

Analysis of Cell Surface Reorganization During Cell Division

by

Nazlı Ezgi Özkan Küçük

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Analysis of Cell Surface Reorganization During Cell Division

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Nazlı Ezgi Özkan Küçük

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

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

Asst.Prof. Elif Nur Fırat Karalar (Koç University)

Asst.Prof. Bilal Ersen Kerman (Medipol University)

Prof. Batu Erman (Sabancı University)

Asst.Prof. Yongsoo Park (Koç University)

Date:



*To my dearest love, ASK
You make me certain, that I can fly...*

ABSTRACT

Plasma membrane is an essential compartment of the cells that contributes to a significant number of cellular events such as development, signal transduction and communication. Thus, understanding the changes in the plasma membrane is crucial to understand the regulation of these events. Cell division is one of these key processes in which the plasma membrane is physically re-arranged yet underlying biochemical regulations remains to be elucidated.

Mass spectrometry based identification of the surface proteins is a powerful approach to quantify the changes in the surface proteome however runs into limitations related with the low abundance of the surface proteins. Thus, establishment of the new approaches that can improve the plasma membrane protein identification is essential.

In the first part of my thesis, I developed an alternative strategy to enrich the cell surface proteins. In this method, carboxylic acid groups on the proteins are targeted with a carboxyl reactive biotin reagent enabling the streptavidin based affinity capture. I used SILAC method to quantitatively compare this new approach with a well-established one, amine reactive labeling. My analysis revealed that carboxyl labeling can effectively enrich the surface proteins and can be used as an orthogonal approach to amine reactive labeling.

In the second part of my thesis, I focused on two specific plasma membrane proteins; PCDH7 and PCDH1 which have mitotic surface localization. I tackled to understand the mechanism of cell cycle dependent translocation of PCDH7&1 and their roles in mitosis. I used proximity dependent biotinylation method and revealed a possible mechanism of the cell cycle dependent surface localization of PCDH7. Depleting the PCDH7 by CRISPR-Cas9 system resulted in abnormalities in the cell division implicating an important role of the PCDH7 during mitosis.

ÖZET

Plazma zarı, hücrenin temel bileşenlerinden biridir ve gelişim, sinyal iletimi, iletişim gibi birçok hücresel olayda önemli rol oynar. Bu nedenle plazma zarında meydana gelen değişiklikleri anlamak, bu hücresel olayların işleyişini anlamak için kritik öneme sahiptir. Hücre bölünmesi, plazma zarının fiziksel olarak yeniden düzenlendiği kilit süreçlerden birdir ancak bu düzenlemelerin ardındaki biyokimyasal regülasyonlar hala aydınlatılmayı beklemektedir. Kütle spektrometresi (MS) temelli analizler, plazma zarı proteinlerinin tanımlanması ve hücre yüzey proteomundaki değişikliklerin niceliksel takibinin yapılabilmesi için güçlü bir yaklaşımdır. Ancak yüzey proteinlerin görece az miktarı, MS temelli analizlerin uygulanabilirliğini kısıtlamaktadır. Bu nedenle, hücre yüzey proteinlerin izolasyon verimliliğini arttırabilecek yeni yöntemlerin geliştirilmesi bir gerekliliktir.

Tezimin ilk bölümünde, hücre yüzey proteinlerinin zenginleştirilmesi için alternatif bir yaklaşım geliştirdim. Bu yöntemde, proteinlerdeki karboksilik asit grupları, karboksil-reaktif bir biyotin bileşiği ile işaretlenmekte ve biyotin-streptavidin çekimi kullanılarak izole edilebilmektedir. Geliştirdiğim bu yeni yöntemi, yüzey proteini izolasyonu için halihazırda kabul gören bir yöntem olan amin-reaktif işaretleme yöntemi ile SILAC (hücre kültüründe aminoasitleri izotop ile etiketleme) metodu kullanarak kıyasladım. Yaptığım niceliksel kıyaslamaya göre, karboksil-reaktif işaretleme yöntemi başarılı bir şekilde hücre yüzey proteinleri zenginleştirebilmektedir ve amin-reaktif işaretleme yöntemine alternatif/tamamlayıcı olarak kullanılabilir.

Tezimin ikinci kısmında, daha önce mitoz-spesifik yüzey lokalizasyonu gösterdiğini belirlediğimiz iki protein; PCDH7 ve PCDH1'e yoğunlaştım. Bu proteinlerin nasıl hücre-döngüsüne bağlı bir şekilde yüzeye gittiklerini anlamaya ve hücre bölünmesi sırasındaki rollerini belirlemeye çalıştım. Yakınlığa-bağlı biyotinleme yöntemi kullanarak, mitoz-spesifik hücre yüzey lokalizasyonunun olası mekanizmasını ortaya çıkardım. CRISPR-Cas9 sistemi kullanılarak PCDH7 protein seviyesi azaltıldığında hücre bölünmesinde anormallikler meydana gelmektedir. Bu durum, PCDH7'nin hücre bölünmesi için önemli bir rolü olduğunu işaret etmektedir.

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NOMENCLATURE

17-ODYA	17-Octadecynoic Acid
2BP	2-Bromopalmitate
AGC	Automatic gain control
Asp	Asparagine
ATP	Adenosine triphosphate
BG	Background
BioID	Proximity dependent biotin identification
BSA	Bovine Serum Albumin
C	Cysteine
CAK	CDK-activating kinase
CC	Cellular component
CCD	Charge-coupled device
CDK	Cyclin-dependent kinase
cGMP	Cyclic guanosine monophosphate
CKI	CDK inhibitor
CM	Conserved motif
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4',6-diamidino-2-phenylindole
DDA	Data dependent acquisition
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
DTT	Dithiothreitol
E. coli	Escherichia coli
EC	Extracellular cadherin
ECL	Enhanced chemiluminescence
EDC	1 ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EEA1	Early endosome antigen 1
eGFP	Enhanced Green Fluorescence Protein
EGFR	Epidermal Growth Factor Receptor
EMCCD	Electron Multiplying Charge-coupled device
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ERM	Ezrin-Radixin-Moesin proteins

FBS	Fatal Bovine Serum
FDR	False discovery rates
FRAP	Fluorescence Recovery After Photobleaching
G1	Gap1
G2	Gap2
GFP	Green Fluorescence Protein
Glu	Glutamine
GO	Gene ontology
HA	Hemagglutinin
HCD	Higher-energy collisional dissociation
HEK293	Human Embryonic Kidney Cells
HeLa	Human cervical cancer cell line S3
HPLC	High performance liquid chromatography
HRP	Horseradish Peroxidase
LAMP1	Lysosome-associated membrane glycoprotein 1
LB	Lysogeny broth medium
LC	Longer chain
LC-MS/MS	Liquid Chromatography-MS/MS
LTQ	Linear Triple Quadrupole
Lys	Lysine
M	Mitosis
MES	2-ethanesulfonic acid
MS	Mass Spectrometry
NA	Numerical apparatus
NCE	Normalized collision energy
NHS	N-Hydroxysuccinimide
NP40	Nonyl phenoxypolyethoxylethanol
PALM	Palmitate
PBS	Phosphate buffered saline
PCDH1	Protocadherin 1
PCDH7	Protocadherin 7
PCDH7-BAC-GFP	Protocadherin 7-Bacterial Artificial Chromosome GFP
PCDH7cytdel	Protocadherin 7 cytoplasmic deleted
Pcdhs	Protocadherins
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
Pen-Strep	Penicillin-Streptomycin
PFA	Paraformaldehyde

PHH3	Phospho-Histone H3
PP1α	Protein Phosphatase 1 alpha
PP2A	Protein phosphatase 2A
Pro	Prodomain
Rb	Retinoblastoma
RNA	Ribonucleic acid
ROI	Region of Interest
Rpm	Round per minute
S	Synthesis
S	Signal peptide
SAX	Strong Anion Exchange
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
sgNT	Non targeting sgRNA
sgRNA	guide RNA
SILAC	Stable isotope labeling with amino acids in cell culture
SNP	Single nucleotide polymorphisms
STC	S-Trityl-L-cysteine
TBS	Tris buffered saline
TBTA	Tris((1-benzyl-4-triazolyl)methyl)amine
TCEP	(tris(2-carboxyethyl)phosphine)
TFRC	Transferrin Receptor
Tx	Triton X
Ub	Unbound
W	Tryptophan
WCL	Whole cell lysate
WST1	Water Soluble Tetrazolium Salt-1

Chapter 1

INTRODUCTION

Plasma membrane is one of the most important compartments of the cells. It acts as a physical barrier that protects and isolates individual cells from their surroundings while, providing a hub for the signal transduction to communicate with the environment. Apart from being a barrier or the communication center, plasma membrane participate a variety of the cellular events such as development, differentiation, immune response and cell division. However, role of the plasma membrane in cell division is relatively understudied.

Dividing cells undergoes dramatic surface re-arrangements; they retract their margins, loosen their contacts and gain a spherical shape. This process is known as the mitotic cell rounding and was shown to be important for the fidelity of the division since disturbance of it caused chromosomal segregation errors. Unlike other master events that control the cell division, we only have limited knowledge about the molecular mechanism and regulation of the cell rounding and its relation with the regulator of the cell division machinery.

One possible reason for this lack of information could be the challenges for working with proteins that localize to plasma membrane. Enrichment of the plasma membrane proteins is already challenging itself, making quantitative comparisons even more difficult.

In a previous research from our laboratory, we analyzed the cell surface proteome of dividing cells and identified a group of proteins that selectively localize to the cell surface during mitosis [1]. Protocadherins PCDH7 and PCDH1 are enriched in the mitotic plasma membrane and were shown to be important for the cell rounding process. Therefore

understanding their role in the plasma membrane during cell division is important to understand the mitotic rounding process.

The overall aim of my study is to reveal the mechanism of cell cycle dependent localization of PCDH7&1, and uncover their role in cell cycle progression. At the same time, I aim to develop an alternative strategy to isolate cell surface proteins that can be used as an orthogonal approach to increase the efficiency and sensitivity of proteomic analysis.

Chapter 2 covers my studies on the development of an alternative method to isolate cell surface proteins. A more detailed introduction is represented in Section 2.1, while review of the current literature is represented in Section 2.2. Details of the methods and the materials are explained in Section 2.3. Results and related discussions are represented in Section 3.4 and the conclusion is represented in Section 2.5.

Chapter 3 covers my studies about the mechanism of the cell cycle dependent plasma membrane localization of PCDH7 and its role in mitosis. A more detailed introduction is represented in Section 3.1, while review of the current literature is represented in Section 3.2. Details of the methods and the materials are explained in Section 3.3. Results are represented in Section 3.4. Detailed discussion and conclusion is represented in Section 2.5.

Chapter 2

DEVELOPMENT OF AN ORTHOGONAL STRATEGY TO ISOLATE CELL SURFACE PROTEINS

2.1 INTRODUCTION

Plasma membrane proteins are involved in key cellular events. Besides, they have great potential as the drug targets. Thus, understanding the changes in the plasma membrane proteome composition is critical to understand the mechanism of many cellular events and the pathological conditions related to them. Hence, proper identification and the quantification of the cell surface proteins are critical.

Mass spectrometry based identification of the plasma membrane proteins is a powerful approach however it runs into some limitations, mostly related to the hydrophobic nature and the low abundance of the plasma membrane proteins. Plasma membrane covers only a small proportion of the cell mass, when compared to the cytoplasm and the inner membranes. Accordingly, abundance of the plasma membrane proteins is much lower than their cytoplasmic counterparts. This difference decrease the identification possibility of the plasma membrane proteins especially with the data dependent acquisition (DDA) methods in which the most abundant peptides are selected for further fragmentation and identification. In addition, hydrophobicity and low solubility of the plasma membrane proteins add another level of difficulty for their identification.

Thus, different enrichment strategies for the plasma membrane proteins have been developed over the years. However, further improvement is needed to increase the yield. In this study, I aim to develop an alternative strategy to isolate cell surface proteins that can be used as an orthogonal approach to increase the efficiency and sensitivity of proteomic analysis.



2.2 LITERATURE REVIEW

2.2.1 Importance and the Challenges of the Cell Surface Proteome Studies

The cell surface is one of the most important cellular compartments due to its central role in nutrient uptake, cell-cell communication, homeostasis, signaling and tissue development. Not surprisingly, many of the approved drugs for different cancer types and autoimmune diseases target cell surface proteins [2]. Characterizing the composition of cell surface proteins and their dynamic regulation, is therefore critically important for understanding many cellular processes and diseases.

Conventional antibody based methods have been helpful, yet limited for the comprehensive studies to cover whole cell surface proteome [3]. In contrast, quantitative proteomics allows genome-scale multiplexed protein detection, though it currently requires a larger amount of cells to achieve this. Despite their importance, large-scale proteomic analysis of cell surface proteins has been challenging. The hydrophobic nature and poor solubility are the main obstacles in the purification and mass spectrometry (MS) based-proteomic analysis of plasma membrane proteins [4-6]. Even though two-dimensional gel-based separation methods, coupled with the LC-MS/MS have been successfully used for the comprehensive characterization of the membrane proteome in earlier studies, poor recovery of the hydrophobic peptides from the gel matrix has limited the capacity of this approach [4, 6]. Development of different sample separation methods and improved instrumentation has partly compensated the limitations that arise from the hydrophobic nature of the plasma membrane proteins [7, 8]. However, hydrophobicity is not the only problem. In addition to their insoluble nature, their relatively low abundance, when compared to cytosolic and other membrane-bound proteins, makes the identification of plasma membrane proteins even more challenging. Highly abundant cytoplasmic proteins

tend to mask the signals from less abundant plasma membrane proteins, and thereby cause their under-representation in standard proteomic data [4, 9].

2.2.2 Cell Surface Isolation Methods

Different plasma membrane protein isolation methods have been developed throughout the years to overcome these problems. Earlier approaches used centrifugation methods to fractionate the different compartments of the cells (reviewed in [10]). Especially, density gradients created by ultracentrifugation methods provided a better resolution for the separation of different organelles. In continuous and discontinuous gradients, different particles are sequestered in the specific zones in respect to their densities. When combined with high speed centrifugation (e.g. at 100,000xg), sucrose gradients can successfully separate the crude membrane fraction into different organelle membrane fractions such as plasma membrane, endoplasmic reticulum and Golgi [10] [11-13].

