that 193 and 308 proteins are significantly enriched in the two biological replicates. Subcellular compartment analysis showed that plasma membrane proteins comprised the most abundant category with 77% and 60%.

Table 2 presents the subcellular localization of significant proteins and their abundances.

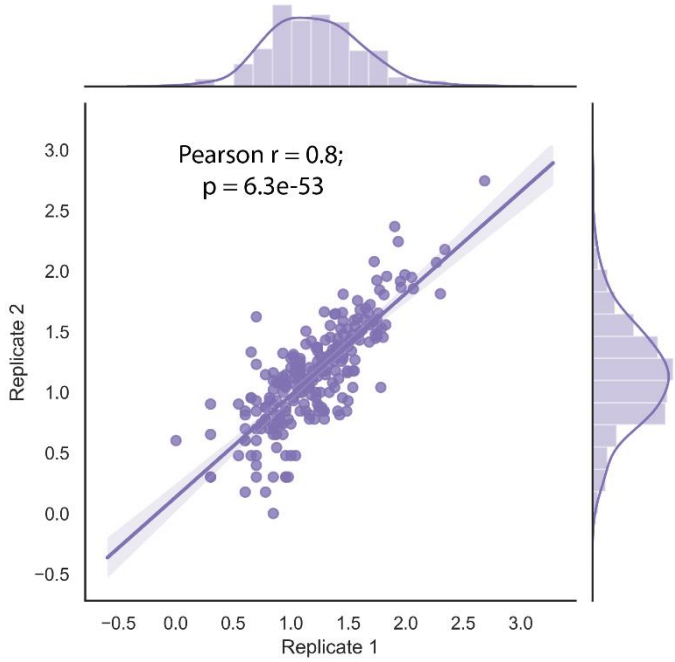

Figure 14 Intensity comparison of the identified proteins in biological replicates.

Pearson correlation of the BioID analysis of membrane fraction of Myrpalm-BirA cell line. (R^2 = 0.8, p = 6.3e-53)

Table 2 Subcellular localization of the significantly enriched proteins

	Myrpalm-BirA-BioID		Myrpalm-BirA-MF-BioID	
	Replicate-1	Replicate-2	Replicate-1	Replicate-2
Plasma Membrane	74.5 %	51.2 %	77.2 %	60.7 %
Membrane	12.7 %	29 %	11.9 %	27.2 %
Cytoplasm	4.8 %	5.4 %	4.1 %	4.2 %
Nucleus	3.1 %	7.5 %	1 %	2.3 %
Others	4.9 %	5.9 %	5.8 %	5.6 %

Then to increase the significance, we pooled together the replicates and analyzed all identified proteins together in SAINTexpress. We obtained 99 significant proteins in Myrpalm-BirA-BioID and 177 proteins in Myrpalm-BirA-MF-BioID (**Figure 15**).

A

	Myrpalm-BirA-BioID	Myrpalm-BirA-MF-BioID
Plasma Membrane	83.8 %	81.4 %
Membrane	9.1 %	11.9 %
Cytoplasm	2 %	2.8 %
Nucleus	4 %	0.6 %
Others	1.1 %	3.3 %
Total	100 %	100 %

B

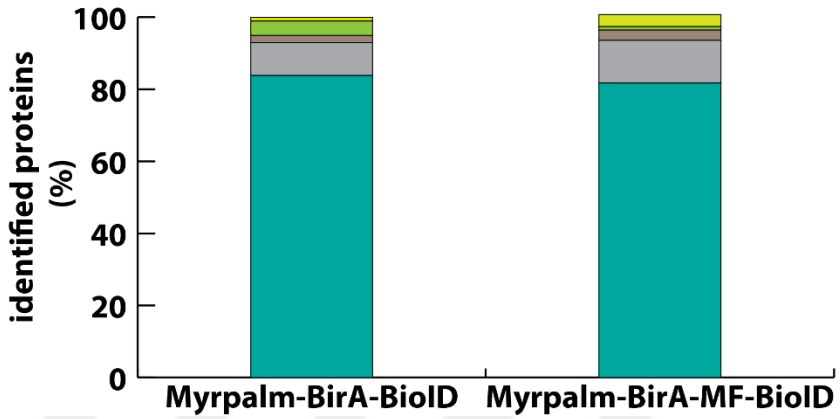

Figure 15 Comparison of significantly enriched proteins of BioID and Centrifuge & BioID methods

A- All identified proteins from two biological replicates are pooled together and the SAINTexpress significance test was applied. The number of significantly enriched proteins and their subcellular localization are presented.

B- Abundances of subcellular localizations were plotted as a bar graph.

Then to gain more insight about the significantly enriched proteins, we applied GO-CC analysis by using g: Profiler database[31]. Top hits for both methods are listed below.

According to GO-CC analysis, most of the top hits constitutes of cell membrane related groups such as plasma membrane, cell periphery, extracellular vesicles, and junctions. As it can be seen from **Figure 16**, membrane fractionated samples showed higher significance values compared to BioID. In both methods, plasma membrane, plasma membrane part and cell periphery categories are the most significantly enriched proteins. Although extracellular compartments such as extracellular exosome, extracellular vesicle, and extracellular organelle subgroups are enriched in both methods they are more enriched in the membrane

fractionated samples. Similarly, the integral component of plasma membrane and intrinsic component of plasma membrane proteins more enriched in membrane fractionated samples.

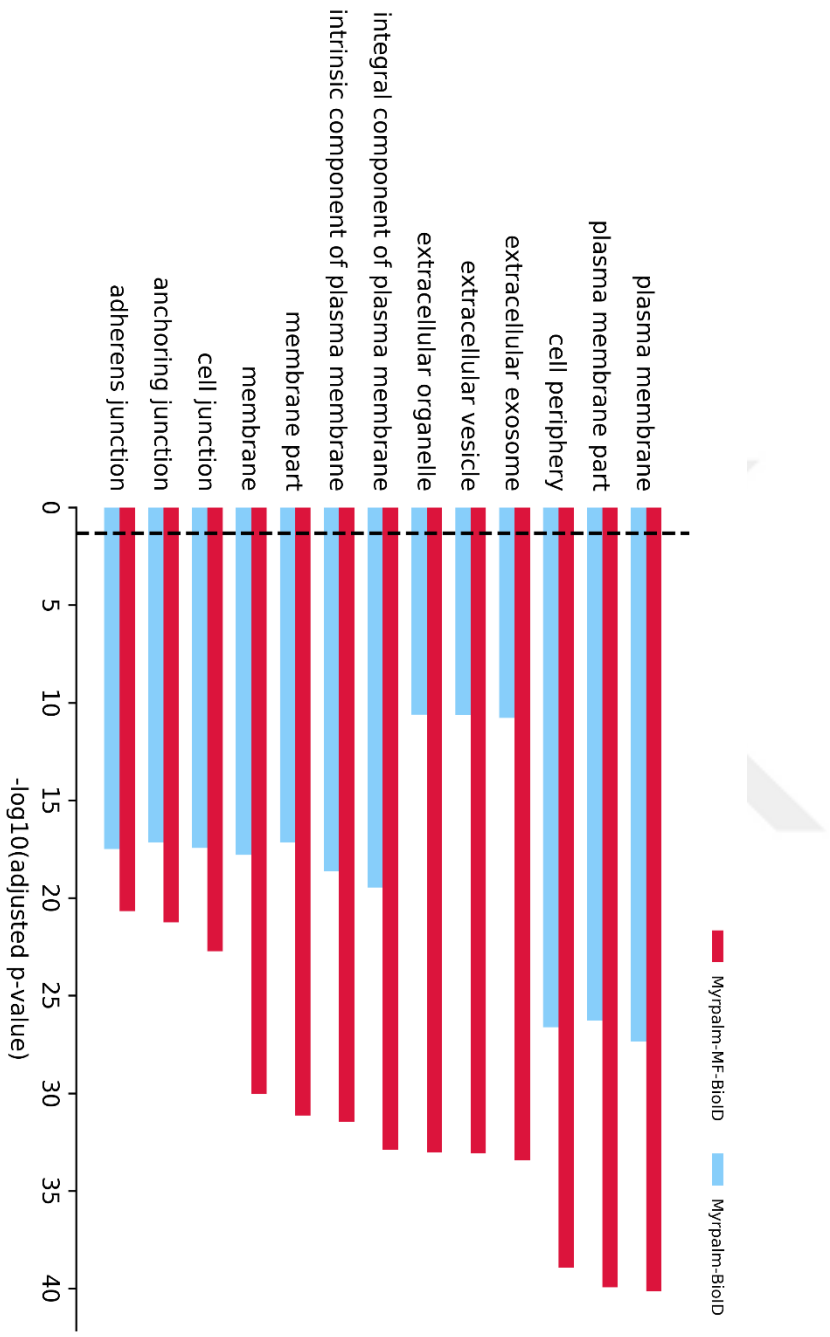

Figure 16 Gene Ontology Cellular Compartment analysis of significantly enriched proteins of Myrpalm-BirA-BioID (green), and Myrpalm-BirA-MF-BioID (yellow).

($p < 0.05$)

We applied g:Profiler Gene Ontology Molecular Function (GO-MF) and Biological Process (GO-BP) analysis to gain more insight about identified plasma membrane proteins. Top 10 significant hits for GO-MF (**Figure 17**) and GO-BP (**Figure 18**) are presented below respectively.

These analysis reveals that most of the significantly enriched plasma membrane proteins in our analysis are related with transmembrane transporter activity and cell adhesion activity. Additionally, these plasma membrane proteins are involved in biological processes such as localization, nutrient and ion exchange and movement of subcellular compartments. Most of these important cellular reactions occur by the activity of the integral membrane. Therefore, we used SOSUI secondary structure prediction database to find transmembrane domain containing proteins in our protein list [32]. According to that database 66% of the significantly enriched proteins contain transmembrane domain when centrifugation and BioID used together (**Figure 19**).