Although this is a relatively simple and robust method, it requires large quantities of the starting material and is highly perturbing to ongoing cellular processes such as post-translational modifications. Moreover, organelles with similar densities and other organelle membranes cause contamination between the different fractions and under-representation of isolated plasma membrane fractions. Therefore, in practice, use of ultracentrifugation in the plasma membrane protein enrichment is often restricted to the enrichment of the total membrane fraction. This crude membrane fraction is further enriched for the plasma membrane proteins by different strategies [9, 10, 14, 15].

Another approach to enrich the plasma membrane proteins is to strip the weakly associated proteins from membranes. Incubation of the samples with carbonate in basic pH and delipidation using ethanol or methanol/chloroform can be combined with various methods such as centrifugation to increase the plasma membrane enrichment efficiency [4]. Similarly, proteolytic digestion can also be used to “shave” the plasma membrane proteins.

Incubation of the intact cells with trypsin for short duration can be used to capture the surface exposed domains of the membrane proteins [4]. Alternatively, enzymatic digestion after cell lysis can be used to enrich the membrane spanning domains of the transmembrane proteins [4, 16]. Enzymatic shaving omits the need for membrane solubilization and advantageous especially for the rigid membranes [4].

In order to enrich the plasma membrane proteins more selectively, chemically targeted cell surface capture strategies have been developed. These methods either use cell-impermeable chemical labeling reagents, such as biotin-reactive esters, or chemically modified coating materials to capture plasma membrane proteins.

A sophisticated method that combines the centrifugation and the chemical targeting uses cationic colloidal silica-beads to capture the plasma membrane proteins. In this method, negatively charged plasma membranes of intact cells are coated with cationic silica beads. Further crosslinking of the silica beads with an anionic polymer results formation of plasma membrane sheets which increase the density of the plasma membrane fraction and enable more efficient collection by centrifugation [17]. This protocol is further adapted for different cell types including suspension and adherent tissues as well as in vivo labeling of primary tissues which are in direct contact with the vasculature system [10, 18-21]. Use of colloidal silica-beads provides a powerful tool for the capture of plasma membrane proteins, especially for the in vivo studies, yet only advantageous for the soft tissues which are easier to homogenize [4, 10].

A similar approach uses superparamagnetic nano particles to enrich plasma membrane proteins. Plasma membrane proteins of intact cells are labeled with phospholipid-coated superparamagnetic nano particles and separated from the cytoplasmic proteins using magnetic field following the cell lysis and centrifugation [4, 22]. Together with the high recovery of the plasma membrane proteins, the ability to isolate membrane protein complexes enable lipidomic and glycomic analyses [4].

2.2.3 Biotinylation of the Cell Surface Proteins

Diverse chemically labeling strategies reagents have been directed at surface exposed plasma membrane proteins. Among these methods, biotin labeling of the cell surface proteins is one of the most popular approaches. Despite their broad diversity, nearly all biotinylation reagents are composed of three common segments: a biotin moiety to be used for the subsequent interactions with avidin/streptavidin-based reagents, a linker to provide required space between the biotin and reactive moiety, and finally, a reactive group to covalently bind the reagent to proteins [23].

Different reactive groups can be utilized to target plasma membrane proteins in different aspects. The assumption that most plasma membrane proteins are glycosylated leads to the usage of sugar moieties of the glycoproteins to capture surface proteins [10, 24-26]. Free sulfhydryl groups of the cysteine residues can also be targeted for the protein biotinylation [23]. However, the most commonly targeted groups are primary amines which can be found in the lysine (Lys) residues and the N-termini of the proteins. First and the most widely used amine-reactive biotin derivative is the N-hydroxysuccinimide (NHS) esters of biotin [23] (**Figure 1**). Membrane-impermeable derivatives of these amine-reactive biotinylation reagents are broadly used to enrich cell surface proteins in different studies [1, 27-32].

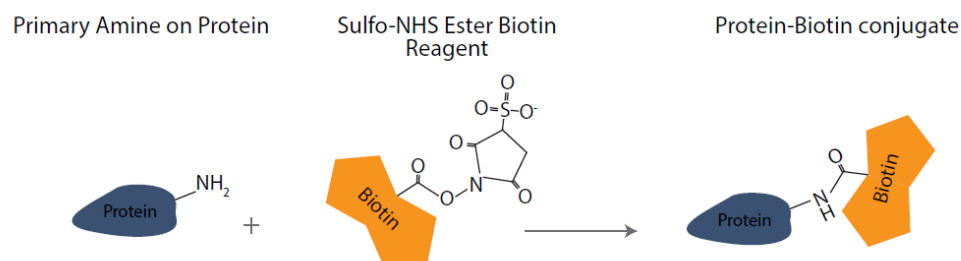


Figure 1. Amine reactive biotinylation reaction.

Linker regions of the biotinylation reagents can also be modified to fulfill different needs and improve the efficiency of plasma membrane protein isolation. Longer chain (LC) homolog of the NHS-biotin (NHS-LC-biotin) has been developed to increase the accessibility of the biotin molecule to avidin/streptavidin reagents. This reagent was further modified to its water soluble analog Sulfo-NHS-LC-biotin and became one of the most popular biotinylation reagents. Another analog with the increased spacer length (Sulfo-NHS-LC-LC-biotin) was also proposed yet; hydrophobic nature of the LC linker limits the availability of the biotin molecule for the avidin/streptavidin reagents. Thus, PEG-based spacers have been developed as the hydrophilic alternatives of the LC-linkers with higher spacer lengths. Cleavable linkers have also been introduced for easy release of the biotinylated proteins subsequent to the avidin/streptavidin capture. Different cleavage strategies such as photo-cleavage or utilization of the reducing agents have been employed in various different reagents [23].

In spite of this vast diversity of the present biotinylation reagents, coverage of the plasma membrane proteins is often below the desired levels for MS-based analysis necessitating alternative method developments to improve cell surface isolation efficiency. Thus, different approaches are often compared, and sometimes combined to find the optimal level of plasma membrane protein enrichment [33-35].

In this study, we aimed to establish an alternative cell surface labeling and isolation method to improve the proteomic analysis of cell surface proteins. Although it is not commonly targeted for modification in proteomics, carboxyl groups on aspartic acid (Asp), glutamic acid (Glu) and C-termini are able to react efficiently and selectively with water-soluble carbodiimides to form active esters that can be trapped using an appropriate nucleophile. An amine-derivative of biotin can be used to target carboxyl groups activated by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)[23] (**Figure 2**). Although, this is well known in bioconjugate chemistry of purified peptides and proteins,

this strategy has not, to our knowledge, been reported for surface labeling of plasma membrane proteins. The frequencies of Asp/Glu residues in the proteins, especially in the extracellular region of transmembrane proteins, make this method appealing. According to the recent databank entries, the frequency of Lys, Asp and Glu residues in the proteins are 5.84, 5.45 and 6.75, respectively [36]. For the membrane proteins, the frequency of Lys is reported as 4.4, while the sum of Asp and Glu is 8.4 [37]. 13% of the extracellular regions of those transmembrane proteins are estimated to have Asp and Glu residues, while Lys residues are reported as 5.25% [38]. Thus, labeling the carboxyl groups has the potential of increasing the yield of cell surface protein enrichments.

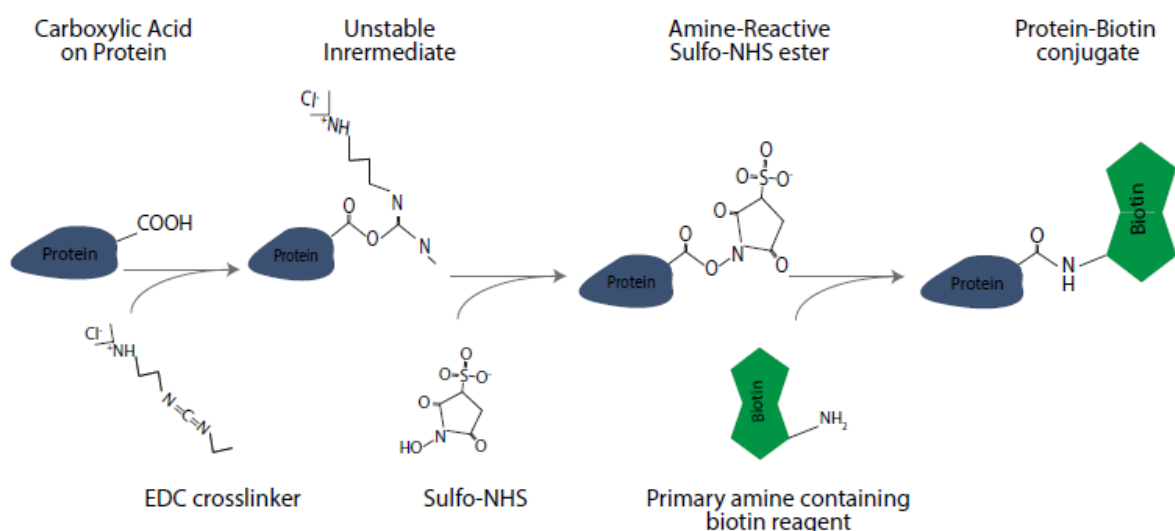


Figure 2: Carboxyl reactive biotinylation reaction.

We optimized biotinylation of cell surface-exposed carboxyl groups, then adapted this to living cells and quantitatively compared the amine- and carboxyl-reactive biotinylation methods using SILAC [39]. Our data suggests that the carboxyl-reactive biotinylation approach can effectively label and isolate cell surface proteins. It requires more steps but appears to be more selective than amine reactive biotinylation. Taken together, we present the carboxyl-reactive biotinylation as an orthogonal approach for amine-reactive biotinylation to cast a wider net for a comprehensive identification of cell surface proteins.

2.3 MATERIALS AND METHODS

2.3.1 Cell Culture

HeLa S3 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). For SILAC experiments, HeLa S3 cells were grown as described previously [1, 40]

2.3.2 Antibodies and Western Blot

For immunostaining or Western blotting, the following primary antibodies and reagents were used: Streptavidin, Alexa Fluor 488 (s-32354; Invitrogen), EGFR (SC-03; Santa Cruz), TFRC (SC-32272; Santa Cruz) and Actin (ab6276; Abcam).

For Western blot analyses, samples were separated by molecular weight using 10% SDS-PAGE gels, and transferred to a nitrocellulose membrane. The membrane was blocked with 4% w/v nonfat dry milk in TBS-0.1% Tween-20 and probed with 1 µg/ml of the described primary antibody diluted in 2% BSA TBS-0.1% Tween-20. The signal was visualized using ECL (32106; Pierce) detection of the HRP-conjugated secondary antibodies (7074S, 70765; Cell Signaling).

2.3.3 Immunostaining and Microscopy

HeLa S3 cells grown on cover slips (~80% confluence) were biotinylated with either carboxyl or amine group labeling. Cells were fixed with 3% paraformaldehyde in PBS for 10 minutes at room temperature, blocked with 2% BSA in PBS for 30 minutes and then incubated with 2 µg/ml fluorescence streptavidin in 2% BSA in PBS for 30 minutes at room temperature. After nuclear staining with DAPI (10 µg/ml), the cover slips were mounted and sealed. Confocal images were taken by Nikon-Eclipse 90i confocal microscope.

2.3.4 Cell Viability Assay

The cell viability was assessed using the WST1 (Water Soluble Tetrazolium Salt-1) (5015944001; Roche) assay according to the manufacturer's instructions.

2.3.5 Biotinylation of the Cell Surface Proteins Using Amine Reactive Labeling

HeLa S3 cells (~80% confluence) were rinsed twice with PBS⁺ (Phosphate buffered saline supplemented with 0.1 mM CaCl₂, 1 mM MgCl₂) pH 7.4 and once with PBS⁺ pH 8.0 and then incubated with 5 mM S-NHS-SS-biotin (21331; Pierce) in PBS⁺ pH 8.0 for 30 minutes at 4°C with gentle shaking. Residual biotin was quenched with 100 mM glycine in PBS pH 8.0 and then washed twice with PBS pH 7.4. Cells were scraped from plates in cold PBS pH 7.4 and snap frozen.

2.3.6 Optimization of the Carboxyl Reactive Labeling for Purified Proteins

1.5 mg/ml Bovine Serum Albumin (BSA) (SC-2323; Santa Cruz) was used in all reactions. PBS⁺ or MES buffers were used in biotinylation reactions.

Two-Step Reaction with PBS/MES: BSA was pre-incubated with 10 mM NHS, 5 mM EDC in PBS⁺ pH 5.7 or MES pH 5.4 for 15 minutes at 4°C and then incubated with 5 mM Amine PEG2 biotin in PBS⁺ pH 8.0 or MES pH 5.4 for 30 minutes at 4°C.

One-Step Reaction with MES: BSA was simultaneously incubated with 10 mM NHS, 5 mM EDC, 5 mM Amine PEG2 biotin in MES pH 5.4 for 40 minutes at 4°C.

Amine reactive biotinylation: BSA was incubated with 5 mM S-NHS-SS-biotin (21331; Pierce) in PBS⁺ pH 8.0 for 30 minutes at 4°C.

2.3.7 Optimization of the Carboxyl Reactive Labeling for Live Cells

Two-Step Reaction with PBS/MES: HeLa S3 cells (~80% confluence) were rinsed twice with PBS⁺ pH 7.4 and once with PBS⁺ pH 5.7 or MES. Then they were pre-incubated with 5 mM EDC/10 mM NHS in PBS⁺ pH 5.4 or MES for 15 minutes at 4°C with gentle shaking. After pre-incubation, cells were directly incubated with 5 mM carboxyl reactive

biotin (in-house synthesized or commercially available (21346; Thermo, BT3761; Interchim) in PBS⁺ pH 8.0 for 40 minutes at 4°C with gentle shaking. Residual biotin was quenched with 100 mM glycine in PBS pH 8.0 and then washed twice with PBS pH 7.4. Cells were scraped from plates in cold PBS pH 7.4 and snap frozen.

One-Step Reaction with PBS/MES: HeLa S3 cells (~80% confluence) were rinsed twice with PBS⁺ (supplemented with 0.1 mM CaCl₂, 1 mM MgCl₂) pH 7.4 and once with PBS⁺ pH 5.4. Then they were simultaneously incubated with 10 mM NHS, 5 mM EDC, 5 mM Amine PEG2 biotin in pH 5.4 for 40 minutes at 4°C with gentle shaking. Residual biotin was quenched with 100 mM glycine in PBS pH 8.0 and then washed twice with PBS pH 7.4. Cells were scraped from plates in cold PBS pH 7.4 and snap frozen.

2.3.8 Biotinylation of the Cell Surface Proteins Using Carboxyl Labeling

HeLa S3 cells (~80% confluence) were rinsed twice with PBS⁺ pH 7.4 and once with PBS⁺ pH 5.7. Then they were pre-incubated with 5 mM ECD/10 mM NHS in PBS⁺ pH 5.4 for 15 minutes at 4°C with gentle shaking. After pre-incubation, cells were directly incubated with 5 mM carboxyl reactive biotin (in-house synthesized or commercially available (21346; Thermo, BT3761; Interchim) in PBS⁺ pH 8.0 for 40 minutes at 4°C with gentle shaking. Residual biotin was quenched with 100 mM glycine in PBS pH 8.0 and then washed twice with PBS pH 7.4. Cells were scraped from plates in cold PBS pH 7.4 and snap frozen.

2.3.9 Isolation of Biotinylated Proteins

Biotinylated and frozen cells were lysed in a buffer (10 mM TrisCl pH 7.6, 0.5% SDS, 2% NP40, 150 mM NaCl, 1 mM EDTA, 10 mM Iodoacetamide) containing protease inhibitors (88666; Pierce). Cell lysates were centrifuged at 7,500 rpm for 15 minutes at 4°C and supernatants were incubated with pre-conditioned Streptavidin Plus UltraLink Resin (53117; Pierce) overnight at 4°C.

Unbound samples were collected and beads were washed with lysis buffer three times. For the Western blot analysis, biotinylated proteins were eluted by boiling at 70°C for 20 minutes in SDS sample buffer including 100 mM DTT with agitation. For the mass spectrometry analysis, samples were preceded to on-bead digestion.

2.3.10 On-Bead Tryptic Proteolysis

Streptavidin agarose beads were washed extensively with 8 M urea in 0.1 M Tris/HCl pH 8.5 followed by reduction and alkylation steps. Finally, the beads were washed with 50 mM ammonium bicarbonate and incubated with trypsin overnight in 50 mM ammonium bicarbonate at 37°C. The resulting peptides were collected via centrifugation at 1,000g for 5 min. The beads were then rinsed with 50 mM ammonium bicarbonate and this second tryptic fraction was pooled with the first one. After acidification, tryptic peptides were desalted with stage tips as described in [41, 42], and subsequently fractionated with the Strong Anion Exchange (SAX) method [43].

2.3.11 Data Acquisition

Peptides were analyzed by online C18 nanoflow reversed-phase nLC (NanoLC-II, Thermo Scientific) combined with orbitrap mass spectrometer (Q Exactive Orbitrap, Thermo Scientific) or C18 nanoflow reversed-phase HPLC (Eksigent nanoLC 2D) combined with a linear ion trap/orbitrap mass spectrometer (LTQ-Orbitrap, Thermo Scientific). Samples were separated in an in-house packed 100 μm i.d. $\times$ 23 cm C18 column (Reprosil-Gold C18, 5 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 300–1,500 m/z ; automatic gain control (AGC) target 1e6; maximum injection time 32 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 85 ms). The isolation window for MS/MS was 2.0 *m/z*.

2.3.12 Data Processing

Raw files were processed with MaxQuant version 1.5.2.8 [44]. 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 20 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 2015) containing 21,039 entries using the Andromeda search engine integrated into the MaxQuant environment [45]. For the analysis of the SILAC dataset, arginine-10 and lysine-8 were assigned as the heavy modifications. 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 MaxQuant results. Identified proteins were annotated using Gene Ontology terms. Annotations were downloaded from Uniprot [46].

2.3.13 GO Enrichment Analysis of Plasma Membrane Proteins

Percentages of identified plasma membrane proteins were calculated using the annotated proteins in each biological replicate of carboxyl- and amine-reactive methods. Proportions z-test was employed to test the significance of the difference between plasma membrane protein percentages in carboxyl- and amine-reactive biotinylation. The Gene Ontology (GO) enrichment analysis was performed both for carboxyl- and amine-reactive biotinylation methods. Top 10 cellular component (CC) enriched annotations for total and

plasma membrane proteins of carboxyl and amine labeling methods were identified via Enrichr [47]. Reported scores are the p-values converted to $-\log_{10}(\text{p-value})$.

2.3.14 SILAC Data Analysis

For the SILAC experiments, Amine/Carboxyl ratios of proteins were normalized by dividing each ratio by the population median, followed by converting the ratios to $\log_2$ scale. Perseus's "Significance A" method was used to determine outlier ratios [48]. In this method, right and left standard deviations are defined according to the distance of the percentiles 15.87 and 84.13 in relation to the 50 percentile of the data. Ratios which fall outside of the normal distributions with right and left standard deviations around the right and left percentiles are defined as the outliers. Based on the outlier ratios, proteins were separated into three sets. Proteins with negative outlier ratios are the proteins that were identified in higher quantities via carboxyl labeling. Similarly, proteins with positive outlier ratios are the proteins that were identified in higher quantities via amine labeling. Proteins with non-outlier ratios are the proteins that were identified in similar abundances in both methods. These three sets of proteins were named in the figures as 'Carboxyl', 'Amine' and 'In both', respectively. For each set of protein, percentages of plasma membrane, inner membrane and cytoplasm annotated proteins were calculated.

2.4 RESULTS AND DISCUSSION

2.4.1 Labeling Cell Surface using Carboxyl-Reactive Biotinylation

To bring an alternative cell surface isolation strategy, we aimed to develop and evaluate a novel approach that targets Asp, Glu residues and the C-terminus of cell surface proteins. In this labeling strategy, amine derivatized biotin molecules react with the carboxylic acid groups on the Asp, Glu residues and the C-terminus of cell surface proteins by activating them to reactive esters with carbodiimides such as EDC. Activated carboxyl groups then react with an amine derivative of biotin reagent. Activation of carboxylate groups has often been used for protein and peptide modification, but has not been used before, to our knowledge, for labeling live mammalian cells. To evaluate the labeling efficiency, we compared our approach with a proven one from the literature; surface labeling of Lys residues with a non-permeable amine-reactive biotinylation reagent, Biotin-linker-SNHS ester [23]. After cell surface labeling with biotin, we performed affinity isolation of biotinylated proteins using streptavidin to enrich cell surface proteins.

Activation of the carboxyl groups and subsequent biotinylation reactions are mostly performed in the MES buffer with pH 5 for purified protein labeling [49]. However, these conditions are not optimal for living cells. Thus, we tested different buffers and reaction conditions for both purified proteins and live cells to minimize cell death during labeling. Among them, PBS supplemented with calcium and magnesium gave the best result, therefore, we replaced MES buffer with it.

Typically for the purified proteins, activation of carboxylates with EDC and sulfo-NHS ester and reaction of active esters with amines are performed in a one-step reaction at pH 5 [49]. To maximize the labeling efficiency, we performed a two-step reaction where carboxylates were activated with EDC and sulfo-NHS ester at pH 5.4 followed by the

reaction of active esters with amines at pH 8. We tested both one-step (**Figure 3A-III**) and two-step reactions (**Figure 3A-II**) for the biotinylation efficiency using purified proteins and living cells. Purified proteins were biotinylated at higher levels in the one-step reaction using MES buffer compared to the two-step reaction (**Figure 3B**). In parallel, to evaluate the labeling efficiency and selectivity in live cells, we pulled down biotinylated proteins and immunoblotted for the surface protein EGFR (Epidermal Growth Factor) and a cytoplasmic protein, actin (**Figure 3C**). One-step reactions did not enrich the cell surface protein EGFR in living cells (**Figure 3C lanes 6 and 9**). This indicates that although biotinylation of purified proteins was higher using the one-step reaction, the two-step reaction was more efficient in terms of biotinylation of cell surface proteins in living cells. Therefore, for carboxyl-reactive biotinylation of surface proteins, we continued with the two-step reaction protocol (**Figure 3A-II**).

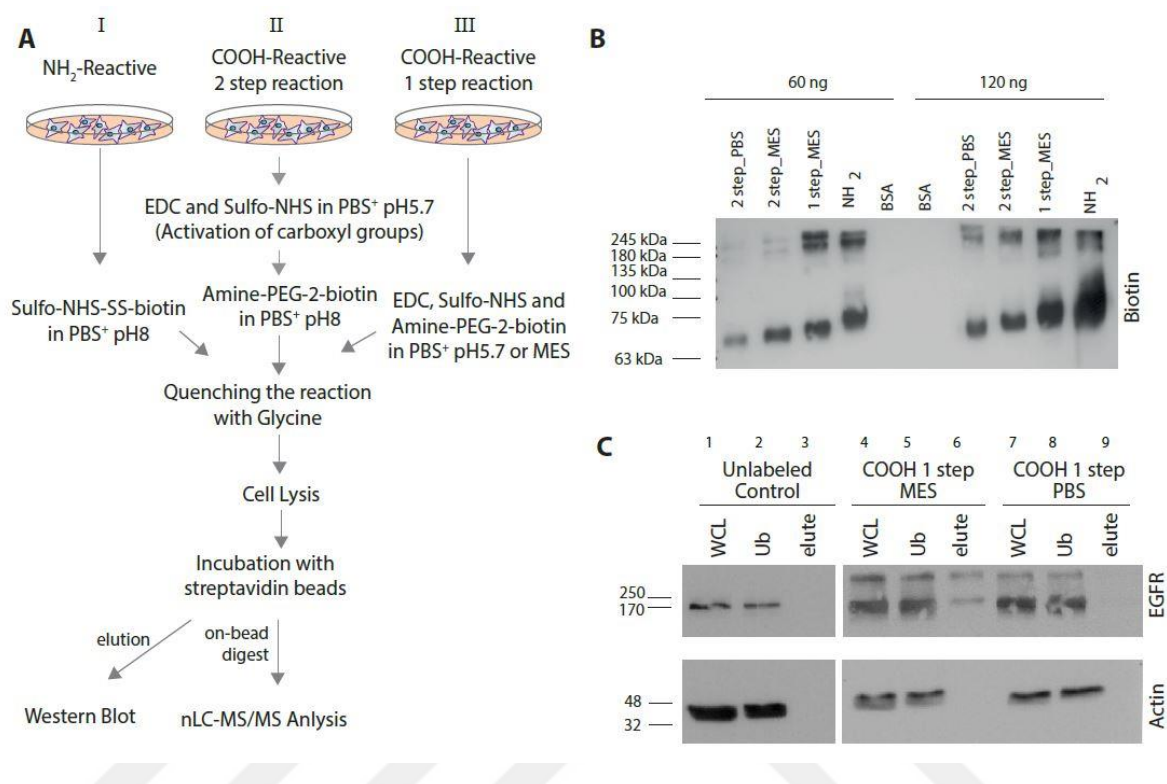


Figure 3: Optimization of the carboxyl reactive biotinylation conditions for live cells.

A. Experimental outline for the optimization of the carboxyl reactive biotinylation. Different reaction conditions are indicated with numbers I, II and III. Workflow of amine reactive biotinylation was illustrated in reaction I. One- and two-step reactions for carboxyl reactive biotinylation are illustrated in reactions III and II, respectively. **B.** Western blot of biotinylated BSA with different reaction conditions. Biotinylated BSA protein was detected using a biotin antibody. Note that increased amount of biotinylation causes a bigger shift on the molecular weight of the BSA protein (molecular weight of the BSA is approximately 66 kDa). Not biotinylated BSA was loaded as negative control. **C.** Western blot analysis to control the efficiency of the surface biotinylation. The biotin-labeled cells were lysed and biotinylated surface proteins were enriched with streptavidin bead incubation followed by elution. Unlabeled cells were used as negative control. WCL: Whole cell lysate, Ub: Unbound, elute (cell surface) fractions were blotted against a cell surface marker, EGFR, and a cytoplasmic marker, actin. [50]

Besides the carboxyl reactive biotinylation, we also performed surface labeling of lysine residues with an amine-reactive biotinylation reagent. Both reactions selectively labeled the surface proteins EGFR and TFRC (Transferrin Receptor Complex) as expected, but the amine-reactive biotinylation recovered more cell surface proteins than the carboxyl labeling approach (**Figure 4B**-lanes 3 and 6). The absence of actin among pulled proteins from both carboxyl and amine reactions suggests that both protocols are well optimized for the cell surface biotinylation and minimization of cytoplasmic contamination (**Figure 4B**). Next, we assessed cell surface labeling using fluorescently labeled soluble streptavidin by confocal microscopy. Both amine and carboxylate biotinylation decorated the cell surface efficiently (**Figure 4C**). As carboxyl-reactive biotinylation is performed in living cells for the first time, we tested its possible side effects on the cell viability with the WST-1 assay. Both carboxyl- and amine-reactive biotinylation did not have major effects on the cell viability (**Figure 4D**).