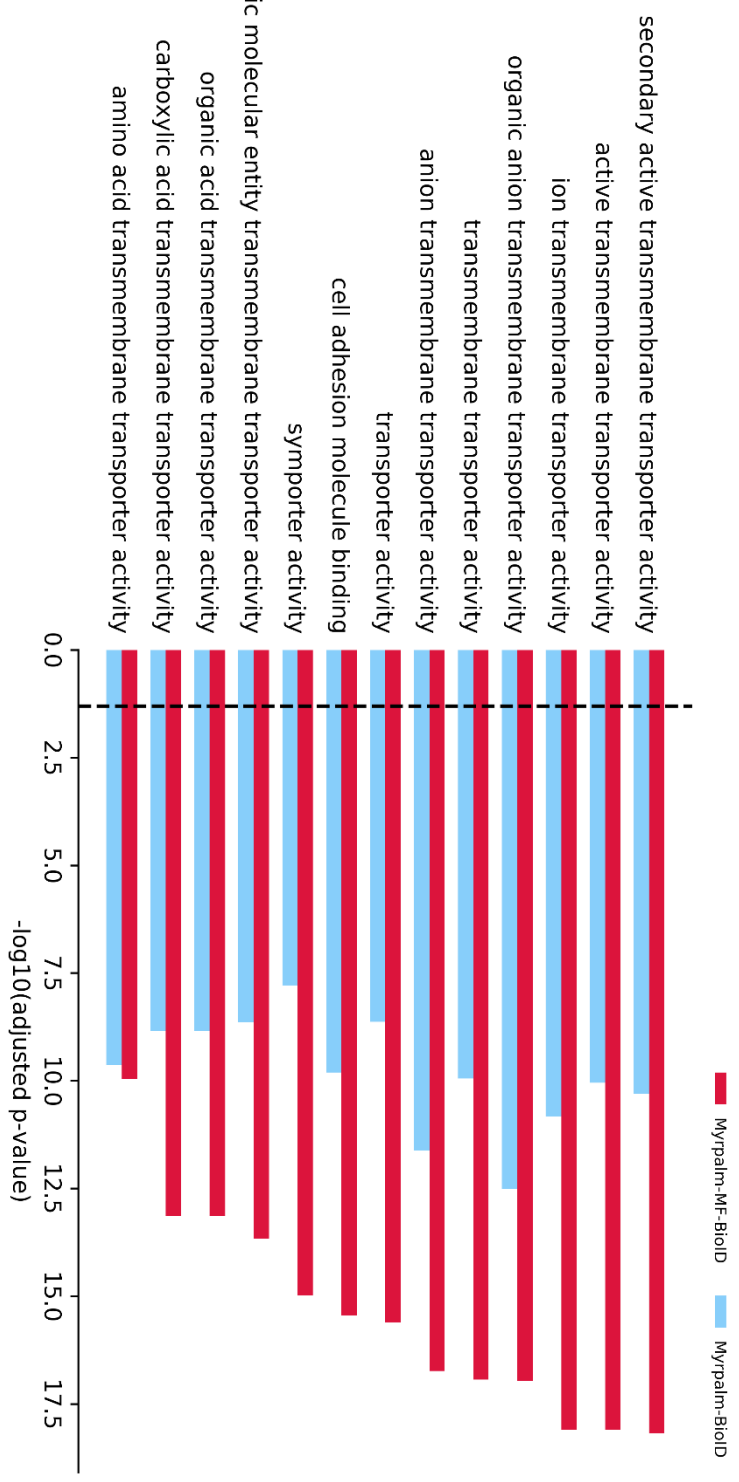

Figure 17 Gene Ontology Molecular Function analysis of significantly enriched proteins in Myrpalm-BirA-BioID (green), and Myrpalm-BirA-MF-BioID (yellow) analysis.

($p < 0.05$)

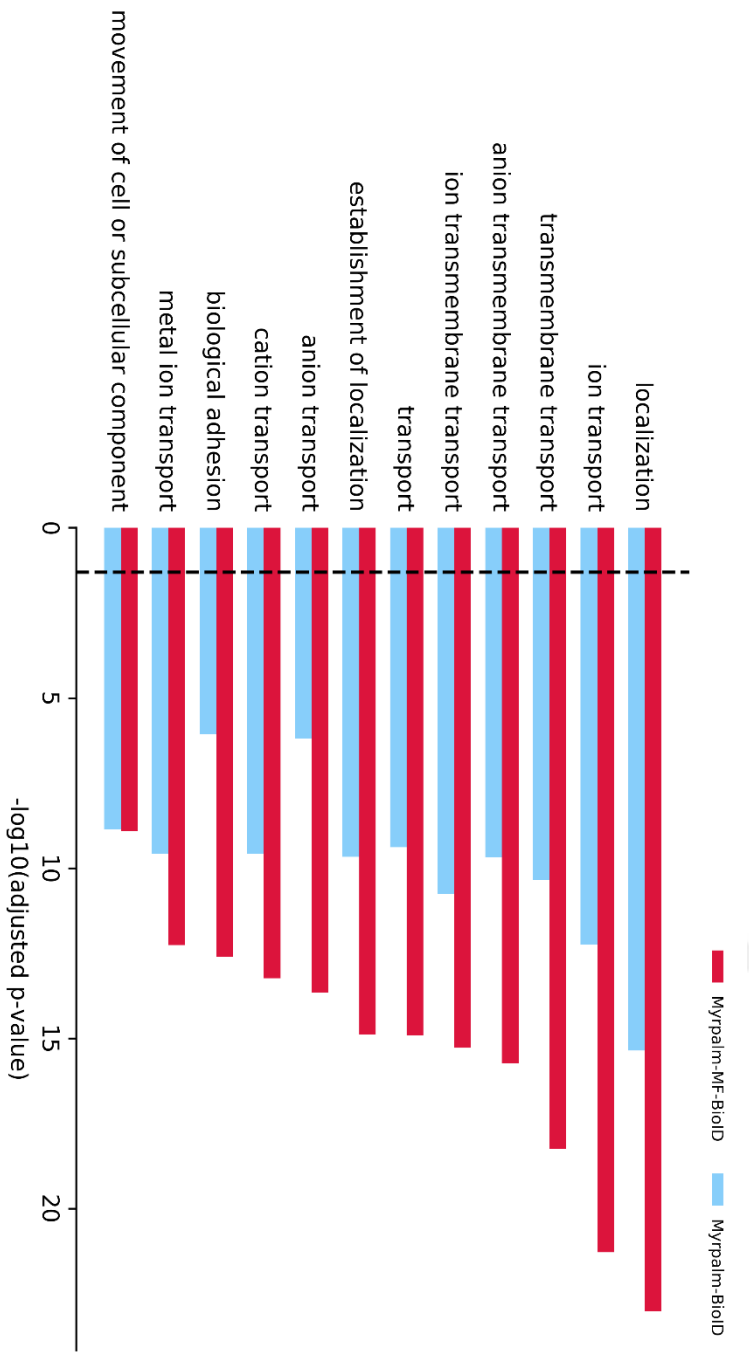

Figure 18 Gene Ontology Biological Processes analysis of significantly enriched proteins in Myrpalm-BirA-BioID (green), and Myrpalm-BirA-MF-BioID (yellow) analysis.

($p < 0.05$)

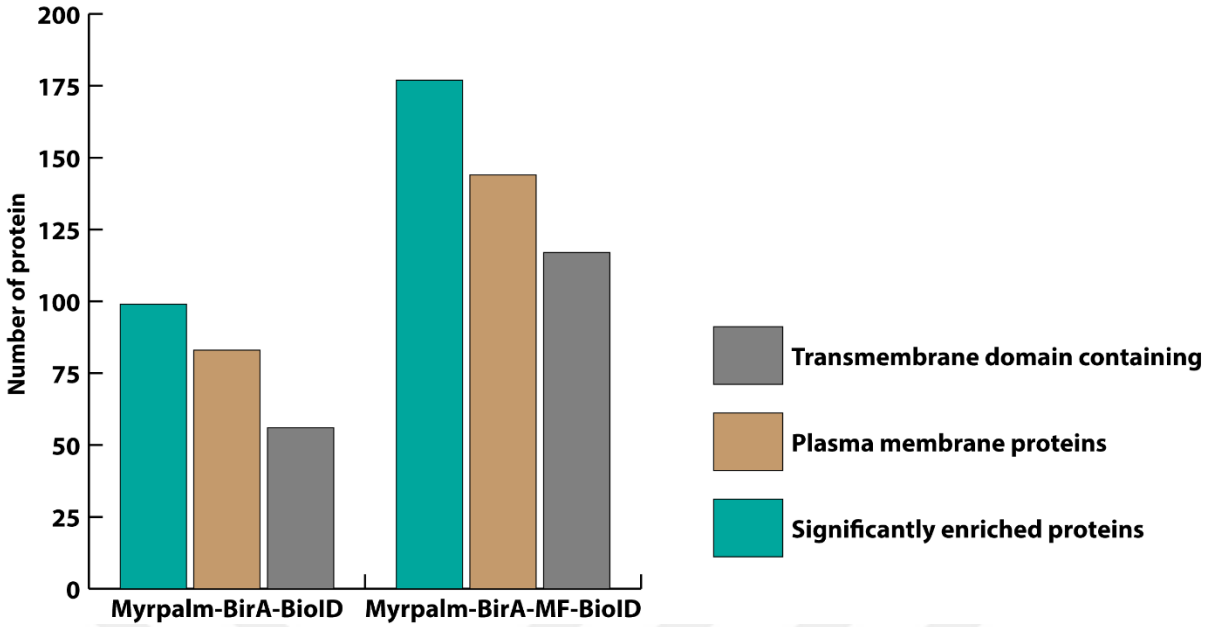

Figure 19 Comparison of structural analysis of significantly enriched proteins

Significantly enriched proteins, plasma membrane proteins and transmembrane domain containing proteins are given. Plasma membrane proteins are identified by GO annotation and transmembrane domain containing proteins are identified by using SOSUI database.

1.4. DISCUSSION

In this study, we aimed to develop an alternative plasma membrane protein enrichment method to obtain comprehensive cell membrane proteomics analysis. Traditional membrane enrichment methods rely heavily on density gradient ultracentrifugation methods. Centrifuge based methods can increase membrane protein levels up to 3-fold [23]. Yet low abundancy and hydrophobicity of the plasma membrane proteins still cause underrepresentation of cell membrane proteins. To elucidate molecular mechanisms of plasma membrane proteins, we need more efficient enrichment methods.

Our method provides two-layered enrichment by using sucrose gradient density fractionation and biotinylation. Centrifugation isolates molecules according to their sizes and densities, while biotinylation provides additional specificity. We generated a stable cell line expressing a myristoylated and palmitoylated version of the biotin ligase enzyme BirA, for biotinylation of cell membrane specific proteins. Microscopic analysis revealed plasma membrane localization of our construct. Functional analysis showed increased biotinylation activity in the presence of biotin and ATP. On the other hand, cytosolic BirA enzyme showed decreased biotinylation activity and localized all over the cytosol.

Based on our preliminary data, we used two-step centrifugation for membrane enrichment. In the first step, we used 10% and 50% sucrose gradient to fractionate the cell lysate and in the second centrifuge, we extracted and pelleted the proteins from the suspension of the first zonal centrifugation. Although we obtained enriched plasma membrane proteins in both pellets, enrichment efficiency of the second centrifuge is much higher with 3.75-fold membrane protein enrichment. Additionally by using two-step fractionation, we can remove any background caused by nuclear proteins (**Figure 11**).

To show biotinylation based membrane enrichment efficiency, we applied BioID analysis to Myrpalm-BirA and BirA cell lines. We categorized identified proteins according to their subcellular localization and our results show that 0.8% of identified proteins are plasma membrane proteins in control group while 38.8% of identified proteins were classified as plasma membrane proteins in Myrpalm-BirA BioID analysis. The increase in plasma membrane proteins suggests that BioID method is applicable for identification of plasma

membrane proteins, if the enzyme is targeted to specifically plasma membrane. Additionally, myristoylation and palmitoylation can recruit the enzyme to the plasma membrane without tampering its activity.