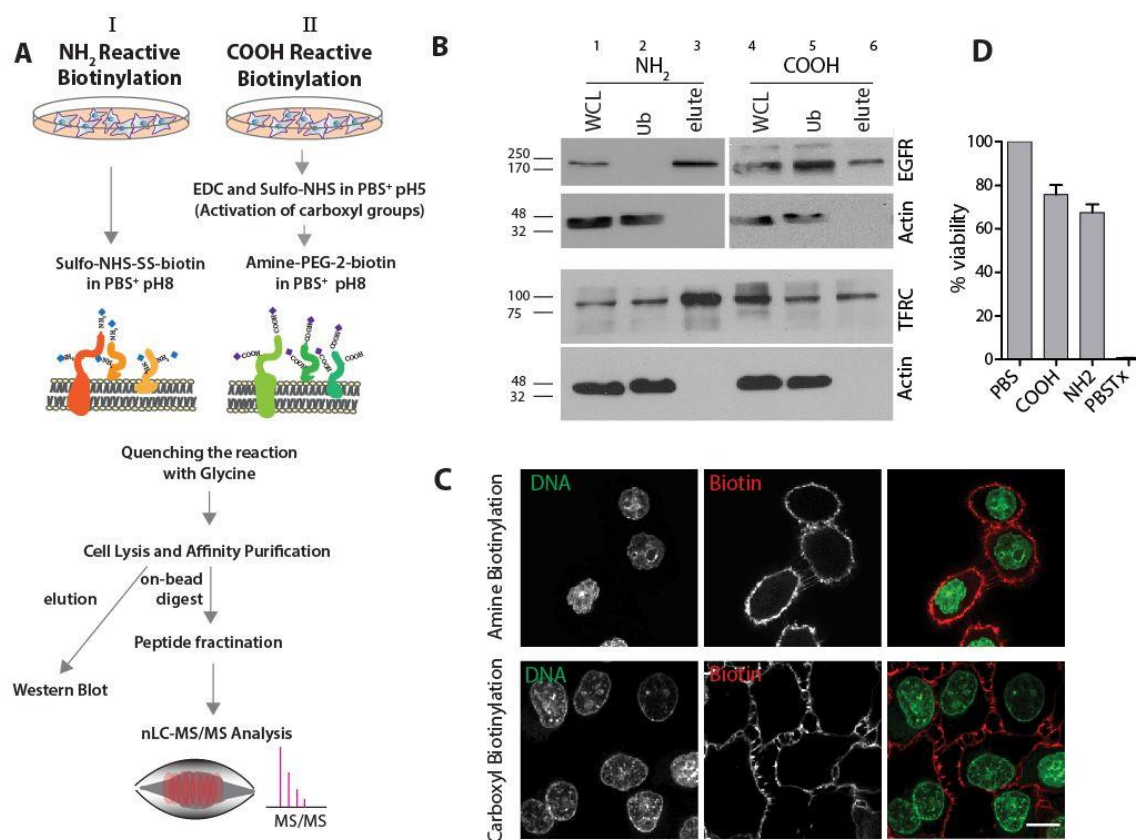


Figure 4: Experimental workflow and the efficiency of the plasma membrane labeling. **A.** Experimental outline for the carboxyl- and amine-reactive biotinylation. **B.** Western Blot analysis to control the efficiency of the surface biotinylation. The biotin-labeled cells were lysed and biotinylated surface proteins were enriched with streptavidin bead incubation followed by elution. WCL: Whole cell lysate, Ub: Unbound and elute (cell surface) fractions were blotted against cell surface markers, EGFR, TFRC, and a cytoplasmic marker, actin. **C.** Immunostaining of HeLa cells following the surface biotinylation. Surface labeling was visualized using FITC-labeled streptavidin (red) and merged with DNA (green). Cell surface was labeled using amine biotinylation (top panel) and the carboxyl biotinylation (bottom panel). Scale bar, 10 μ m. **D.** WST-1 cell viability assay. PBS-Tx treated cells were used as negative control and PBS treated cells were used as positive control.[50]

2.4.2 Comparison of Amine versus Carboxyl Biotinylation using Mass Spectrometry

We next compared our biotinylation protocols comprehensively using mass spectrometry. Approximately 8×10^6 cells were labeled with either amine- or carboxyl-reactive biotin reagents. Following the cell lysis, biotinylated proteins were pulled down using streptavidin beads. Subsequent to extensive washing steps, proteins on beads were digested. The obtained peptides were fractionated using SAX columns[51] and identified in LC-MS/MS (**Figure 4A**). We performed two biological replicates of the amine- and carboxyl-reactive biotinylation methods in HeLa cells. **Figure 5A** shows the correlation of identified proteins in both replicates with a Pearson correlation of 0.91 for the amine- and 0.96 for the carboxyl-reactive biotinylation method. High correlation indicates the reproducibility of our methods. The numbers of identified proteins using each method and their enrichment for cellular components are compared in **Figure 5B**. Among these, the carboxyl labeling approach yielded better plasma membrane enrichment: 179 (61.51%) proteins were annotated as “Plasma membrane” for the carboxyl method, whereas the amine-reactive method identified 163 (30.75%) plasma membrane proteins (**Figure 5B-left**). The observed increase in the plasma membrane enrichment with carboxyl labeling was significant for all biological replicates (**Figure 5C**).

More than half of the identified plasma membrane proteins were common in both methods. Importantly, the rest of the identified cell surface proteins were unique to one method suggesting that using both methods might cast a wider net for a comprehensive identification of cell surface proteins (**Figure 5D-left**). When the sub-categories of the plasma membrane proteins were analyzed, amine-reactive biotinylation recovered slightly more extracellular plasma membrane proteins while carboxyl-reactive biotinylation recovered more integral plasma membrane proteins (**Figure 5D-right**).

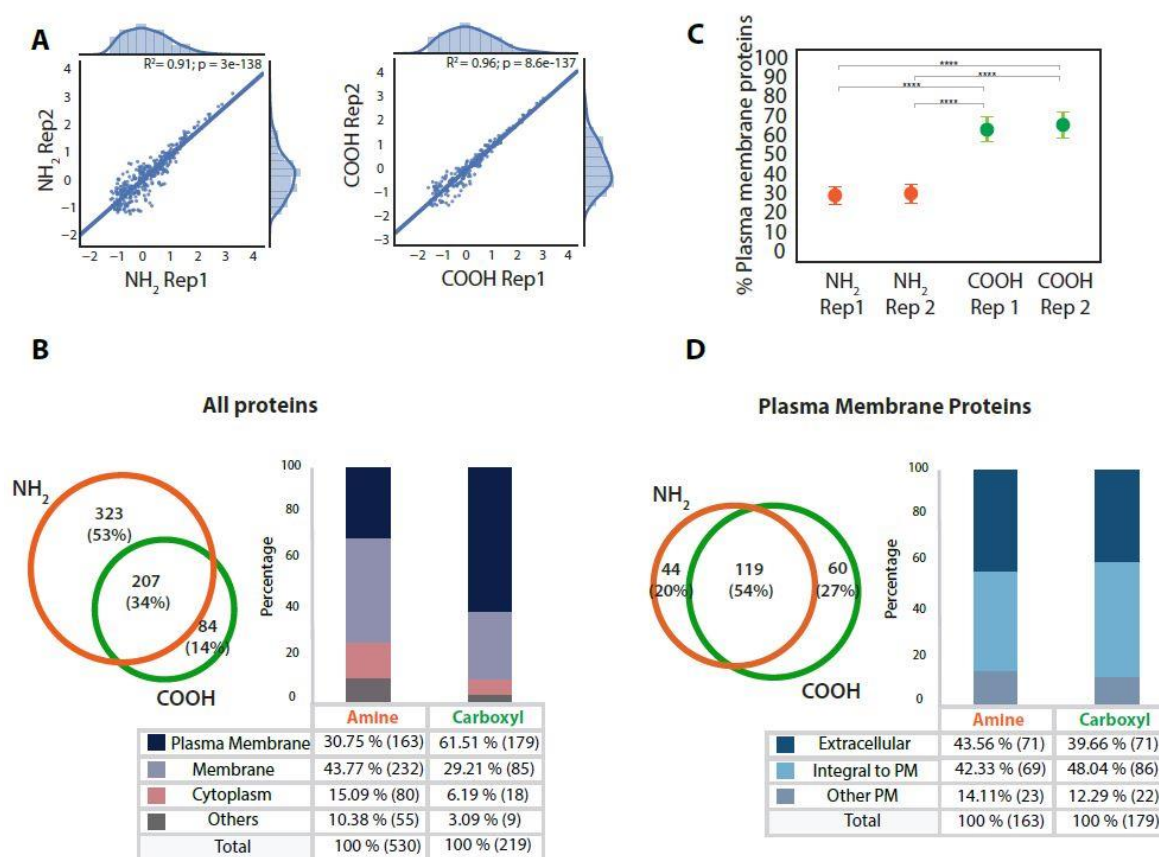


Figure 5: Comparison of the identified proteins with amine- (orange) and carboxyl- reactive (green) biotinylation methods. A. Intensity comparison of the identified proteins in two biological replicates of amine- (left) and carboxyl- reactive (right) biotinylation. **B.** Comparison of the total proteins identified in carboxyl- (green) and amine- reactive (orange) biotinylation methods (left). Distribution of the identified proteins according to their subcellular localizations (right). **C.** Comparison of the plasma membrane protein enrichment in biological replicates. Percentages of plasma membrane proteins identified in amine biological replicate 1, 2 and carboxyl biological replicate 1, 2 are shown with standard errors. Four asterisks illustrate the significant difference between groups with $p < 0.0001$ (Proportions z-test). **D.** Comparison of plasma membrane proteins identified in carboxyl- (green) and amine- reactive (orange) biotinylation methods (left). Distribution of the identified plasma membrane proteins based on their GO annotation (right).[50]

Next, we used cellular component gene ontology term enrichment analysis to compare both methods globally. The top-ten annotations for all proteins identified in carboxyl and amine biotinylation are represented in **Figure 6A**. Plasma membrane related categories like cell surface, junctions, adhesions and membrane rafts are significantly enriched in both methods. Then, we specifically focused on the identified plasma membrane proteins to reveal any possible method-based selectivity against a specific protein subgroup. The most significantly enriched groups of amine biotinylation were stable structures such as cell junctions and adherens junctions (**Figure 6B**-orange bars), whereas groups that are more significantly enriched in carboxyl-reactive biotinylation were cell surface, integral component of plasma membrane and membrane rafts (**Figure 6B**-green bars).

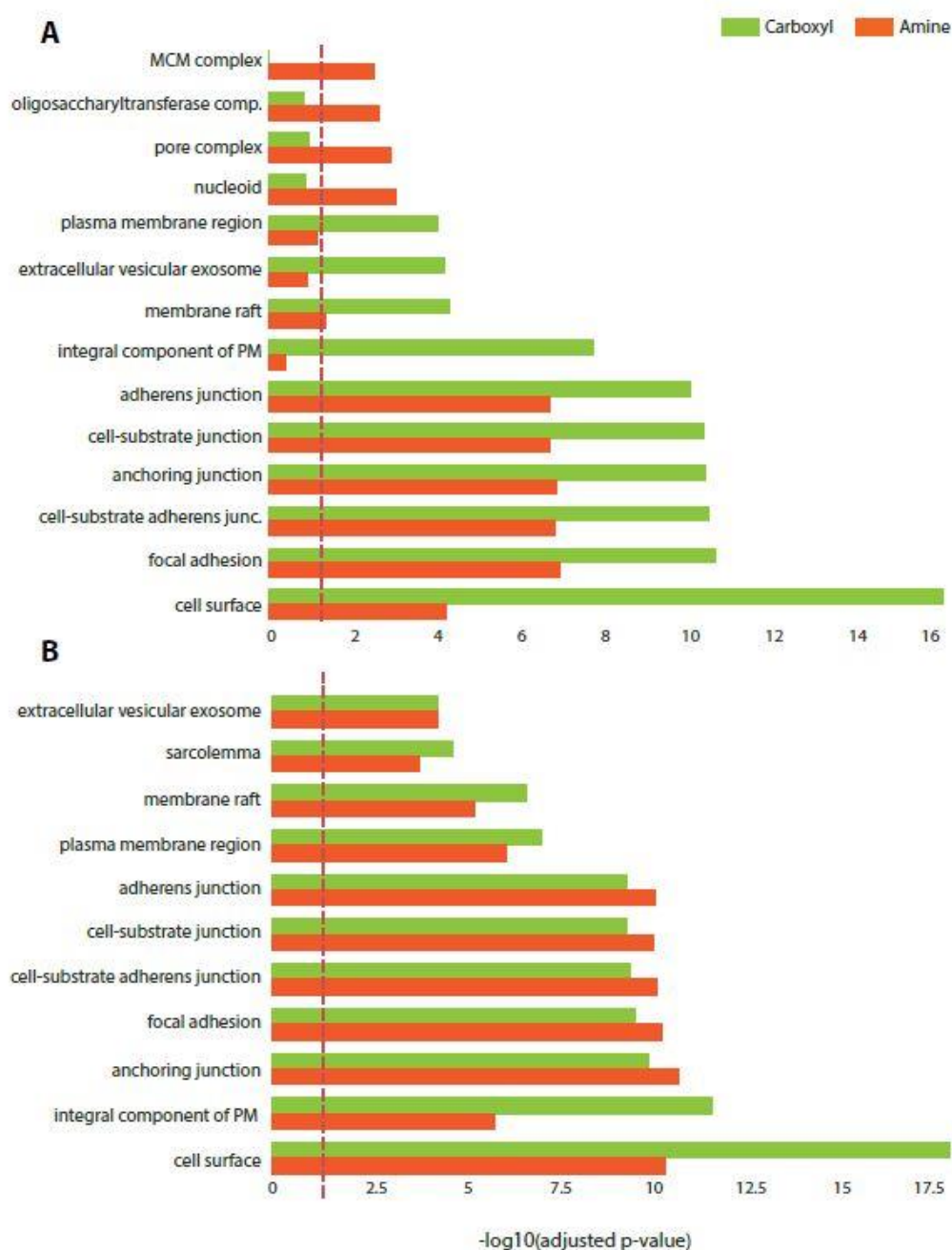


Figure 6: Gene Ontology (GO) Cellular Compartment analysis. **A.** GO Cellular Compartment analysis of the top ten protein groups for all identified proteins in carboxyl- (green bars) and amine-reactive (orange bars) biotinylation methods. Red line illustrates the significance threshold. **B.** GO Cellular Compartment analysis of the top ten protein groups for the plasma membrane proteins identified carboxyl- (green bars) and amine-reactive (orange bars) biotinylation methods. Red line illustrates the significance threshold.[50]

2.4.3 Quantitative Comparison of Amine versus Carboxyl Biotinylation using Mass Spectrometry

Next, we quantitatively compared the two surface biotinylation protocols using SILAC [39]. One cell population was grown in medium containing heavy lysine and arginine (^{15}N and ^{13}C labeled) for several generations, and a parallel culture in ordinary light medium. We labeled the surface of heavy cells with amine-reactive biotin and the same amount of light cells with carboxyl-reactive biotin. We also performed another set of experiments in which we swapped the labels. After the surface labeling and cell lysis, we pooled the two cell populations in equal ratios and affinity-purified the biotinylated proteins with streptavidin beads. Captured proteins were then digested on-beads and resulting peptides were eluted, fractionated using SAX [51] and analyzed in LC-MS/MS (**Figure 7**).

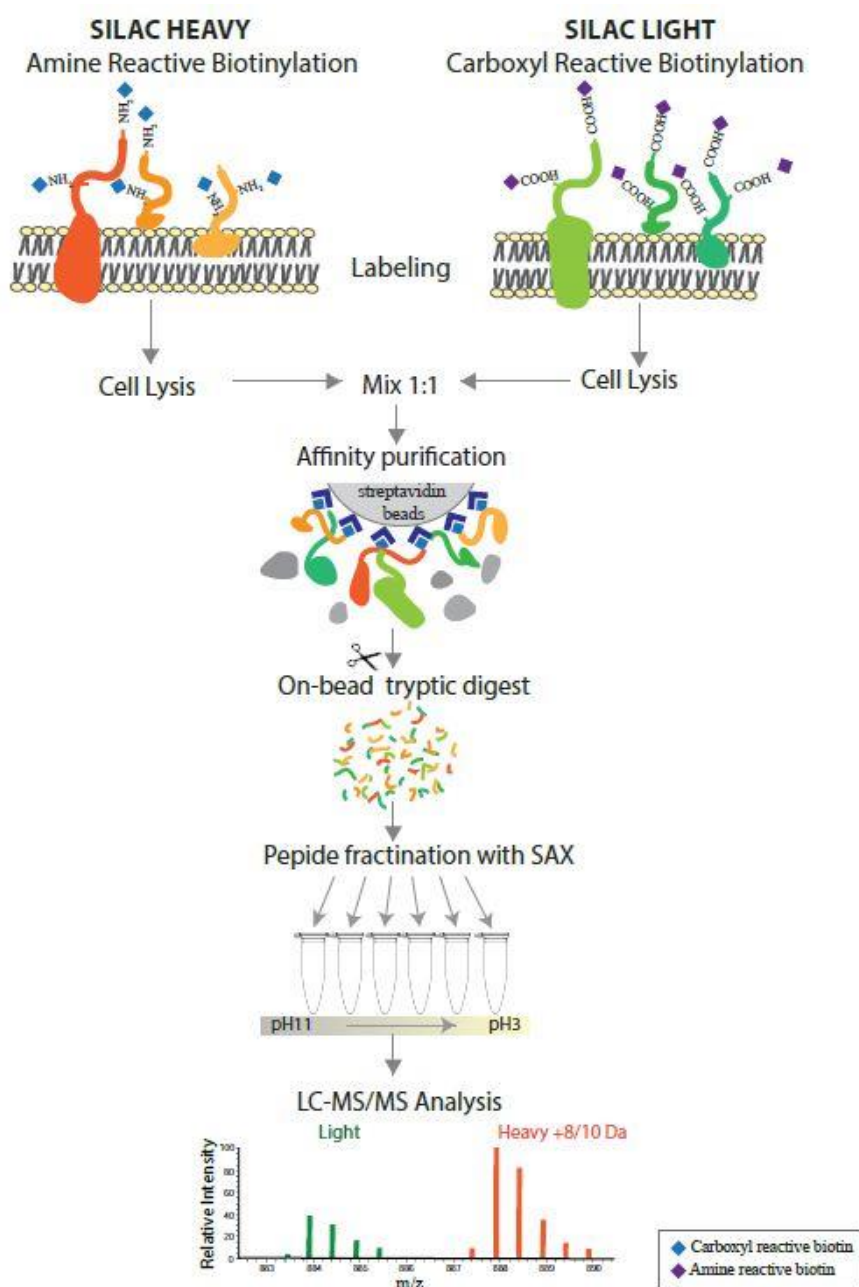


Figure 7: Experimental outline for the quantitative comparison of the amine- and carboxyl-reactive biotinylation methods. The surface proteins of heavy isotope containing cells were labeled with amine-reactive biotinylation (left panel) and light isotope containing cells were labeled with carboxyl-reactive (right panel) biotinylation. Following the cell lysis, two populations were pooled in 1:1 ratio and processed together.[50]

The SILAC ratios of amine versus carboxyl biotinylated proteins were analyzed. **Figure 8A** shows the distribution of the identified cytoplasmic and membrane protein numbers based on their isotopic ratios in amine versus carboxyl biotinylation. The left panel represents the experimental set in which the heavy cells were labeled with amine-reactive biotin and light cells were labeled with carboxyl-reactive biotin while the right panel represents the label-swapped counterpart. For the cytoplasmic proteins, the curve (blue) is notably skewed to the amine biotinylation side implying that identified proteins were more enriched in amine biotinylation preparation than carboxyl biotinylation. In other words, the majority of commonly identified cytosolic proteins gave higher ratios for amine biotinylation (**Figure 8A left**, blue curve). In contrast, the plasma membrane proteins' curve is shifted towards carboxyl biotinylation (**Figure 8A left**, red curve) implying that commonly identified plasma membrane proteins are more enriched in carboxyl biotinylation than amine. The technical replicate of the SILAC measurements demonstrated high reproducibility with a Pearson correlation coefficient of 0.99 (**Figure 9**).

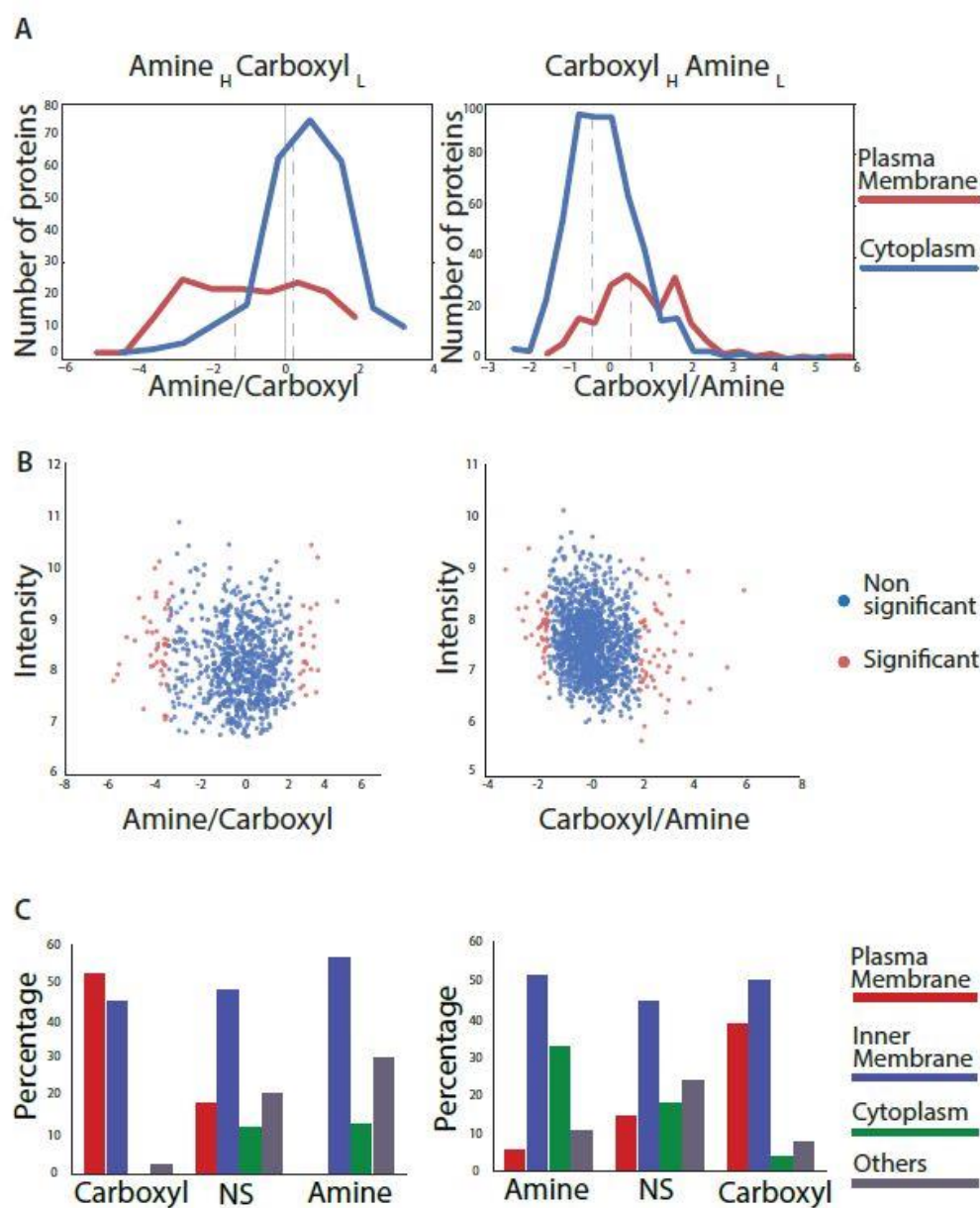


Figure 8: Quantitative comparison of proteins identified with amine- and carboxyl-reactive biotinylation. Left panel shows experiment I, in which the heavy cells were labeled with amine and light cells were labeled with carboxyl. Right panel shows experiment II, in which the heavy cells were labeled with carboxyl and light cells were labeled with amine. **A.** Distribution of the identified “plasma membrane” and “cytoplasm” annotated protein numbers according to their SILAC ratios. Note that distribution patterns of the proteins show a mirror-like trend in label swapped experiments as expected. (Continued)

(Continued) **B.** Distribution of protein intensities according to SILAC ratios. SILAC ratios are normalized according to median and converted to $\log_2$ scale. Outlier proteins which have significantly higher ratio for either of the methods are represented with red dots. **C.** Annotations of the protein sub-groups according to significance of the SILAC ratios. NS, non-significant group corresponds to the proteins with no significant change in the ratio. Carboxyl and amine groups correspond to the proteins with significant change in the ratio.[50]

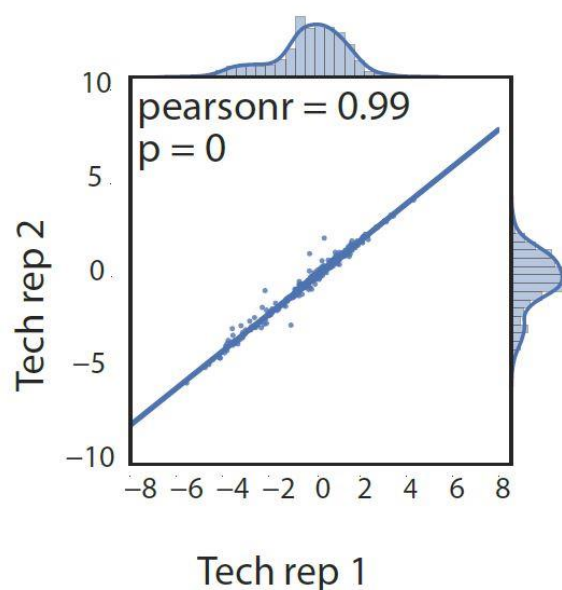


Figure 9: Pearson correlation of the SILAC ratios of the technical replicates.[50]

The second biological replicate, where the labels were swapped, showed a mirror-like pattern demonstrating high reproducibility of these results (**Figure 8A** right). Taking all together, we suggest that carboxyl biotinylation is more cell-surface selective than amine labeling. This could be due to more penetration of the amine-labeling reagent through the plasma membrane, or possibly due to more cell lysis. GO annotation analysis of outliers that are significantly up-regulated in carboxyl or amine biotinylation methods also confirmed this notion. The distribution of the proteins according to their isotopic ratios in amine- versus carboxyl-reactive biotinylation is represented in **Figure 8B**. When we individually analyzed significantly regulated proteins in each method (**Figure 8B**-labeled in red) based on their GO cellular compartment annotations, 50-40% of carboxyl labeling selective proteins were plasma membrane proteins in both replicates (**Figure 8C** red bars), whereas amine labeling selective ones do not have a significant portion of plasma membrane proteins.

2.5 CONCLUSION

In summary, this study was carried out to identify novel approaches to identify cell surface-exposed proteins. We investigated a new cell surface labeling method using a conventional approach of EDC coupling with a biotin derivative where we activated exposed carboxylic acids with EDC and SNHS, and then let them react with a biotinylated amine. This approach has been much used for protein and peptide labeling, but less used for proteomics, and has not been previously used for cell surface labeling. By using one of the carboxyl-reactive biotin reagents, here we present the first example of targeting the carboxyl groups of plasma membrane proteins. In the future, the carboxyl labeling method can be further optimized by using variable carboxylate activation reagents and linker regions (e.g.: PEG linkers) in amine-derivative biotin reagents.

We compared our method for the selective biotinylation of the surface-exposed proteins with labeling amines – a standard approach. Both methods worked for selective cell surface labeling. Each method has pros and cons. Standard amine labeling was faster and required fewer steps, whereas carboxyl group labeling led to more selective cell surface enrichment and less cytoplasmic contamination and was still straightforward. More importantly, both methods identified unique proteins in addition to common ones. Therefore, labeling both amine and carboxyl groups in separate reactions casts the widest net. Applying both methods simultaneously does not require much extra effort: both labeling reactions can easily be performed in parallel and combined in the same purification step. This combined cell surface labeling approach is recommended for more comprehensive cell surface proteomics studies, if the number of cells is not limiting.

Chapter 3

CELL CYCLE DEPENDENT PLASMA MEMBRANE LOCALIZATION OF PCDH7

3.1 INTRODUCTION

Plasma membrane is the key compartment for mediating cells' communication with their environment. Therefore its function is critical to respond to the intra- and extracellular alterations. During these responses, the cell surface changes its composition to receive or give signals that activate downstream pathways. The adherent cells undergo dramatic surface rearrangements also during cell division. They retract their margins and transiently abolish their adhesion to substrate that leads to round shaped morphology. During this process, retraction fibers keep these loosely attached cells anchored to the substrate. Actin cytoskeleton is completely reorganized, forming a more rigid and thinner cortex, which increases the stiffness of cells. This whole process is known as mitotic cell rounding and has been shown to be important for spindle formation and chromosome capture [52]. The mechanism of cell rounding is not completely understood but thought to be partly driven by the regulators of actin cytoskeleton and down regulation of cell adhesion [53]. We also have a limited knowledge about the biochemical changes behind the physical rearrangement during mitotic cell rounding and their connections with the master regulators of the mitotic progression.

In our previous study, we compared the cell surface proteome as a cell undergoes cell division, and identified a group of proteins that show mitosis specific localization to cell surface. Protocadherins (PCDHs), specifically PCDH7 and PCDH1, selectively localize to the cell surface during division [1]. PCDHs are a subgroup of cadherin superfamily proteins. Cadherins are well-known cell adhesion molecules; however there is only limited knowledge about PCDHs' functions. Considering their homology with classical cadherins, the role of PCDHs was proposed as hemophilic adhesion molecules. However, recent studies reveal their possible roles in both hemophilic and heterophilic interactions suggesting a more complex regulatory function in adhesion, yet underlying mechanisms have remained puzzling [54]. Besides, elevated PCDH levels in specific cancers are reported, indicating possible role in cancer progression [55]. Their proposed adhesive function and involvement in cancer progression makes PCDHs interesting targets to study and to understand the biochemical changes of the cell surface during cell division. The overall aim of my study is to understand the mechanism of cell cycle dependent localization of PCDH7&1, and reveal their role in cell cycle progression by defining the interaction partners.