After the optimization of centrifugation and BioID methods, we then combined these two methods to develop a more efficient plasma membrane enrichment method. For this two-stepped enrichment method, stable cell lines generated, and the cells were cultured in biotin containing medium. As the first layer of the enrichment plasma membrane proteins were enriched by centrifugation. Then biotinylated proteins were purified from the fractionated sample by affinity-based pulldown techniques. Proteins were digested and analyzed by mass spectrometry. Our proteomics results showed that there was no enrichment of plasma membrane proteins in BirA cell line, while about 60% (209 of 348) identified proteins were plasma membrane in Myrpalm BirA cell line. Using multiple isolation techniques causes decrease in identified protein number yet desired proteins enriched in the analysis. However, the cell surface localization of Myrpalm-BirA enzyme and subsequent biotinylation of plasma membrane proteins lead to increase in both identified protein number and plasma membrane proteins ratio compared to cytosolic BirA.

A comparison of different techniques revealed the efficiency of our approach. We compared significantly enriched proteins of Myrpalm BirA cell line in BioID and our combined method. 99 proteins were significantly enriched in BioID analysis while 177 significantly enriched proteins were identified when centrifuge and BioID used together. Subcellular localization analysis revealed that most of the significantly enriched proteins are plasma membrane related proteins with 83% for BioID and 81% for centrifuge and BioID. Although membrane enrichment ratios are quite similar, there is an about 78% increase in significantly enriched proteins with our combined approach.

These enrichments efficiencies are quite high compared to literature. Recently, Joost et al. (2019) suggested a protocol for the identification of brain tissue samples and 31.7% (369 of 1161 protein) of total identified protein were plasma membrane related in their study [33]. In another study, Bernfur et al. (2013) identified 188 integral membrane proteins in 703 identified proteins (26.7%) [34]. Also, recently Ozkan Kucuk et al. (2017) proposed a carboxyl group labeling based plasma membrane identification method and they reported that they identified 179 plasma membrane protein in 291 identified proteins (61%). They also reported

that they identified 86 integral proteins (29%)[35]. When compared to these techniques our approach promises great potential with 80% plasma membrane protein enrichment.

GO Cellular Compartment analysis of significantly enriched proteins showed that all the top hits were related to the plasma membrane and cell periphery related compartments which indicate the high specificity of our method. GO Molecular Function and GO Biological Process analysis showed that most of the proteins have roles in molecule and ion exchange and cell adhesion. These functions are usually carried out by transmembrane and integral proteins. Our analysis showed that two-third of the significantly enriched proteins constitute of integral plasma membrane proteins. Therefore, our method can be applied for identification and analysis of the integral plasma membrane proteins.

As future perspective, our approach can be used for mapping plasma membrane proteins of different cell lines and tissues to discover unique plasma membrane markers. These markers can be useful for targeted drug developments. Our strategy can be quite efficient for mapping the interactome of a drug or a ligand as well. Mechanism of drugs and cells response to its binding can be investigated by using our proteomic approach. Our methodology is quite useful especially integral proteins which have cytosolic domain. Therefore, to obtain more comprehensive plasma membrane proteomic analysis, chemical labelling techniques can be incorporated into our method. By this way both extracellular and cytosolic part of the plasma membrane can be analyzed.

1.5. Conclusion

In summary, this study was carried out to propose a novel approach for the identification of plasma membrane proteins in proteomics studies. To achieve that we developed a two-step enrichment method. We generated a myristoylated and palmitoylated BirA (Myrpalm BirA) biotin ligase enzyme. This enzyme localizes to the cell membrane and labels its surroundings. Our microscopy results showed that palmitoylation and myristoylation tags can target recombinant proteins to cell membrane and this approach can be used for other membrane targeting studies.

Then we used centrifugation and streptavidin pulldown to isolate and enrich plasma membrane proteins. Isolated proteins are analyzed by mass spectrometry and identified proteins are categorized according to their subcellular localization by using gene ontology analysis.

We applied the same procedure to cytosolic BirA expressing cells. Cytosolic BirA does not bear any modification to localize itself to a specific cellular compartment.

We compared the proteomics analysis of Myrpalm BirA and BirA. We showed that usage of ultracentrifugation and BioID together increases the identification efficiency of plasma membrane proteins. 60% of all identified proteins were plasma membrane proteins in our analysis. 81% of the significantly enriched proteins were constituted of plasma membrane proteins. Also, our analysis showed that most of the plasma membrane proteins enriched in our protein list are composed of integral plasma membrane proteins. Thus, we suggest using our proposed method for identification of integral membrane proteins.

2. CHARACTERIZATION OF CHLORIDE INTRACELLULAR CHANNEL PROTEINS IN CELL CYCLE DEPENDENT MANNER

2.1. Literature Review

2.1.1. Cell cycle

All living cells rely on cell division mechanisms to grow and to reproduce. Cell division is a vital machinery for a cell to transfer its genetic material to offspring cell. In a healthy cell, cell must fulfill all the requirements of cell cycle to complete cell division. The cell cycle comprised of mainly two phases which are interphase and mitosis. Interphase is the preparation phase of mitosis, it consists of three stage G1, S, and G2. During Gap phase 1 (G1), cell grow and prepare itself to DNA replication. There is another subphase in G1 which is called G0 or resting phase. If a cell loses its ability to divide or it does not commit itself into dividing, then the cell enters G0 phase. Some terminally differentiated human cells such as nerve cells and heart muscle cells enter G0 phase and do not divide afterward. S phase or synthesis phase is the phase where cell duplicates its genomic materials. During G2 phase, cell prepares itself for mitosis phase by growing and producing necessary proteins for mitosis[5].

Upon completion of the interphase, the cell enters mitosis. Mitosis composed of five steps which are prophase, prometaphase, metaphase, anaphase, and telophase (**Figure 20**). Prophase is the first step of the mitosis and chromosome condensation take place during prophase. Centrosomes move to different poles to generate mitotic spindles in prophase. In prometaphase, cell nucleus diffuses, and chromosomes become available for mitotic spindles. In metaphase, chromosomes aligned into metaphase plane. In anaphase, centrosomes separate sister chromatids by pulling them through mitotic spindles, separated chromatids then move to different poles. In telophase, two new nuclei assemble around separated chromosomes. Once cell completes all these steps, daughter cells physically separated from each other in cytokinesis[36].

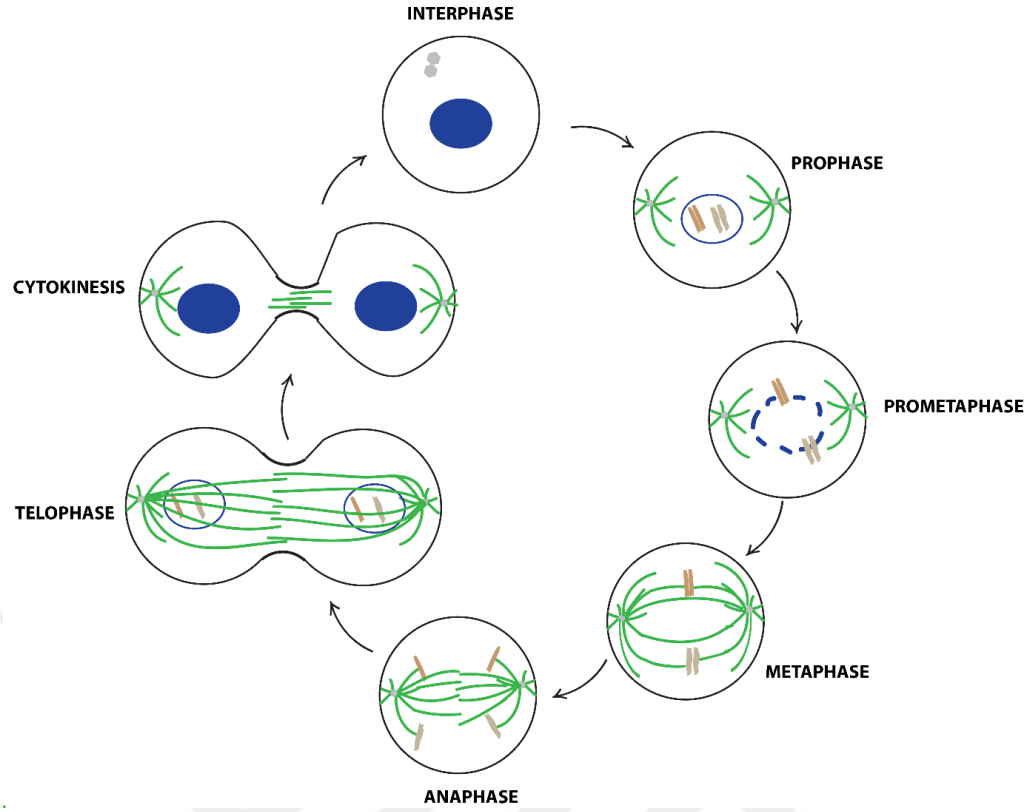

Figure 20 Overview of the eukaryotic cell cycle.

DNA replication and duplication of the centrosome (gray) occurs in interphase, DNA condensation and transportation of centrosomes to different poles occurs in prophase. Nuclear lamina dissociates and chromosomes can attach to microtubules at prometaphase. Chromosomes aligned in equatorial plane during metaphase, sister chromosomes get separated in anaphase. Formation of new nucleus and decondensation of chromosomes in telophase. Cytokinesis initiates after anaphase and cells physically separated from each other. Adapted from [5].

2.1.2. Regulation of the Cell Cycle

The cell cycle is one of the most significant machinery for the cell, therefore it is strictly controlled by several mechanisms in various phases of cell cycle. The cell cannot proceed to next phase without meeting the necessary criteria of each phase. There are three main checkpoints in cell cycle which are G1/S checkpoint, G2/M checkpoint, and mitotic spindle checkpoint. G1/S checkpoint controls if the environment is suitable for division and if there is any DNA damage present on genome. In S phase genomic DNA replicated and then G2/M checkpoint controls if there is any chromosome that is not replicated or if there is any damage on replicated DNA. G1/S and G2/M checkpoints halt the cell cycle progression if there is any problem and recruit other mechanisms to fix it. Once the cell satisfies these two checkpoints,

cell enters mitosis and the cell cycle proceeds. There is another checkpoint in metaphase which controls if all chromosomes aligned correctly and if the mitotic spindles attached to all sister chromatids. Any mistake in this step will cause daughter cells to have abnormal number of chromosomes, therefore it is critical for cell to segregate its genetic material equally[37, 38].

These checkpoints regulate cell cycle through cyclins and cyclin-dependent kinases (CDK) proteins. CDK regulates the cell cycle by phosphorylating their target proteins while cyclins are the activator elements of CDK's. There are several cyclins and CDK proteins present in the cell. Active cyclin and CDK proteins change in different stages of the cell cycle. Cyclin levels oscillate through cell cycle while level of CDK remains same. Once the level of cyclin increases, it activates its counterpart CDK and active CDK phosphorylates other proteins. Thus, several downstream processes get activated while some other pathways get inhibited by activation of same CDK[37].