3.2 LITERATURE REVIEW

3.2.1 Cell Cycle and Cell Rounding

Life cycle of an eukaryotic cell can be divided in four consecutive stages: A gap phase for cell growth (G1); a synthesis phase in which the DNA is replicated (S); a second gap phase in which the cells get prepared for the division (G2) and mitosis (M) phase that results with the production of two daughter cells. Combination of these successive phases is known as the mitotic cell cycle, which is probably the most complicated and most regulated event during the lifespan of the cell (**Figure 10**).

Success of the cell cycle is established by the strict control on the “Check points” in the different phases which ensure the proper duplication and the segregation of the genetic material. If a cell cannot pass through a check point, cell cycle is held until the problem is fixed or cell is destined to apoptosis to prevent the generation of mutant progeny [56]. Loss of this control mechanism is the leading cause of the cancer which indicates the importance of the regulation of the cell cycle [57].

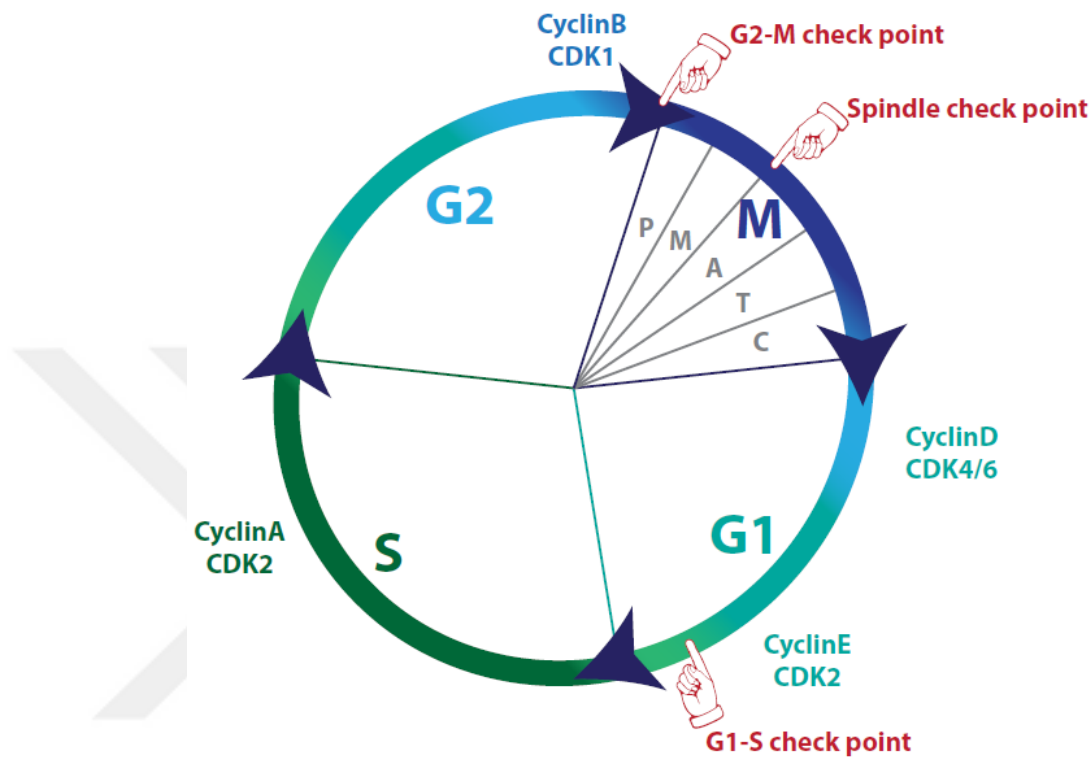


Figure 10: The cell cycle. Cell cycle phases, check points and corresponding Cyclin/CDK couples. (Adapted from [58, 59])

Progression and the regulation of the cell cycle are mainly governed by the cyclin-dependent kinase (CDKs) and their activators cyclin family proteins. Levels of the different cyclin proteins oscillate throughout the cell cycle, activating the specific family of the CKDs. D-type cyclins are expressed in the mid-G1 phase and activate their partner kinases CDK4/6 and CDK2. These kinases phosphorylate and inactivate the retinoblastoma protein (Rb). Inactivation of the Rb liberates and activates the transcription factors such as E2F which activates the transcription of the S-phase genes. Subsequently, expression of Cyclin E and related CDK activity promotes and required for the G1/S transition. Further, cyclin A and cyclin B dependent activity of the Cdc2 regulates the entry to M phase [57].

Even though the cyclins and the CDKs are defined as the major regulators of the cell cycle progression, activity of cyclin-CDK complexes are regulated in various levels. In addition to binding of the partner cyclin, CDK activation also requires an activating phosphorylation by CDK-activating kinase CAK. Negative regulation of the CDKs may also be achieved by the inhibitory phosphorylation of specific sites. This type of control is crucial to keep the Cdc2-cyclin B complex in its inactive state until G2/M transition [60]. Another level of negative regulation of the CDK activity is provided by CDK inhibitor protein CKIs which bind and inhibit their kinase activity. On the other side, CDK-dependent phosphorylation of CKIs also play critical role in the proteasome dependent degradation of the CKIs [57]. Control of sub-cellular localization of the CDKs and their regulators provide another level for the regulation of cell cycle progression [61].

Along with the function of all these molecular regulators in the biochemical level, progression of the cell cycle is also controlled in the mechanical level. The most popular example of the mechanical control of the cell cycle progression is probably the spindle assembly checkpoint. If the mitotic spindle is not properly oriented and attached to the metaphase chromosomes, the lack of the physical tension between the sister chromatids triggers the check point and delay the entry to the anaphase until all the chromosomes are attached to the spindle [62]. The mechanism and the players that translate this physical signal into complex biochemical response are mostly revealed through the years (reviewed in [62, 63]). However, another kind of physical control, shape of a dividing cell, has just begun to gain attraction.

On the onset of mitosis, adherent cells change their geometry by retracting their margins to acquire a spherical shape. This retraction results in the formation of the “retraction fibers” which are the actin-rich tubular cytoplasm strands that keep the rounding cells attached to the substratum [64]. This process is called as “mitotic cell rounding” (**Figure 11**) and it is highly conserved through the evolution. Judged by this conservation,

mitotic cell rounding was considered to have an important role during cell division however, clues about the mechanism came out with the quite recent studies [52]. Those studies revealed that rounding up for the mitosis is indeed essential for the proper chromosome segregation during cell division and it requires the coordinated regulation of the multiple signaling pathways in order to disassemble focal adhesion complexes [65], regulate the osmotic pressure within the cell [66] and re-arrangement of the actin cytoskeleton to form a rigid actomyosin cortex underneath the plasma membrane [52, 67, 68]. This actomyosin cortex is connected to the plasma membrane through activated ERM (Ezrin-Radixin-Moesin) proteins and provides the mechanical stiffness to the rounded mitotic cells [64, 66, 69, 70].

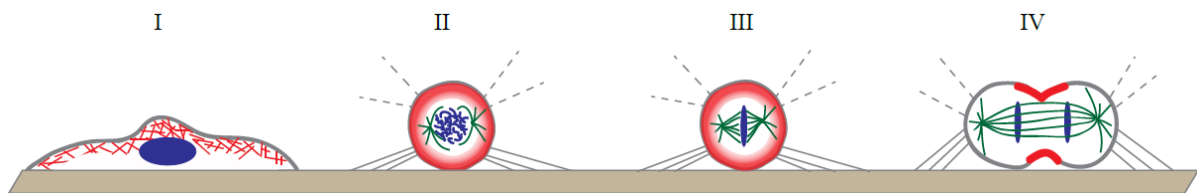


Figure 11: Mitotic cell rounding. Adherent interphase cells (I) retract their margins and gain a spherical shape (II). Branched actin network is rearranged, forming stiff mitotic cortex (II and III-red) than gets concentrated in the furrow as cell goes to cytokinesis (IV). Cell rounding ensures the correct positioning of spindle. (Adapted from [53])

Cell rounding provides the required geometric space to allow the formation and the stability of the mitotic spindle and for the chromosome capture. Hence, inhibition of the cell rounding causes spindle instability and inefficient chromosome capture leading chromosome segregation errors [52, 64, 71]. Spherical geometry that is provided by the cell rounding ensures that chromosomes are positioned in a perfectly-sized area that is optimal

for the “search and capture” by the mitotic spindle [52]. This geometry also provides homogeneity for the spindle-cortex distance and ensures that the astral microtubules which emanate from the both poles can reach to the cortex and keep the bipolar spindle in stable position [52]. In addition to ensuring the fidelity of the division, mitotic rounding may also effect the positioning of the division plane thus regulate the asymmetrical division of the cytoplasm [52].

3.2.2 Cadherin Superfamily Proteins

Cadherin superfamily is a large group of cell surface proteins that are characterized by the presence of EC (Extracellular cadherin) repeats at their extracellular regions, which are composed of about 110 residues. Number and the arrangement of the EC domains define the subfamilies of this superfamily (**Figure 12**). They are involved in multiple cellular processes that vary from embryonic development of central nervous system, cell signaling, tissue homeostasis and mechanotransduction [72-80]. However, most of the cadherins are known for their roles in Ca^{2+} dependent cell adhesion. Ca^{2+} ions bind to the conserved sites on the EC domains of the cadherin proteins, strengthen and protect the ectodomain structure [72, 81-83].

Classical type-I and type-II cadherins represent the far most popular group in this aspect. Extracellular domain of the type-I and type-II cadherins are composed of 5 EC repeats. These single pass transmembrane proteins form homophilic trans interactions with the neighboring “alike” cells through their extracellular EC1 domains. Together with the cis interactions that is formed through the EC2 domains, this cadherin interphase mediate formation of adherens junctions and play critical role in the formation and the organization of the solid tissues [72, 78, 84]. On the other hand, their cytoplasmic domains bind to the β -catenin and armadillo proteins (p-120 catenin) which connect these cell surface proteins with the cytoskeleton and the signaling machinery of the cell [72, 85].

Members of the type-I cadherins have different adhesive properties and are usually mutually exclusive. They are expressed in the different embryonic germ layers or tissues and this expression pattern should be strongly regulated [78, 86]. In relation with their roles in adhesion and signaling, cadherin proteins can represent both tumor-suppressive and tumor-promoting activities. The best known examples are the epithelial cadherin (E-cadherin) and the neuronal cadherin (N-cadherin). Replacement of E-cadherin expression with N-cadherin in carcinomas provides less-adhesive, more metastatic abilities to cancer cells and drive epithelial-mesenchymal transition [85, 87]. On the contrary, type-II cadherins show often overlapping patterns of expression especially in the neuronal development [78, 88, 89].

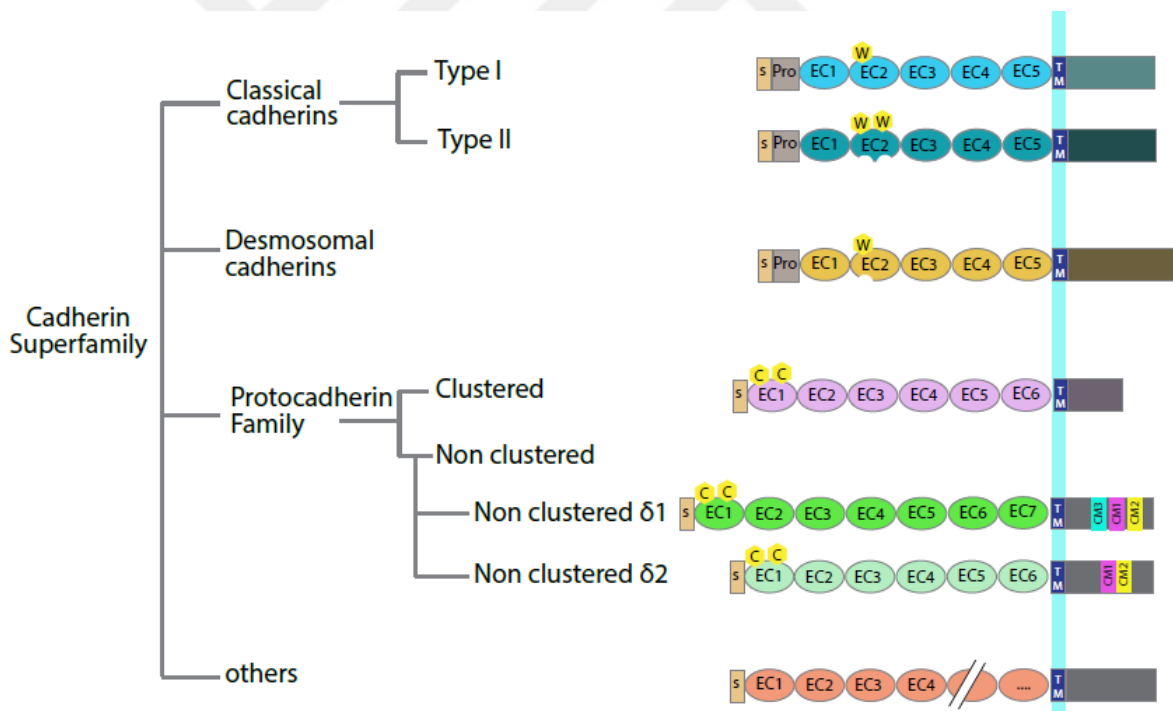


Figure 12: Classification of cadherin superfamily and protocadherins family. S: Signal peptide. Pro: Prodomain. EC: Extracellular cadherin domain. CM: Conserved motif W: Tryptophan C: Cysteine (Adapted from [90] [85])

The second main cadherin subgroup is the desmosomal cadherins which differ from the classical cadherins with their cytoplasmic regions. Desmosomal cadherins interact with the intermediate filaments forming desmosomes; cell-cell junctions that are specialized to resist mechanical stress [72, 91].

Other cadherin groups are more diverse in the structure; 7D cadherins and Flamingo/CELSR cadherins contains more than 5 EC repeats, differ in their conserved tryptophan sequences, transmembrane structures and distinct cytoplasmic domains [72, 84]. Cadherin-related proteins have even more diversity with variable length ectodomains with additional motives and variable cytoplasmic domains [85].

The last cadherin family subgroup, protocadherins forms the largest cadherin subfamily. Protocadherins are further classified into two sub groups, depending on their genomic loci localization, clustered and non-clustered, and will be discussed in details in the next section [90].

3.2.3 Protocadherins

Protocadherin family was first described 25 years ago while in the search for “more classical cadherins” with their structural similarities with the classical cadherins [92]. Similar to classical cadherins, protocadherins are type-I transmembrane proteins and share 6 or 7 EC repeats in their extracellular domains. However, these EC domains share low sequence similarities with those of the classical cadherins. EC1 domain of the protocadherins lacks both the tryptophan residues and the hydrophobic pockets which are essential for the hemophilic interaction interphase of the classical cadherins but, contains a characteristic disulfide-bonded loop [81, 90, 93]. In addition, protocadherins lack the catenin-binding sites and have unique properties in their cytoplasmic domains [92, 94-97].

With more than 70 identified genes, protocadherins (Pcdhs) represents the largest subfamily of the cadherin superfamily proteins and they are predominantly expressed in

the nervous system [90, 96]. Based on their genomic organization, protocadherins are classified in two major groups named clustered and nonclustered protocadherins.

Clustered protocadherin genes are organized in three different genomic loci each encode a subgroup named Pcdh α , β and γ family proteins [90, 98, 99]. These loci have different variable and constant regions whose combinations generate over 50 transcripts from 3 gene clusters [90]. When combined with monoallelic and combinatorial expression patterns that is observed especially for the Pcdh α and γ isoforms, this provides a great variability of the Pcdh expression patterns in the different cell types of the neuronal cells, even within the same type of neurons [90, 100-102]. Thus, clustered protocadherins were proposed to function as molecular barcodes for the self-recognition by the neurons [103]. Adhesive properties of the clustered protocadherins were shown to include homophilic trans interactions as for the classical cadherins however, they form cis-tetramers and these cis-tetramers act as a trans interaction unit. Content of these interaction units can vary greatly since α , β and γ -Pcdhs can form multimeric complexes in cis expanding the broadening the diversity [103-107].

Nonclustered protocadherins are found dispersed in the genome and classified in two groups according their cytoplasmic domains; *Pcdh δ s* and “others”. *Pcdh δ* family proteins contain highly conserved motives (CM1 and CM2) in their cytoplasmic domains. δ -*Pcdhs* are further divided into two sub groups according to their sequence homology: $\delta 1$ -*Pcdhs* contain 7 EC domains in their extracellular site and 3 conserved motives CM1, CM2, CM3 (protein phosphatase-1 α (PP1 α) binding motif) in their cytoplasmic sites while $\delta 2$ -*Pcdhs* contain 6 EC repeats and 2 conserved motives (CM1, CM2) [97, 108, 109].

Even though δ -*Pcdhs* are able to form Ca^{2+} dependent homophilic cell adhesion, their adhesive properties are much less than the classical cadherins. Thus, the adhesive role of Pcdhs was suggested to be related with the intercellular regulations rather than cell-cell adhesion. Homophilic interactions of a number of Pcdhs including PCDH1, PCDH19,

PAPC (*Xenopus* PCDH8 paralog) and PCDH7 were shown to be important for the cell sorting during embryonic development [108, 110-114]. In addition to hemophilic interaction, δ -*Pcdhs* were also shown to form heterophilic cis interaction with the classical cadherins. These direct or indirect interactions were shown to regulate the adhesive properties of the classical cadherins [108]. In addition to the adhesive properties, δ -*Pcdhs* were also shown to interact with the signaling machineries like Wnt signaling and regulate the morphogenesis and brain development during embryonic development [108].

3.2.4 PCDH7 and PCDH1

Both PCDH7 and PCDH1 belong to the δ 1-*Pcdh* family of the non-clustered protocadherins and they share 46-49% sequence homology [115].

PCDH1 is among the first identified protocadherins and reported to mediate cell adhesion and cell sorting during *Xenopus* embryogenesis [92, 110]. Regulated expression of PCDH1 in different organs and tissues was also reported to be crucial during the mouse development from early embryonic stages to late adult stage [116]. PCDH1 was reported to be involved in the regulation of morphogenesis during development. It is required for the notochord development in *Xenopus* embryos [110, 117] and for the proper localization of the neuronal crest cells in chick embryos [108, 118].

PCDH7 is also known as brain hearth (BH)-protocadherin due to its predominant expression in those tissues. It contains 7 EC repeats in the extracellular region, and the EC2 domain has a unique 55 amino acid insertion in the middle. Alternative splicing creates 3 isoforms of PCDH7, named PCDH7a, b and c which differ in their cytoplasmic domains and have 1069, 1072 and 1200 amino acid length respectively. Extracellular regions of the isoforms are almost identical with the exception of PCDH7c, which lack a 47 amino acid region that correspond to Ca^{2+} binding pocket [115, 119, 120].

PCDH7 was shown to mediate weak hemophilic cell-cell adhesion and sorting of *Xenopus* epidermal cells. In addition, PCDH7 activity is required for proper morphogenesis

and ectoderm integrity in the *Xenopus* embryos [108, 114]. This function of PCDH7 is closely related with the cytoplasmic interaction partner TAF1 since over expression of TAF1 can rescue the observed defects [108, 121]. PCDH7 was also suggested to be involved in neuronal development since inhibition of *Xenopus* PCDH7 was shown to reduce the retinal axon and dendrite initiation and elongation [108, 122]. A migratory role of PCDH7 during development was also suggested since it was found to be one of the upregulated genes in migratory neuroblasts in mouse [123]. In addition, PCDH7 was also suggested to effect signaling. In an earlier yeast two-hybrid screen, PCDH7 was shown to interact with the PP1 α and was suggested to effect neurotransmitter receptor pathways [98, 108, 124].

Apart from adhesive properties and functioning as a cell interaction protein, a possible role of PCDH7 in the cancer progression has long been discussed but controversial. Loss of heterozygosity of the PCDH7 gene locus on 4p15 was observed in some head and neck squamous cell carcinomas [115]. Another study reported a gradual decrease in the PCDH7 expression from normal tissue to gastric cancer tissue with lymph node metastasis [125]. In addition, single nucleotide polymorphisms (SNPs) of PCDH7 were also reported to be correlated with the bad prognosis in lung cancer patients [126]. On the contrary, strong upregulation of PCDH7 was reported in brain metastatic breast cancer, and specific sub groups of medullablastoma and breast cancer cells [108, 127-129]. Upregulation of PCDH7 was also observed in androgen-independent prostate cancer cells, primary ovarian tumors and melanomas and an in vitro model of ovarian carcinoma [108, 130-133].

Although PCDH7 expression was reported to be altered in multiple cancer types throughout the years, some mechanistic clues only came out lately. PCDH7 interaction with PP2A and SET was shown to potentiate the MAPK signaling by preventing dephosphorylation of the ERK by PP2A thus promote tumorigenesis [134]. Another recent

study showed that expression of PCDH7 in breast and lung cancers promotes assembly of carcinoma-astrocyte gap junctions through connexin43. Metastatic cancer cells use these junctions to transfer the secondary messenger cGMP to the astrocytes which activate the downstream pathways to support tumor growth and chemoresistance [135]. Another possible link between PCDH7 and the cancer progression could be related with its potential role during cell division. We have previously showed that PCDH7 localizes to cell surface during mitosis and required for the proper mitotic rounding [1]. Considering the importance of the mitotic rounding for cell division, function of the PCDH7 during mitosis might be important for the fidelity of the cell division. Yet the molecular role of PCDH7 during cell division remains to be investigated.

3.3 MATERIALS AND METHODS

3.3.1 Cell Culture and Synchronization

HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin (P/S) and 10% fetal bovine serum (FBS). PCDH7-BAC-GFP (MCP Ky 5914 T457) transgenic cell line was a kind gift from Dr. Ina Poser and were grown in DMEM supplemented with 1% P/S, 10% FBS and 400ug/ml G418.

Cells were synchronized to mitosis, interphase [136] and cytokinesis [137, 138] as described previously. Briefly, HeLa S3 cells were grown to about 40% confluency and incubated with 2mM thymidine (296542A; Santa Cruz) containing media for 20 hours. At the end of 20 hours, media was removed and cells were released from thymidine by excessive washes with PBS. Fresh complete media was then added and incubated for 8 hours. Second thymidine block was performed by the incubation with 2mM thymidine for another 19 hours. In the end of second thymidine block, interphase cells were collected or fixed for immunofluorescence analysis. For the mitosis synchronization, cells were released from the second thymidine block by PBS wash and incubated with fresh media containing 10 μ M STC (164739-5G164739; Sigma) for 20 hours.

For the monopolar cytokinesis synchronization, cells were released from the thymidine block by PBS wash, incubated with fresh media containing 10 μ M STC for 16-20 hours, than incubated with 100 μ M purvalanol A (1580; Tocris Bioscience) for 15 minutes.

In order to inhibit the vesicular trafficking pathway, cells were incubated with 80 μ M Dynasore (SC-202592; Santa Cruz) overnight in serum free media [139]. In order to

inhibit the palmitoylation, cells were incubated with 100 μ M 2BP (2-Bromopalmitate, 21604; Sigma) overnight [140].

3.3.2 Immunostaining and Microscopy

PCDH7-BAC-GFP cells that stably express PCDH7-GFP near physiological levels were used for the imaging experiments.

For immunostaining, cells were fixed with 3% paraformaldehyde (PFA), blocked and incubated with primary and secondary antibodies in 2% BSA in PBS containing 0.1% Triton-X. As an exception, mild detergent conditions (0.1% saponin (SC-280079A, Santa Cruz)) were used for the immunostaining of the endosomes.

The following antibodies were used in immunostaining experiments: Tubulin (CS-2128S), GFP (noncommercial), EEA1 (BD-610457; BD Biosciences), CD63 (H5C6; DSHP) and LAMP1 (H4A3; DSHP), HA (ab16918; Abcam), Alexa Fluor®-488 and Alexa Fluor®-555, Streptavidin-Alexa Fluor 488 (S32354; Invitrogen). Confocal images were taken by Nikon-Eclipse 90i confocal microscope. Live imaging was performed using Zeiss Cell Observer SD spinning disk microscope.

3.3.3 FRAP

3.3.3.1 Experimental set-up

The microscopy set-up included a frequency doubled femtosecond-pulsed Ti:Sa solid-state tunable laser source (Chameleon Ultra II, Coherent) equipped with a second harmonic generator. Laser output was tuned to 488nm and the beam directed through mirrors and a Keplerian telescope to the inverted microscope (Eclipse TE2000-U; Nikon) equipped with a dichroic mirror (Q495LP; Chroma) and 60X oil-immersion objective (Nikon Apo TIRF, NA=1.49). A 300 mm focal length lens was placed right before the microscope to focus laser at the back focal plane of the microscope objective to obtain wide field illumination.

Microscope was equipped with two different cameras for brightfield and fluorescence image acquisition. Brightfield images were captured by CCD camera (DCU223 M; Thorlabs). Fluorescence images were captured by Hamamatsu EMCCD camera (C9100-13) using the band-pass filter of $530 \pm 60\text{nm}$ to prevent laser light in to the EMCCD.

Photobleaching was performed by removing the neutral density optical filter for 1 second to focus the light at desired area. Image acquisition was done in every five seconds using an automated shutter and minimum 130 frames were captured post-bleaching for each sample.

3.3.3.2 Data Processing

Image analysis was done by using a MATLAB code (created by Nima Bavili) based on the double normalization algorithm [141]. In this method, overall decrease in the fluorescence intensity of the samples is also considered and the normalized intensity of the region of interest (ROI) $I_{normalized}$ is given by:

$$I_{normalized}(t) = \frac{I_{total, prebleached} - BG}{I_{bleached region, prebleached} - BG} \cdot \frac{I_{bleached region}(t) - BG}{I_{total}(t) - BG}$$

In which, $I_{total, prebleached}$ and $I_{bleached region, prebleached}$ are prebleached intensities of the whole cell and the ROI respectively. $I_{bleached region}(t)$ and $I_{total}(t)$ are corresponding intensities at time t . BG is the intensity of the background region.

Total number of frames and the first post bleached frame was defined for the each sample. Size of the selected background and the ROI areas in each samples were fixed to 14x14 pixels [142]. Unhealthy cells and the motile cells that obstruct the tracking of the ROI thought the frames are discharged from the data. Obtained $I_{normalized}(t)$ data for each cell then is fitted to $1 - Ae^{-at} - Be^{-bt}$ [141] to get half time values $t_{1/2}$. Recovery

percentage was also calculated for the each cell. The Mann–Whitney U test was applied to reveal statistical differences in the recovery and the half-time between of the samples.

3.3.4 Antibodies and Western Blot

The following antibodies were used in the Western blot analysis: PCDH7 (ab139274; Abcam), EGFR (SC-03; Santa Cruz), Tubulin (3873; Cell Signaling), Actin (ab6276; Abcam), Phospho-Histone H3 (Upstate; 06-570), Biotin (noncommercial).

For Western blot analyses, samples were separated by molecular weight using 10% SDS-PAGE gels, and transferred to a nitrocellulose membrane. The membrane was blocked with 4% w/v nonfat dry milk in TBS-0.1% Tween-20 and probed with 1 µg/ml of the described primary antibody diluted in 2% BSA TBS-0.1% Tween-20. The signal was visualized using ECL (32106; Pierce) detection of the HRP-conjugated secondary antibodies (7074S, 70765; Cell Signaling).

3.3.5 Pulldown of Cell Surface Proteins

Plasma membrane proteins were labelled with sulfo-NHS-SS-biotin for membrane enrichment as previously described [1, 50, 143]. Briefly, cells were incubates with 5 mM S-NHS-SS-biotin (21331; Pierce) for 30 minutes at 4°C with gentle shaking, reaction was quenched with glycine and cells were snap frozen.

Cells were lysed in a buffer (10 mM TrisCl pH 7.6, 0.5% SDS, 2% NP40, 150 mM NaCl, 1 mM EDTA, 10 mM Iodoacetamide) supplemented with protease inhibitors (88666-Pierce). Cell lysates were centrifuged at 7,500 rpm for 15 minutes at 4°C and supernatants were incubated with pre-conditioned Streptavidin Plus UltraLink Resin (53117; Pierce) overnight at 4°C.

Unbound samples were collected and beads were washed with lysis buffer three times. Biotinylated surface proteins were eluted by boiling at 70°C for 20 minutes in SDS sample buffer including 100 mM DTT with agitation. All fraction including whole cell lysate

(WCL), unbound (Ub) and plasma membrane enriched (elute) were analyzed in Western blot.