Some important cyclins during the cell cycle are Cyclin D, Cyclin E, Cyclin A, and Cyclin B while CDK2, CDK4, CDK6, and CDK1 are important CDK proteins during mitosis. The first cyclin produced in cell cycle is Cyclin D. It is active in G1 and activates CDK4 and CDK6 which initiate production of other cyclins and necessary proteins. During S phase and G1 to S transition CDK2 is activated by Cyclin E and Cyclin A. Cyclin A also activates CDK1 in G2 together with Cyclin B and Cyclin A activity cell proceed into mitosis phase[39]. The signal to cell to exit mitosis phase is the degradation of active Cyclin B and Cyclin A. Once these cyclins are degraded, anaphase-promoting complex/cyclosome (APC/C) initiates degradation of mitotic marker and then cell enters the cytokinesis phase[40].

2.1.3. Cytokinesis

Cytokinesis is the final phase of the cell cycle and it terminates the cellular division by physically separating the cell into two daughter cells. After cytokinesis is completed, nascent daughter cells separated from each other by a cellular membrane. However, some cells complete their cell cycle without cytokinesis, such as early *Drosophila* embryonic cells and mammalian heart muscle cells do not undergo cytokinesis at the end of their cell cycle. Additionally, in higher- plant cells cytokinesis takes place by accumulation of cell plates which later form the cellular wall of daughter cells[41].

Unlike plant cells, in animal cells cytokinesis required to complete cell division. Cytokinesis in the animal cell comprised of these four subsequent stages; positioning of the division plane, formation, and ingression of the cleavage furrow, midbody formation, and abscission (**Figure 21**)[42].

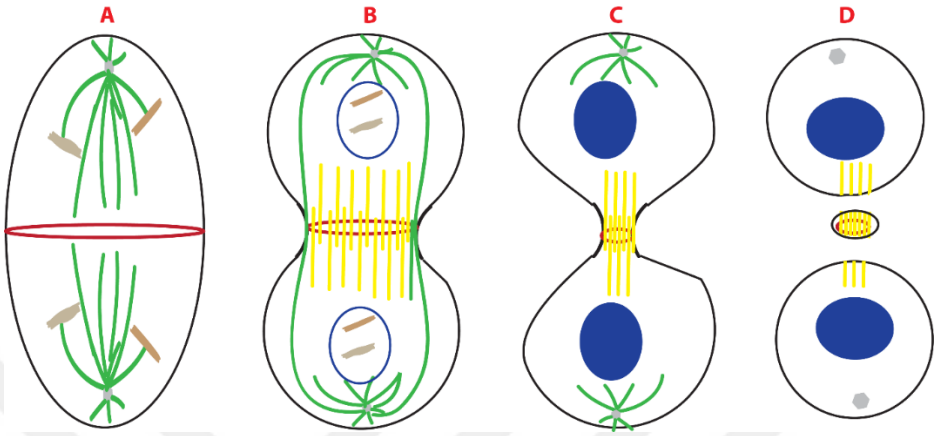

Figure 21 The stages of cytokinesis in animal cells.

Cytokinesis has four main steps. Cytokinesis initiates after sister chromosomes separated from each other after anaphase. Adapted from [43].

A- The division plane is determined after all genetic materials removed from equatorial plane of the cell and by the activity of actomyosin ring (red) cell membrane starts to ingress.

B- While actomyosin ring constricts cleavage furrow formed, and ingression of the cleavage furrows continues until midbody formed.

C- Continues constriction leads to the formation of intercellular bridge from central spindle microtubules (yellow). This structure is called as midbody.

D- Physical separation occurs by severing the midbody.

The initial step of the cytokinesis is the determination of cleavage plane. Since cytokinetic activity is irreversible, spatiotemporal regulation of cytokinesis is crucial. To generate healthy daughter cells, cytokinesis must take place between segregated chromosomes to prevent abnormalities in chromosome number in daughter cells. Microtubules play key roles for determination of division plane. After chromosomes segregated in anaphase, mitotic spindles reorganized such that they form central spindle microtubules between segregated chromosomes. Apart from microtubules many cytoskeletal and signaling proteins also contribute to assembly of central spindle. Anaphase promoting complex ensures that

cytokinesis does not occur before anaphase. APC/C promotes degradation of mitotic cyclins and inactivates mitotic cyclin-dependent kinases, thus gives mitotic exit signal to cell[44-46].

After this step, cleavage furrow assembled in the cleavage plane. Actomyosin filaments and RhoA are the key players for cleavage furrow assembly and ingression. Actomyosin filaments build a structure called contractile ring. Contractile ring position itself perpendicular to the central spindle. Although the exact regulation machinery and how constriction mechanism works is still debated, it is known that RhoA is the main regulator that controls cleavage furrow elements[42, 47].

As the main cytokinesis regulator, RhoA controls contractile ring through two different pathways. RhoA stimulates profilin mediated actin proliferation through binding Diaphanous (Dia) formin homology proteins. The other pathway is, through activation of Rho-associated kinase (ROCK) RhoA stimulates phosphorylation of myosin regulatory light chain and promotes myosin contraction. By these two machinery RhoA regulates both assembly and ingression of cleavage furrow. Progressive constriction of contractile ring causes increase in cell surface area. A sudden increase in cell membrane requires massive amount of transportation. Additionally, RhoA recruits proteins necessary for abscission to cell membrane while cleavage furrow progress[42, 45, 48].

Ingression of the cleavage furrow constricts central spindle and forms a dense structure called the midbody region. Although exact mechanism is not clear, transition between contractile ring to midbody mediated by anillin. Anillin and RacGAP are responsible for linking central spindle to plasma membrane. Midbody is around 1µm diameter and it is a tight bridge between two daughter cells. Midbody can be considered as a bridge comprised of many proteins that are required for organization and recruitment of abscission elements[42].

The final step of cytokinesis is the abscission. During abscission, midbody is severed through enzymatic activity. Endosomal sorting complex required for transport (ESCRT) proteins localize to midbody and catalyze the division. To complete cytokinesis interaction between ESCRT III and Cep55, TSG101 and ALIX complex is required. When midbody region is severed, ESCRT mediated abscission machinery is inherited to one of the daughter cells[49, 50].

2.1.4. CLIC protein family

Chloride intracellular channel (CLIC) proteins family is comprised of seven members which are p64 (CLIC5B) and CLIC1-6. CLICs are evolutionarily conserved proteins among vertebrates. Despite the differences in their lengths, CLIC proteins share a homology domain of around 220 amino acids (**Figure 22**). In these homology domains CLICs have two transmembrane domains and a nuclear localization signal. CLICs have both cytosolic soluble and membrane-associated form. Several diverse physiological functions are associated to CLIC proteins. Some of the biological processes CLICs play roles in are actin-dependent vesicular trafficking, pH regulation of vesicles, membrane remodeling, glutaredoxin like enzymatic activity[51-54].

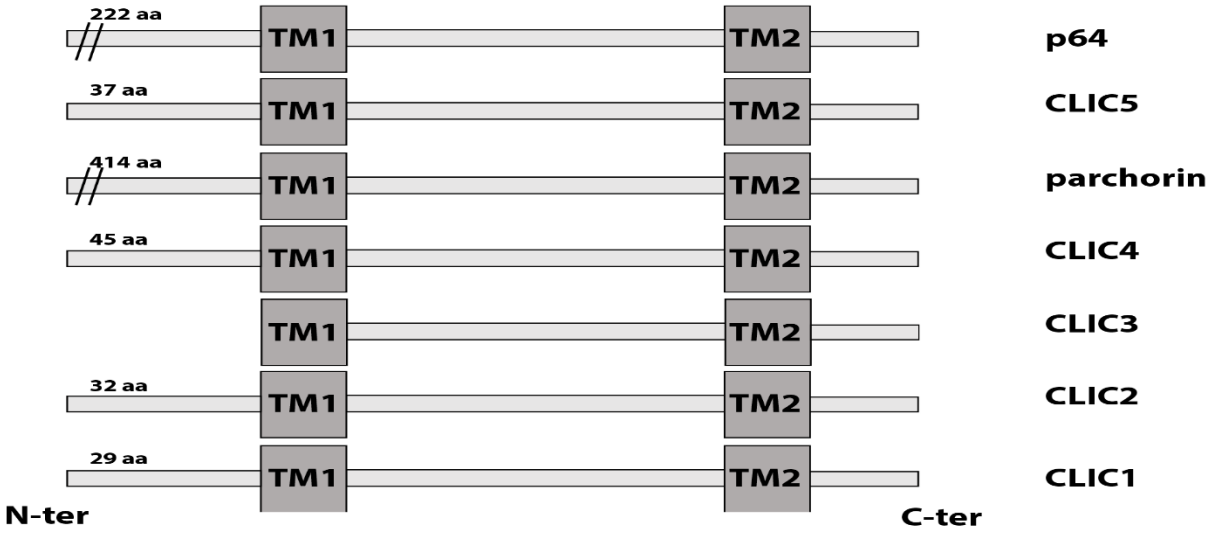

Figure 22 Chloride intracellular channel (CLIC) protein family members.

CLICs can be found in various sizes in the cell. Conserved transmembrane domains are shown as TM. Adapted from [55]

Since their identification in the 90s, electrophysiological characterization of CLICs is still not completed. Ion channel properties of CLICs have been heavily investigated, due to their names. The first CLIC member, CLIC5B, is discovered by its affinity towards a chloride ion channel inhibitors, thus it is treated as one of them. Despite remarkable differences in the protein sequences of CLIC5B and other chloride ion channel proteins, it still functions as chloride ion channels in vitro. However, following studies investigating if CLICs show any selective affinity towards chloride ion, concluded that CLIC4 and CLIC5 proteins showed equal selectivity towards both cation (K^+) and anionic (Cl^-) molecules. Their ion conductance is very

low compared to other ion channels. These studies showed that CLICs are quite different than traditional chloride ion channels[51].

The crystal structure of the soluble form of CLICs showed that they are structurally related to Glutathione S-transferase omega (GSTo) protein family. CLICs and GSTo proteins share homology in their glutathione (GSH) binding site. Since GTSo proteins can modulate the activity of the other chloride ion channels, some studies concluded that CLIC proteins are members of GSTo superfamily proteins. GSTo proteins catalyze the glutathione binding reaction in the cell and the catalytic region of these proteins contains cysteine-proline-phenylalanine-cysteine/serine residue. This catalytic region is conserved among all CLICs[51, 56].

The affinity of CLICs toward glutathione is lower than GSTs, because of the 3D conformation of the GSH binding site. This region is more open in CLICs compared to GSTs; therefore, this region could be used for interaction with other proteins as well. Basic nature of the catalytic cleft makes it possible to target not only other proteins but also phospholipids as well. Therefore, this cleft could help CLICs to associate with membrane[51].

2.1.4.1. CLIC1 and CLIC4

Chloride intracellular channel protein 1 (CLIC1) and Chloride intracellular channel protein 4 (CLIC4) are the two most studied members of the CLIC protein family. Their sizes are 27 kDa and 29 kDa respectively. They are expressed ubiquitously in the cell and they both have soluble and membrane-bound forms. They share conformational similarities with omega class of the glutathione S-transferases, similar to other CLICs. They have thioredoxin-like domains and transmembrane protein (TM) regions in their N terminus and nuclear localization signal (NLS) in their C terminus. Although they have soluble and membrane-associated forms, our knowledge on cytosolic form of CLIC1 and CLIC4 is limited[53, 55].

CLIC1 plays important roles in intracellular vesicle acidification, vesicular transportation, and exocytosis. CLIC1 can form oligomeric integral membrane ion channel proteins and play roles in maintaining homeostasis of membrane potential[57].

CLIC4 is associated with many biological processes such as cell differentiation, cellular adhesion, migration, apoptosis, angiogenesis, intracellular vesicle trafficking, autophagy and signaling pathways[53, 58, 59]. Its expression profile and expression levels are important for

many complications as well, due to its participation in many important cellular systems. CLIC4 is an important hallmark for many cancer cases. The expression profile of CLIC4 protein varies in different cancer cells. Its expression pattern is used as biomarker in kidney cancer and epithelial ovarian cancer cells. CLIC4 expression level increases in metastatic colorectal cancer cells, decreases in malignant squamous cell carcinoma[55, 60, 61].

2.1.5. The motivation of the study

Most of the functions of CLICs are associated to its membrane-bound form. The transition between soluble and membrane-bound forms of CLICs was not revealed fully. However, in a recent quantitative proteomics study from our research group, Ozlu et al. (2015) presented that plasma membrane protein composition shows significant changes between interphase and mitosis. The quantity of several proteins in plasma membrane varies between these phases. According to this study, CLIC protein levels (CLIC1 and CLIC4) in plasma membrane are higher in mitosis, compared to their level in interphase (**Table 3**)[3]. However, currently there is no information about the cell cycle-dependent role of CLIC4 in literature.

According to findings of previous proteomics and microscopic analysis done by Zeynep Cansu Uretmen Kagiali (former Ph.D. in our group), CLIC4 localized to cleavage furrow and its localization last until cytokinesis completed. Also, when CLIC4 protein is knockdown by shRNA, the fidelity of cytokinesis is affected, and cells get more prone to be multinucleated.

However, knockdown studies do not always reflect complete phenotype. Therefore, in this study, I aimed to show the physiological phenotype of CLIC proteins by analyzing its effect on cytokinesis fidelity. To that extent, we generated CRISPR-Cas9 mediated CLIC4 and CLIC1 knockout cell lines and investigated their roles in the cell cycle.

Our data suggest that CLIC4 and CLIC1 proteins get enriched on plasma membrane after anaphase and they remain in the cytokinesis plane until the end of cytokinesis. Additionally, CLIC knockout cell lines showed cytokinesis defects and increased multinucleation.

Table 3 CLIC1 and CLIC4 enrich on the cell surface during mitosis

Gene name	Protein	p-value	Median ratio	Number of peptides	Coverage (%)	Direction
EGFR	Epidermal Growth Factor Receptor	Std	0.80	42	28	No change
IGFR2	Cation-independent mannose 6-phosphate	0.01	0.35	36	17	Interphase
CLIC1	Chloride intracellular channel protein 1	0.01	2.35	8	39	Mitosis
CLIC4	Chloride intracellular channel protein 4	0.01	3.08	6	37	Mitosis
PCDH7	Protocadherin 7	0.01	5.15	5	6	Mitosis

2.2. METHOD

2.2.1. Cell culture

HeLa S3 cells were grown routinely cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 unit/ml Penicillin and 100 µg/ml Streptomycin (Lonza, DE17-602E) at 37°C and 5% CO_2.

2.2.1.1. Cell Synchronization

Following cell synchronization conditions were applied to HeLa S3 cells. Cells were grown until they reach 40% confluency and were treated with 2mM thymidine (Santa Cruz, 296542) for 16 hours and released for 8 hours to block the cell cycle in interphase. To obtain higher efficiency thymidine block step was applied for second time. Then to block cells in mitosis, cells were incubated with nocodazole (Calbiochem, 487928) for 5 hours. To obtain cytokinesis synchronized population after nocodazole treatment cells were released for drugs for 60-90 minutes and cells harvested[62].

2.2.1.2. Single Cell Isolation

To generate homogenous knockout cells, single-cell isolation method was used. Heterogenous HeLa S3 CLIC1-sg1 and CLIC4-sg1 populations were diluted and 10 mL cell suspension with a concentration of 8 cells/mL was obtained. 100 µL of this cell suspension was seeded to each well of the 96 well plate. Cells were grown in the conditions mentioned above and grown colonies transferred into bigger plates. Colonies were screened by immunoblotting to verify CLIC1 or CLIC4 knockout phenotype.

2.2.2. Characterization of knockout colonies

2.2.2.1. Western Blot

Cells were lysed by using EDTA-free protease inhibitor cocktail (Thermo) supplemented NP-40 lysis buffer (1% NP-40, 150mM NaCl, 50mM pH8.0 Tris-Cl). Cell debris was removed by using centrifugation at 14.000 rpm at 4°C for 10 minutes. To asses protein concentration, BCA kit (Pierce, 23227) was used. Proteins were boiled in 2X Laemmli sample buffer (4% SDS, 0.02% bromophenol blue, 100mM dithiothreitol (DTT), 20% glycerol, 120mM pH6.8 Tris-Cl) for 5 minutes at 95°C.

Western blot separates proteins according to their sizes. During this study, I used 10% SDS-PAGE gels and proteins were separated on the gel at 120V for 90-120 minutes.

After that proteins were transferred to the nitrocellulose membrane (GVS, 1215471) from the gel by using the Trans-Blot Turbo Transfer System (BioRad) at 25V, 2mA for 10 minutes. We used wet transfer system for GST pulldown experiments. For that transfer proteins were transferred to membrane by using 45V for 16 hours at 4°C. Transfer efficiencies were controlled by Ponceau staining. Membranes were blocked with 4% non-fat dry milk in TBS-Tween 20 (0.1%) for 60 minutes. Then I incubated membranes with primary antibodies in 2% BSA containing TBS-Tween 20 (0.1%) for 3 hours at RT or 16 hours at 4°C. Excess antibodies and nonspecific bindings removed by washing with excess TBS-Tween 20 (0.1%) washes for 10 minutes. Then membranes incubated with secondary antibodies which are counterparts of the primary antibodies for 1 hour. Secondary antibodies were prepared in 4% non-fat dry milk in TBS-Tween 20 (0.1%). Then excess bindings were removed by washings again. Then membranes were detected by using horseradish peroxidase - luminol based chemiluminescence method (Pierce, 32106 and Pierce, 32132).

1.1.1.1. Immunofluorescence and Multinucleation analysis

HeLa S3-CLIC4 and HeLa S3-CLIC1 knock out cells were grown on coverslips until they reach 60% confluency. Cells were fixed by using 3.2% PFA solution and permeabilized by PBS-Triton-X (0.1%). Fixed cells were blocked by using 2% BSA containing PBS-Triton X (0.1%) for 1 hour. Then cells incubated with primary antibodies for 3 hours at RT or 16 hours at 4°C. Excess antibodies and nonspecific bindings removed by PBS-Triton X (0.1%) washes. Then cells treated with appropriate secondary antibodies for 45 - 60 minutes in dark. Images were taken by using confocal microscopy (Eclipse 90i, Nikon). Quantifications for multinucleation experiments were done by using Image J software[63]. Statistical analysis was done by using Graphpad 5 software.

1.1.1.2. Antibodies

In this study, I used following primary antibodies: anti-CLIC1(Santa Cruz, Sc-81873), anti-CLIC4 (Santa Cruz, Sc-135739), anti-Actin (Abcam, ab6276), anti-β-Tubulin (Cell Signaling, 2128), anti-p-Histone-H3 (S10) (Upstate, 32219), anti-Anillin (Abcam, ab99352), anti-Ezrin (Cell Signaling, 3145), anti-ALIX (Abcam, ab88388).

For secondary, antibodies following antibodies were used: HRP conjugated anti-mouse IgG (Cell Signaling, 7076S) and HRP conjugated anti-rabbit IgG (Cell Signaling, 7074S)antibodies used for western blot while Alexa Flour 488- and 555- conjugated anti-mouse and anti-rabbit (Cell Signaling) antibodies were used for immunofluorescence

Western blot separates proteins according to their sizes. During this study, I used 10% SDS-PAGE gels and proteins were separated on the gel at 120V for 90-120 minutes.

After that proteins were transferred to the nitrocellulose membrane (GVS, 1215471) from the gel by using the Trans-Blot Turbo Transfer System (BioRad) at 25V, 2mA for 10 minutes. We used wet transfer system for GST pulldown experiments. For that transfer proteins were transferred to membrane by using 45V for 16 hours at 4°C. Transfer efficiencies were controlled by Ponceau staining. Membranes were blocked with 4% non-fat dry milk in TBS-Tween 20 (0.1%) for 60 minutes. Then I incubated membranes with primary antibodies in 2% BSA containing TBS-Tween 20 (0.1%) for 3 hours at RT or 16 hours at 4°C. Excess antibodies and nonspecific bindings removed by washing with excess TBS-Tween 20 (0.1%) washes for 10 minutes. Then membranes incubated with secondary antibodies which are counterparts of the primary antibodies for 1 hour. Secondary antibodies were prepared in 4% non-fat dry milk in TBS-Tween 20 (0.1%). Then excess bindings were removed by washings again. Then membranes were detected by using horseradish peroxidase - luminol based chemiluminescence method (Pierce, 32106 and Pierce, 32132).

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1.2. RESULTS

1.2.1. CLIC4 KO colony characterization

CLIC4 proteins enriched on the plasma membrane during mitosis, yet its role in the cell cycle is not known. Therefore, to show its importance for cell cycle, we aimed to generate CRISPR-Cas9 mediated CLIC4 knockout HeLa S3 cell line. Zeynep Cansu Üretmen Kagiali generated five guide RNAs targeting the CLIC4 gene. Among those guide RNAs, sgRNA-1 treated heterogenous population showed decreased CLIC4 expression. To obtain complete phenotype, I generated single cell colonies and characterized them. Single cell colonies were isolated and were expanded, then by using immunoblotting analysis we checked CLIC4 expression of single colonies (**Figure 23**). As control group Hela S3 cells were treated with non-targeting sgRNA. a-Tubulin was used as loading control for western blot analysis.

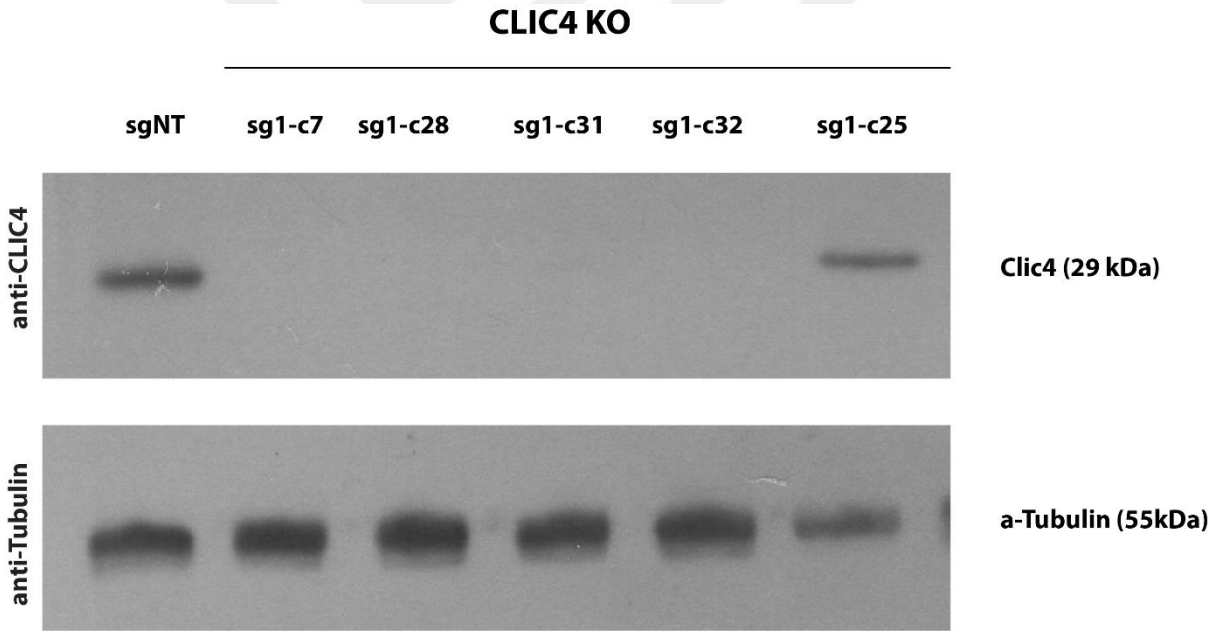

Figure 23 CLIC4 KO colony screening by western blotting.

CLIC4 expressions of single colonies verified by CLIC4 blotting, tubulin blotting was used as a loading control. Endogenous CLIC4 expression is shown in sgNT control group. sgNT is the cells treated with nontargeting guide RNA.

Our results showed that we successfully generated CLIC4 knockout cell lines. sgNT and c25 express comparable amount of CLIC4 protein, while c7, c28, c31, and c32 colonies are showing

CLIC4 knockout phenotype. Knockout colonies were chosen for further characterization studies.

We aimed to determine the cell cycle-dependent role of CLIC4. Our preliminary results were showing CLIC4 localize to the cell membrane after anaphase and remain in the midbody until the cell cycle completed. Therefore, membrane bound CLIC4 should be an important protein for cytokinesis. Our hypothesis is that CLIC4 has an important role in cytokinesis, therefore, we investigated cytokinesis fidelity of the cytokinesis in the wild type and CLIC4 knockout cells by quantifying multinucleated cells. To do that, cells were grown on the coverslips and were fixed the cells and stained them against a-Tubulin and DAPI. Quantifications were done by using Image J software (**Figure 24**). Statistical analysis was done by using GraphPad 5 software.

Control group was showing 3% multinucleation while CLIC4 knockout cells showed increased multinucleation ratios. Colony 31 and colony 32 were the two highest multinucleated cell lines with 8.3% and 10.3% respectively. Increased multinucleation rates indicate that CLIC4 plays important roles for cytokinesis and its depletion would lead to increase in cytokinesis defects.

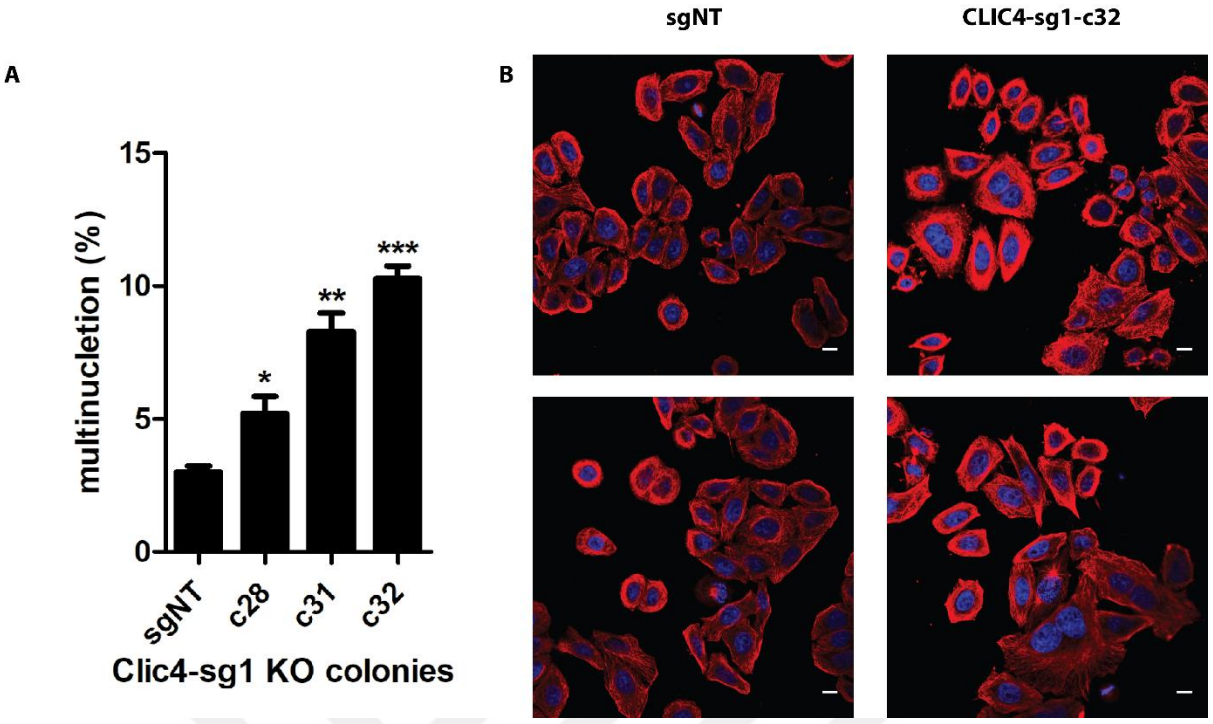

Figure 24 CLIC4 knockout HeLa S3 cells show increased multinucleation in asynchronous population.

A- Quantification of multinucleated cells in asynchronous populations. Data are presented as mean ± SEM (n=3); >300 cells were analyzed for each set of experiments. Unpaired t-tailed Student's Test was applied for significance analysis (*p<0.05, **p<0.01, ***p<0.0001).

B- Representative images for control and CLIC4 knockout colony 32 cell lines. Cells were stained against a-Tubulin (red) and DAPI (blue). Scale bar 10µm.

1.2.2. CLIC1 KO colony characterization

CLIC4 and CLIC1 share structural homology and a huge part of their amino acid sequence are conserved among them. Additionally, CLIC1 also is enriched on the plasma membrane during mitosis, similar to CLIC4 according to proteomics data [3]. Therefore, there could be some redundant pathways where CLIC1 compensates CLIC4's role in CLIC4 knockout cells. To see if CLIC1 also affects the multinucleation rate of the cells, we generated CLIC1 knockout HeLa S3 cell lines by using CRISPR-Cas9 genome editing tool. In order to obtain complete phenotype and generate homologous population, we generated single cell clones from heterogenous CLIC1-sg1 populations. Single cell colonies were characterized by western blotting analysis against CLIC1 antibody (**Figure 25**). Nontargeting guide RNA is treated to HeLa S3 cells to generate a control group. a-Tubulin was used as loading control western blotting.

Our immunoblotting results showed that CLIC1 expression is depleted in most of the colonies. Therefore, we successfully generated CLIC1 knockout HeLa S3 cell lines. Some colonies were expressing slightly lower bands in CLIC1 blot. This could be an artifact of the antibody or a shorter CLIC1 isoform. We used complete knockout colonies for further analysis.

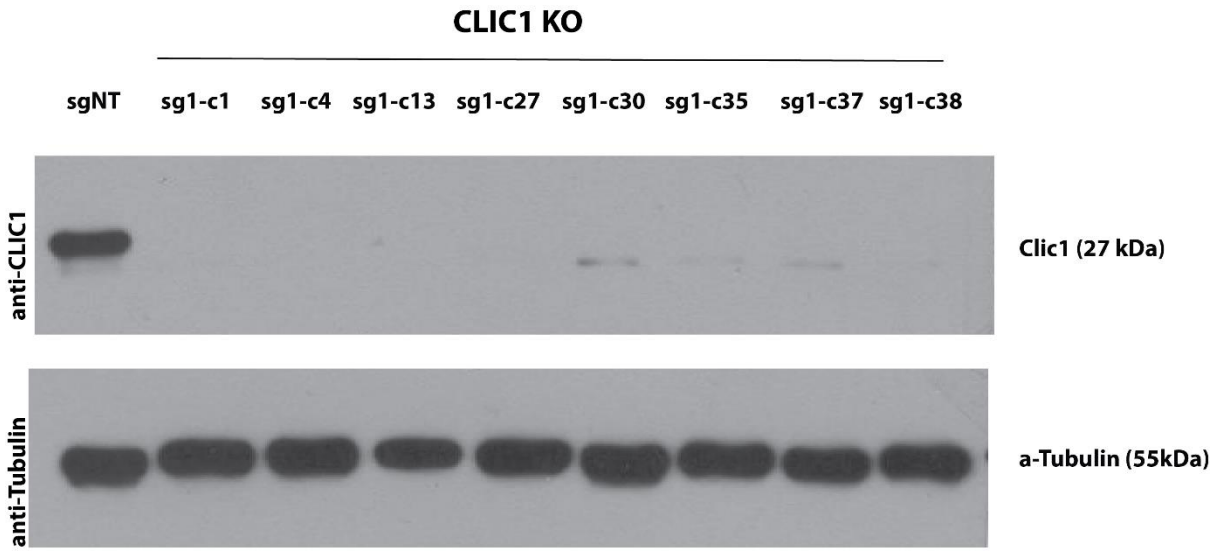

Figure 25 CLIC1-sg1 knockout colony screening by western blotting.

CLIC1 expressions of colonies verified by CLIC1 blotting, tubulin blotting was used as a loading control. Endogenous CLIC1 expression is shown in sgNT control group. sgNT is the cells treated with nontargeting guide RNA.

After verification of colonies, we investigated if CLIC1 knockout HeLa S3 cells have higher multinucleation rates then control group. To show its effect on cytokinesis fidelity, CLIC1 knockout colonies were fixed and stained then analyzed under fluorescence microscopy (**Figure 26**). Quantifications were done by using Image J software and statistical tests were done by using Graph Pad software.

According to our results, CLIC1 knockout cell lines showed increased multinucleation ratios compared to the control group. Non-targeting guide RNA treated control group showed 3.94% multinucleation ratio while CLIC1 knockout colony 1 and colony 27 showed the highest multinucleation ratios with 11.35% and 10.93% respectively. Increased multinucleation rates indicate that CLIC1 also plays important roles in cytokinesis and its depletion would lead to increase in cytokinesis defects.

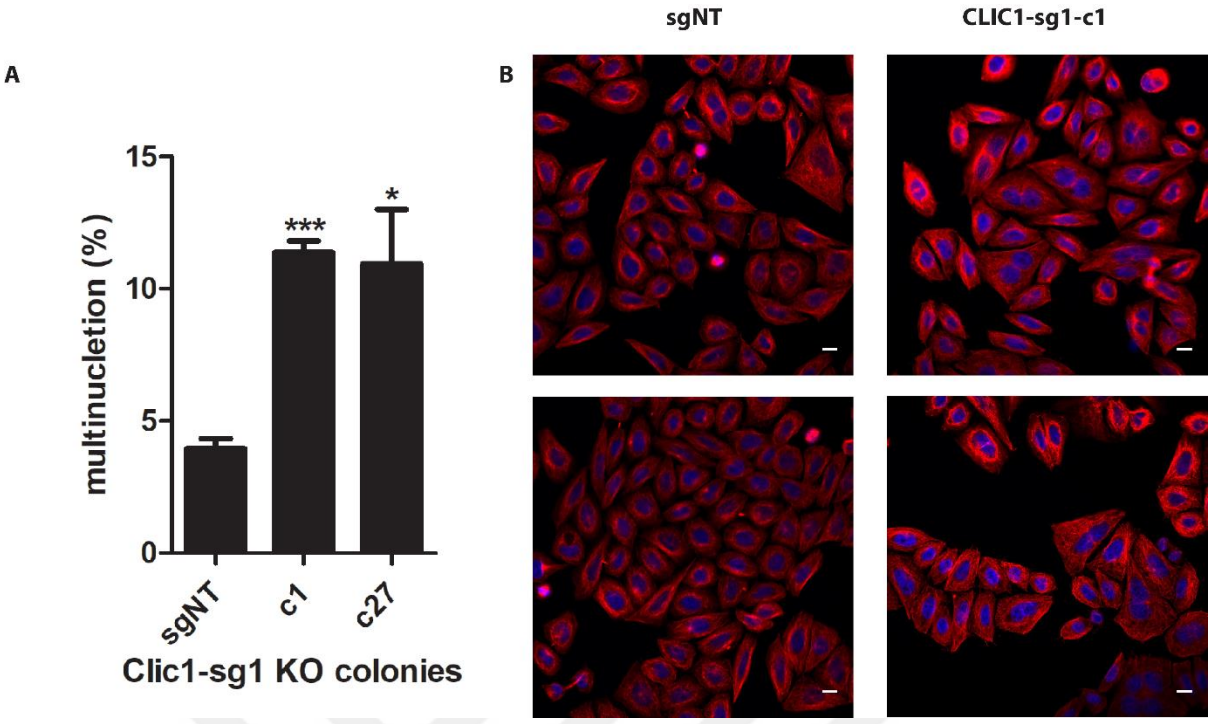

Figure 26 CLIC1 knockout HeLa S3 cells show increased multinucleation in asynchronous population.

A- Quantification of multinucleated cells in asynchronous populations. Data are presented as mean ± SEM (n=3); >300 cells were analyzed for each set of experiments. Unpaired t-tailed Student's Test was applied for significance analysis (*p<0.05, **p<0.01, ***p<0.0001).

B- Representative images for control and CLIC1 knockout colony 1 cell lines. Cells were stained against a-Tubulin (red) and DAPI (blue). Scale bar 10µm

1.3. DISCUSSION

In this study, we examined the cell cycle-dependent roles of CLIC1 and CLIC4 protein. Our previous data was suggesting that CLIC1 and CLIC4 proteins are enriched on plasma membrane during mitosis[3]. Our group previously showed that CLIC1 and CLIC4 proteins localize to cleavage furrow until cytokinesis completed. Despite several studies focused on CLIC proteins, there is not a consensus in literature about their cellular function [51, 53]. There is not any study about cell cycle-related functions of CLIC proteins.

To asses cell cycle related function of CLIC4, we used CRISPR/Cas9 gene-editing technique and generated CLIC4 knockout HeLa S3 cell line. Characterization of these cells revealed that CLIC4 knockout cells undergo abnormal cytokinesis and they show significantly higher multinucleation ratio compared to wild type cells.

CLIC proteins share structural homologies and our proteomics results also suggested that CLIC1 is enriched on the plasma membrane in mitosis. Therefore, there could be functional redundancy between CLIC4 and CLIC1. That is why we generated CLIC1 knockout HeLa S3 cells by using CRISPR/Cas9 system. Microscopic analysis revealed that these cells are also showing increased cytokinetic defects that result in higher multinucleation ratios compared to wild type cells.

In conclusion, we generated CLIC1 and CLIC4 knockout cells by using CRISPR-Cas9 gene editing tool and characterized CLIC1 and CLIC4 knockout HeLa S3 cell line. To test their involvement in cytokinesis we quantified the multinucleated cell ratio of the knockout cells. Then we observed that CLIC1 and CLIC4 depletion lead to increase in multinucleation in HeLa S3 cells which indicates that these proteins have roles in cytokinesis.

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VITA

Mehmet Akdağ was born in Istanbul Turkey on June 25, 1992. He graduated from Istanbul Bahçelievler Dede Korkut Anadolu High School in 2010 and then he received his B.Sc. degree in Molecular Biology and Genetics from Istanbul Technical University, Istanbul, 2015. He worked as a teaching and research assistant at Koç University between February 2017 to September 2019. He received his M.Sc. degree in Molecular Biology and Genetics with the thesis titled as “Development of a Novel Approach for Cell Surface Protein Analysis” under supervision of Assoc. Prof. Dr. Nurhan Özlü.

APPENDIX

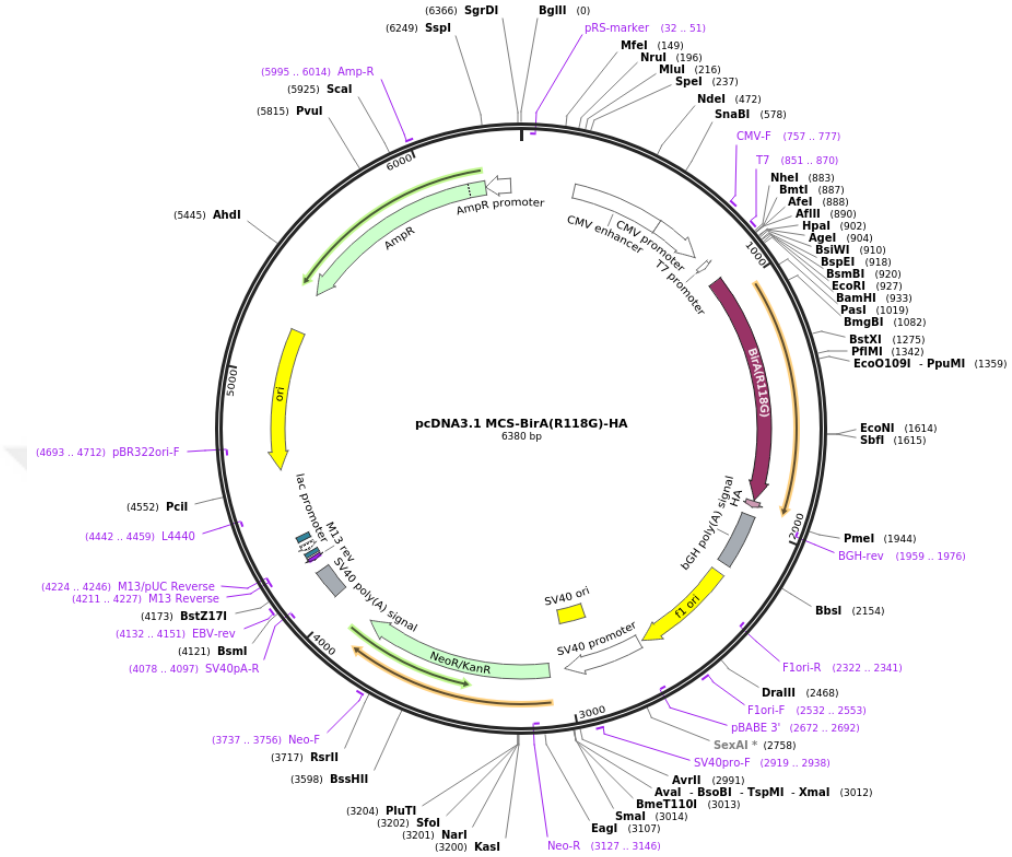

Figure 27 Vector map of pcDNA3.1 MCS-BirA(R118G)-HA

(Addgene #36047) [1]

Table 4 Significantly enriched proteins identified in BioID analysis of Myrpalm-BirA cell line.

Accession	Gene Name	Σ PSM	Avg PSM	FoldChange
Q09666	AHNAK	3194	638.8	6388
O00161	SNAP23	119	23.8	238
P01891	HLA-A	157	31.4	4.19
P08195	SLC3A2	377	75.4	8.62
P02786	TFRC	346	69.2	11.53
P05023	ATP1A1	317	63.4	42.27
P43121	MCAM	265	53	530
Q687X5	STEAP4	148	29.6	8.46
P80723	BASP1	55	11	8.8
P51149	RAB7A	47	9.4	4.7
P78380	OLR1	54	10.8	108
P35613	BSG	83	16.6	166
Q9Y6M5	SLC30A1	73	14.6	146
Q96AC1	FERMT2	148	29.6	14.8
Q15758	SLC1A5	149	29.8	29.8
P05556	ITGB1	120	24	240
O15498	YKT6	74	14.8	148
Q9Y666	SLC12A7	327	65.4	654
O95721	SNAP29	31	6.2	62
Q01650	SLC7A5	125	25	5.56
O43491	EPB41L2	192	38.4	384
P07948	LYN	69	13.8	138
Q16181	SEPT7	66	13.2	132
P20020	ATP2B1	150	30	300
P46108	CRK	56	11.2	112
Q00587	CDC42EP1	58	11.6	116
P01893	HLA-H	44	8.8	11.73
Q9Y5Y0	FLVCR1	48	9.6	96
O60716	CTNND1	60	12	120
P33527	ABCC1	152	30.4	304
Q9H3Q1	CDC42EP4	35	7	70
P21333	FLNA	260	52	3.53
P14209	CD99	32	6.4	64
P27105	STOM	21	4.2	42
P16070	CD44	75	15	150
Q13393	PLD1	117	23.4	234
P00533	EGFR	157	31.4	314
P53985	SLC16A1	76	15.2	8.69
Q13596	SNX1	31	6.2	24.8
P55011	SLC12A2	106	21.2	212
Q07960	ARHGAP1	21	4.2	42
Q96QD8	SLC38A2	33	6.6	66

Q9C0B5	ZDHHC5	50	10	100
P53794	SLC5A3	40	8	80
Q9C0C2	TNKS1BP1	63	12.6	126
Q8TAA9	VANGL1	45	9	90
P11171	EPB41	90	18	180
P21730	C5AR1	22	4.4	44
Q9GZU7	CTDSP1	12	2.4	24
Q9H2H9	SLC38A1	54	10.8	108
P42166	TMPO	60	12	120
Q9Y6M7	SLC4A7	122	24.4	244
Q5T2T1	MPP7	48	9.6	96
Q9ULF5	SLC39A10	52	10.4	104
Q9UNH7	SNX6	24	4.8	9.6
Q15043	SLC39A14	33	6.6	66
Q7Z2W4	ZC3HAV1	49	9.8	98
Q658P3	STEAP3	40	8	80
Q9Y6N7	ROBO1	112	22.4	224
P78310	CXADR	57	11.4	114
Q7KZI7	MARK2	45	9	90
Q9BXB4	OSBPL11	34	6.8	68
Q14677	CLINT1	98	19.6	196
P31641	SLC6A6	18	3.6	36
O95297	MPZL1	11	2.2	22
O00592	PODXL	12	2.4	24
O60503	ADCY9	55	11	110
Q96TA1	FAM129B	39	7.8	78
Q9ULH7	MKL2	24	4.8	48
Q6NZI2	PTRF	13	2.6	26
Q96RD7	PANX1	10	2	20
Q9BYI3	FAM126A	27	5.4	54
Q9UPY5	SLC7A11	16	3.2	32
Q96C19	EFHD2	31	6.2	62
Q01974	ROR2	30	6	60
Q12965	MYO1E	53	10.6	106
P52569	SLC7A2	23	4.6	46
Q14160	SCRIB	19	3.8	38
P43003	SLC1A3	24	4.8	9.6
P48029	SLC6A8	21	4.2	42
Q9UHN6	TMEM2	62	12.4	124
O95466	FMNL1	27	5.4	54
Q9BZF1	OSBPL8	32	6.4	64
Q5T5U3	ARHGAP21	126	25.2	252
P11717	IGF2R	88	17.6	176
Q96BD0	SLCO4A1	42	8.4	84
P11166	SLC2A1	19	3.8	38
Q92692	PVRL2	14	2.8	28

Q14118	DAG1	21	4.2	42
O75116	ROCK2	37	7.4	74
P11532	DMD	55	11	110
Q96RT1	ERBB2IP	29	5.8	58
Q9ULH0	KIDINS220	38	7.6	76
P10586	PTPRF	63	12.6	126
Q31612	HLA-B	36	7.2	72
P49757	NUMB	19	3.8	38
Q92536	SLC7A6	24	4.8	48
Q13740	ALCAM	16	3.2	32
P98082	DAB2	11	2.2	22

Table 5 Significantly enriched proteins identified in BioID analysis of membrane fraction of Myrpalm-BirA cell line

Accession	Gene Name	Σ PSM	Avg PSM	FoldChange
O00161	SNAP23	147	36.75	367.5
P62834	RAP1A	36	9	7.2
P80723	BASP1	95	23.75	237.5
Q09666	AHNAK	2080	520	5200
P02786	TFRC	383	95.75	13.21
P08195	SLC3A2	605	151.25	15.12
P10809	HSPD1	186	46.5	5.17
P43121	MCAM	375	93.75	937.5
P27105	STOM	101	25.25	252.5
P78310	CXADR	142	35.5	355
P51153	RAB13	35	8.75	87.5
P35613	BSG	97	24.25	242.5
P01891	HLA-A	178	44.5	14.83
Q687X5	STEAP4	168	42	28
P05023	ATP1A1	403	100.75	1007.5
Q9Y6M5	SLC30A1	121	30.25	302.5
P07437	TUBB	138	34.5	345
P51149	RAB7A	38	9.5	38
P78380	OLR1	97	24.25	242.5
O43491	EPB41L2	190	47.5	475
P49411	TUFM	86	21.5	8.6
Q9NW97	TMEM51	80	20	200
P07948	LYN	107	26.75	267.5
P62820	RAB1A	36	9	90
Q15836	VAMP3	33	8.25	82.5
P20020	ATP2B1	329	82.25	822.5
P00533	EGFR	258	64.5	645
O75955	FLOT1	51	12.75	127.5
Q9Y666	SLC12A7	523	130.75	1307.5
Q10589	BST2	23	5.75	57.5
P33527	ABCC1	344	86	860
P50454	SERPINH1	40	10	100
Q15758	SLC1A5	205	51.25	41
P00338	LDHA	43	10.75	4.78
Q9BV40	VAMP8	12	3	30
Q96RQ3	MCCC1	107	26.75	2.97
P08754	GNAI3	77	19.25	192.5
Q15043	SLC39A14	55	13.75	137.5
Q9C0B5	ZDHHC5	134	33.5	335
Q96RD7	PANX1	47	11.75	117.5
O15498	YKT6	27	6.75	67.5
Q5T2T1	MPP7	69	17.25	172.5

P11171	EPB41	117	29.25	292.5
P53985	SLC16A1	116	29	8.29
P24752	ACAT1	78	19.5	195
Q9Y5Y0	FLVCR1	53	13.25	132.5
P07947	YES1	89	22.25	222.5
P05556	ITGB1	171	42.75	427.5
O00522	KRIT1	55	13.75	137.5
P09543	CNP	51	12.75	127.5
Q14126	DSG2	131	32.75	327.5
O95297	MPZL1	26	6.5	65
Q06830	PRDX1	28	7	70
Q13740	ALCAM	85	21.25	212.5
O15440	ABCC5	138	34.5	345
Q13393	PLD1	191	47.75	477.5
Q96QD8	SLC38A2	63	15.75	157.5
O95372	LYPLA2	31	7.75	77.5
P21730	C5AR1	21	5.25	52.5
Q9UHN6	TMEM2	150	37.5	375
P40939	HADHA	94	23.5	235
Q5M775	SPECC1	56	14	140
Q9H7F0	ATP13A3	94	23.5	235
Q12959	DLG1	60	15	150
Q9Y6M7	SLC4A7	206	51.5	515
Q08357	SLC20A2	40	10	100
Q8N350	DOS	32	8	80
P42892	ECE1	25	6.25	62.5
Q01650	SLC7A5	182	45.5	8.67
Q9HCJ1	ANKH	27	6.75	67.5
Q8WUM9	SLC20A1	39	9.75	97.5
Q95604	HLA-C	56	14	140
P53794	SLC5A3	58	14.5	145
Q8WTV0	SCARB1	49	12.25	122.5
P10586	PTPRF	157	39.25	392.5
P05787	KRT8	84	21	8.4
P05362	ICAM1	143	35.75	357.5
P55011	SLC12A2	150	37.5	375
O75044	SRGAP2	45	11.25	112.5
Q658P3	STEAP3	74	18.5	185
Q8TAA9	VANGL1	129	32.25	322.5
P39023	RPL3	111	27.75	4.27
O43175	PHGDH	41	10.25	4.56
O60669	SLC16A7	18	4.5	45
O60266	ADCY3	76	19	190
Q99569	PKP4	81	20.25	202.5
P98172	EFNB1	30	7.5	75
Q8IUW5	RELL1	27	6.75	67.5

Q9Y6N7	ROBO1	115	28.75	287.5
P14923	JUP	109	27.25	272.5
Q9UIQ6	LNPEP	83	20.75	207.5
P18084	ITGB5	37	9.25	92.5
Q8NFJ5	GPRC5A	52	13	130
O60503	ADCY9	121	30.25	302.5
Q99832	CCT7	38	9.5	95
Q9BXW7	CECR5	22	5.5	55
Q9Y3L5	RAP2C	18	4.5	45
O15427	SLC16A3	79	19.75	19.75
O00592	PODXL	44	11	110
Q9NSE4	IARS2	111	27.75	277.5
P36776	LONP1	43	10.75	107.5
P54886	ALDH18A1	61	15.25	152.5
P01893	HLA-H	44	11	110
O60716	CTNND1	70	17.5	175
Q31612	HLA-B	41	10.25	102.5
P52569	SLC7A2	50	12.5	125
P29317	EPHA2	83	20.75	207.5
Q9P1T7	MDFIC	24	6	60
Q92536	SLC7A6	70	17.5	175
P28288	ABCD3	44	11	6.29
P54760	EPHB4	76	19	190
Q99714	HSD17B10	18	4.5	9
Q01974	ROR2	91	22.75	227.5
Q14160	SCRIB	80	20	200
Q9NZM1	MYOF	133	33.25	332.5
Q9ULF5	SLC39A10	68	17	170
Q96QT4	TRPM7	168	42	420
Q13444	ADAM15	52	13	130
O15031	PLXNB2	81	20.25	202.5
P61073	CXCR4	23	5.75	57.5
Q96BD0	SLCO4A1	42	10.5	105
P16070	CD44	70	17.5	175
Q9Y5Z9	UBIAD1	12	3	30
Q03135	CAV1	8	2	20
Q9HD45	TM9SF3	48	12	120
P35610	SOAT1	15	3.75	37.5
Q9H2J7	SLC6A15	24	6	60
P48029	SLC6A8	52	13	130
Q9BXS9	SLC26A6	40	10	100
P26012	ITGB8	18	4.5	45
P04920	SLC4A2	32	8	80
Q8IW52	SLITRK4	52	13	130
P43007	SLC1A4	22	5.5	55
P43003	SLC1A3	19	4.75	47.5

Q14156	EFR3A	46	11.5	115
P30411	BDKRB2	30	7.5	75
P51798	CLCN7	22	5.5	55
Q99808	SLC29A1	27	6.75	6.75
Q9HCM3	KIAA1549	59	14.75	147.5
Q92692	PVRL2	52	13	130
Q13433	SLC39A6	70	17.5	175
Q96AC1	FERMT2	44	11	110
O75054	IGSF3	317	79.25	792.5
P11717	IGF2R	129	32.25	322.5
Q86X29	LSR	28	7	70
Q9Y4F1	FARP1	55	13.75	137.5
P15529	CD46	31	7.75	77.5
Q9H2H9	SLC38A1	91	22.75	227.5
P30825	SLC7A1	28	7	70
P11166	SLC2A1	27	6.75	67.5
Q9GZU7	CTDSP1	15	3.75	37.5
P31641	SLC6A6	51	12.75	127.5
O76082	SLC22A5	52	13	130
P14209	CD99	24	6	60
Q6PJF5	RHBDF2	21	5.25	52.5
O14828	SCAMP3	23	5.75	11.5
Q9H015	SLC22A4	13	3.25	32.5
Q96J84	KIRREL	26	6.5	65
P46531	NOTCH1	50	12.5	125
Q6UVK1	CSPG4	29	7.25	72.5
Q92930	RAB8B	30	7.5	75
P22695	UQCRC2	26	6.5	65
Q9UHR4	BAIAP2L1	28	7	70
P0DMV8	HSPA1A	43	10.75	7.17
P29966	MARCKS	25	6.25	62.5
Q9ULC8	ZDHHC8	18	4.5	45
Q14204	DYNC1H1	128	32	320
O60488	ACSL4	61	15.25	152.5
Q9ULH0	KIDINS220	92	23	230
O60779	SLC19A2	10	2.5	25
P08238	HSP90AB1	34	8.5	85
Q96TA1	FAM129B	29	7.25	72.5
P23470	PTPRG	42	10.5	105
Q8NE01	CNNM3	16	4	40