3.3.6 CRISPR

3.3.6.1 Cloning of CRISPR Vectors

Different sgRNAs that target the PCDH7 and PCDH1 (see Appendix B) were designed using online “CRISPR design toll” and cloned into lentiCRISPR plasmid (pXPR_001, 49535; Addgene) as described [144]. Briefly, lentiCRISPR plasmid was digested with BsmBI (R0580; New England Biolabs) restriction enzyme at 37°C for 30 minutes, visualized in 1% agarose gel and the corresponding band were extracted using Nucleospin Gel and PCR Clean-up Kit (740609; Macherey Nagel). Oligonucleotides that contain the sgRNA sequences were phosphorylated and annealed in a 10 µl reaction that contain 100 µM of each oligonucleotide (1 µl), 10X T4 ligation buffer (1 µl), T4 PNK (M0201; New England Biolabs) (0.5 µl). Reaction was performed in a thermocycler with an initial incubation at 37°C for 30 minutes, followed by 95°C 5 minutes, and then ramp down to 25°C at 5°C/minutes rate. Next, digested plasmids and the oligonucleotide duplexes were ligated in a 10 µl reaction that contain 50 ng of plasmid, 100 ng oligonucleotide duplex, 2X Quick Ligase Buffer (New England Biolabs) and 1 µl Quick Ligase (M2200S; New England Biolabs) at room temperature for 10 minutes. Quick ligation products were then used to transform the chemically competent *E.coli* Stbl3 bacteria cells. For transformation, 5 ng of the plasmid were incubated with the 50 µl of the bacteria on ice for 30 minutes. Following the 1 minute heat shock at 42°C and 2 minutes incubation on ice, 450 µl of LB was added and samples were incubated at 37°C for 90 minutes with 300 rpm mixing. At the end of pre-incubation, bacteria were concentrated with centrifugation at 8,000 rpm for 10 minutes, excess media was removed and suspended pellet was plated onto LB agar plated with the appropriate selective antibiotic. Next

morning, colonies were selected and the plasmid DNA was isolated using NucleoSpin Plasmid (740588; Macherey Nagel) kit.

CRISPR cell lines were generated either with lentiviral packaging and viral transduction of the CRISPR vector or transient transfection of the CRISPR plasmid followed by the antibiotic selection.

3.3.6.2 Stable Cell Line Generation

The lentiviruses that contain the CRISPR plasmids were packaged in HEK293 cells that are plated to 100 mm dishes with 80-90 % confluency using PEI transfection. Briefly, 30 μ l of 1 mg/ml PEI was diluted in reduced serum media (OPTIMEM, 31985047; Invitrogen) and incubated for 5 minutes at room temperature. Next, DNA mix (PEI/DNA ratio; 3:1) that contains the 5 μ g of the CRISPR plasmid that contains the target sgRNA; 3.5 μ g of the packaging vector (psPAX2, 12260; Addgene) and 1.5 μ g of the envelope vector (pVSVg, 8454; Addgene) was prepared; added onto PEI/OPTIMEM mix and incubated for 30-45 minutes at room temperature. Next day, transfection media was replaced with the fresh culture media. Culture media that contains the viruses were collected at the 48th and the 72nd hours of the transfection and centrifuged at 3,500 rpm for 10 minutes at 4°C to pellet the cell debris. Viral supernatants were then filtered through sterile 0.45 μ m PES filters and stored at -80°C until transduction.

Prepared lentiviruses were used to infect the HeLa S3 cells that are plated in 6-well cell culture plates. Briefly, cells were incubated with a 1.5 ml of the infection media that contains the 500 μ l of the culture media, 3 μ l of the 8 mg/ml protamine sulfate and 1 ml of the viral supernatant for 48 hours. After the first incubation, a second viral infection was performed and incubated for another 24 hours. Next day, infection media was replaced with the culture media and left for the recovery for 24 hours. Then, transduced cells were selected by incubating with 4 μ g/ml selective antibiotic puromycin. PCDH7 levels in the

transduced cells were controlled using western blotting and promising pool populations were used for the single colony isolation.

Since transient expression of PCDH7_lentiCRISPR plasmids is enough for the genome editing, stable cell lines can be generated by transient transfection as an alternative to viral transduction. PCDH7-CRISPR cells lines were also generated with PEI transfection follow by antibiotic selection by Mohammed Haroon Qureshi, another graduate student from our laboratory.

Rescue experiments were performed by viral transduction of PCDH7-eGFP plasmid (created by Gürkan Mollaoğlu and Mohammed Haroon Qureshi) to PCDH7-CRISPR cell lines. The lentiviruses that contain the PCDH7-eGFP were packaged in HEK293 cells using pLenti PCDH7-eGFP (created by Mohammed Haroon Qureshi), packaging vector (psPAX2, 12260; Addgene) and envelope vector (pVSVg, 8454; Addgene) as previously described. Resulting viruses were used to infect the PCDH7-CRISPR cells.

3.3.7 BioID

BioID constructs for a partial sequence of the PCDH7 gene and a cytoplasmic domain deleted mutant (PCDH7cytdel) were previously prepared by former graduate student Gürkan Mollaoğlu [145]. In addition to these constructs, a more complete sequence of PCDH7, PCDH1 and the eGFP were cloned into BioID vector (pcDNA3.1 MCS-BirA (R118G) Addgene; 36047).

3.3.7.1 Cloning of BioID Vectors

PCDH7, PCDH1 and eGFP sequences were amplified from the PCDH7-eGFPN1, PCDH1-eGFPN1 and empty-eGFPN1 vectors respectively. Appropriate restriction enzyme cut sites were introduced during amplification using specific primers (see Appendix A). Insert sequences were amplified from the template vectors using 20 pmol of the specific forward and reverse primers (see Appendix A) that introduce the cut sites for the restriction

enzymes, 10 nM dNTP, Q5 High GC Enhancer Buffer, and Q5 high fidelity DNA polymerase (New England Biolabs; M0491). PCR reaction was performed as following. Initial denaturation: 98°C, 30 seconds. Amplification: denaturation 98°C 10 seconds, annealing 72°C 20 seconds, extension 72°C 2 minute (30 cycles). Final extension: 72°C 2 minutes.

PCR product was visualized on 1% agarose gel and corresponding band was extracted using Nucleospin Gel and PCR Clean-up Kit (Macherey Nagel; 740609) according to manufacturer's instructions. PCR products and the recipient vectors were digested with the corresponding restriction enzymes. Following the restriction digest, vectors was dephosphorylated by incubating with Antarctic Phosphatase enzyme (New England Biolabs; M0289) in 37°C for 1 hour. Digestion products were visualized on 1% agarose gel and corresponding band was extracted using Gel Extraction Kit according to manufacturer's instructions.

Gel purified inserts and the vectors were ligated using T4 DNA ligase (New England Biolabs; M0202). The optimal vector to insert ratio (1:3) was calculated using "Ligation Calculator" online tool (New England Biolabs). Ligation was performed in 16°C with 14-16 hours of incubation. Reactions were then heat inactivated in 65°C for 10 minutes. Ligation products were used to transform the chemically competent *E. coli* DH5 α . For transformation, 5 μ l of the plasmid were incubated with the 50 μ l of the bacteria on ice for 30 minutes. Following the 1 minute heat shock at 42°C and 2 minutes incubation on ice, 450 μ l of LB was added and samples were incubated at 37°C for 90 minutes with 300 rpm mixing. At the end of pre-incubation, bacteria were concentrated with centrifugation at 8,000 rpm for 10 minutes, excess media was removed and suspended pellet was plated onto LB agar plated with the appropriate selective antibiotic. Next morning, colonies were selected, plasmid DNA was isolated using NucleoSpin Plasmid (Macherey Nagel; 740588) kit and success of the cloning was tested with a control restriction digest. Positive colonies that gave the expected band pattern were further validated by sequencing.

3.3.7.2 Synchronization and Transfection

Double thymidine and double thymidine followed by STC methods, for synchronization of the cells to interphase and mitosis respectively, were modified for transfection of BioID vectors.

HeLa S3 cells were grown to about 40% confluency. First, cells were washed once with PBS and incubated for 20 hours in DMEM high glucose (10%FCS, 1% Pen-Strep, 1% Glutamine) (complete media) containing 2mM thymidine (296542A; Santa Cruz). At the end of 20 hours, media was removed and cells were washed 3 times with PBS. Fresh complete media was added to release cells from the excess thymidine. Transfection mixtures were prepared in Opti-MEM reduced serum medium (31985047; Invitrogen) using PEI with 60 µg DNA in 3:1 ratio and added onto cells that contain fresh complete media after 30 minutes of incubation. Cells were incubated in thymidine free media that contain transfection mix for 8 hours. After incubation for 8 hours, 2mM thymidine was added onto cells while they are still in media containing transfection mixture. Cells were incubated for another 19 hours in second thymidine. Then, for the interphase cells, media was changed with fresh media containing 50 µM D-Biotin (B1595; Invitrogen) and 1 mM ATP (A2383; Sigma) and 2 mM thymidine (6060; Calbiochem). For the mitosis cells, thymidine was released by washing with PBS than fresh media containing 10 µM STC (164739-5G164739; Sigma), 50 µM D-Biotin and 1 mM ATP was added. Both group was collected and pelleted after 20 hours of incubation.

3.3.7.3 Streptavidin Affinity Capture of Biotinylated Proteins for Western Blot

Cell pellets were lysed in lysis buffer (50 mM Tris, pH 7.4; 500 mM NaCl; 0.4% SDS; 5 mM EDTA; 2% TritonX; 1 mM DTT; Protease Inhibitor) using 26G needle and incubated on ice for 15 minutes, than centrifuged at 4°C, 7,500 rpm (Hettich-Micro200R) for 15 minutes.

A fraction of the whole cell lysate (WCL) was taken and kept at -20°C for further analysis. Rest of the samples were loaded on Streptavidin beads (53117; Pierce) and incubated overnight at 4°C in a tube rotator.

Next morning, unbound fraction was collected and kept at -20°C until analysis. Beads were washed twice with Wash Buffer 1 (2% SDS in dH₂O) for 10 minutes. Then, beads were washed once with Wash Buffer 2 (2% deoxycholate; 1% TritonX; 50 mM NaCl; 50 mM Hepes, pH 7.5; 1 mM EDTA) for 10 minutes, once with Wash Buffer 3 (0.5% NP-40; 0.5% deoxycholate; 1% TritonX; 500 mM NaCl; 1 mM EDTA; 10 mM Tris, pH 8.1) for 10 minutes, and once with Wash Buffer 4 (50 mM Tris, pH 7.4; 50 mM NaCl) for 30 minutes, respectively and all washing steps were carried out at room temperature.

Bound proteins were eluted from the streptavidin beads with 50 µl of Laemmli-DTT sample buffer containing 500 nM D-Biotin at 98°C by 1,000 rpm mixing for 10 minutes.

3.3.7.4 Streptavidin Affinity Capture of Biotinylated Proteins and Identification of Interactors

Cell pellets were lysed with lysis buffer (50 mM Tris, pH 7.4; 500 mM NaCl; 0.4% SDS; 5 mM EDTA; 2% TritonX; 1 mM DTT; Protease Inhibitor) using 26G needle and incubated on ice for 15 minutes, then centrifuged at 4°C, 7,500 rpm (Hettich-Micro200R) for 15 minutes.

Lysates were incubated with streptavidin beads overnight at 4°C to capture the biotinylated proteins. Next morning, beads were washed twice with Wash Buffer 1 (2% SDS in dH₂O) for 10 minutes. Then, beads were washed once with Wash Buffer 2 (2% deoxycholate; 1% TritonX; 50 mM NaCl; 50 mM Hepes, pH 7.5; 1 mM EDTA) for 10 minutes, once with Wash Buffer 3 (0.5% NP-40; 0.5% deoxycholate; 1% TritonX; 500 mM NaCl; 1 mM EDTA; 10 mM Tris, pH 8.1) for 10 minutes, and once with Wash Buffer 4 (50 mM Tris, pH 7.4; 50 mM NaCl) for 30 minutes and all washing steps were carried out at room temperature. After the final wash with wash buffer 4, beads were washed with urea

buffer (8M urea in 0.1 M Tris/HCl pH 8.5). Then, bead-bounded proteins were reduced with 100 mM DTT in urea buffer in 56°C for 30 minutes. After reduction, cysteine residues were alkylated using 100 mM iodoacetamide in urea buffer with 20 minutes incubation in dark. After alkylation, beads were washed with 50 mM ammonium bicarbonate and incubated with Trypsin (25247; Thermo Scientific) at 37°C overnight (14-16 hours) in ThermoMixer (Eppendorf™) with 1,000 rpm shaking. Next morning, digested peptides were collected and desalted as described [18] and analyzed in Thermo Scientific Q-Exactive LC-MS/MS mass spectrometer.

3.3.8 Site Directed Mutagenesis

CSS-Palmitoylation Site Predictor [30] tool was used to determine the candidate palmitoylation sites on PCDH7. Seven of the cysteine residues; C14, C17, C18, C99, C108, C460 and C904 were selected as candidate palmitoylation sites. Mutations were introduced with the site directed mutagenesis strategy as described previously [31]. Briefly, PCDH7 sequence was amplified from PCDH7-eGFPN1 vector using the specific primers to introduce the desired mutation (see Appendix A). PCR reaction mix contained Pfu Turbo DNA polymerase (600250; Agilent), Pfu Turbo Reaction mix, 2.5 mM dNTP, 5% DMSO, 0.4 μ M of each primer and 100 ng template DNA. PCR reaction was performed as following. Initial denaturation: 95°C, 2 minutes. Amplification: denaturation 95°C 20 seconds, annealing 55°C 1 minutes, extension 68°C 14 minutes (19 cycles). Final extension: 68°C 10 minutes. PCR reaction was then incubated with the DpnI (R0176; New England Biolabs) restriction enzyme to digest the methylated parental plasmid and remaining mutant plasmid was used for the transformation of the chemically competent DH5 α bacteria cells. Resulting colonies were sequenced and inserted mutations were confirmed.

3.3.9 Click Chemistry for PCDH7 Palmitoylation

Palmitoylated proteins can be determined using a method that combines the metabolic labeling with click-chemistry and LC-MS/MS analysis [146, 147]. In this method, cells are metabolically labeled with a palmitate analog, 17-ODYA which gets incorporated into the palmitoylation sites of the proteins. Unlike the palmitate group, 17-ODYA can perform a click reaction with the azide group in an azido-biotin in the presence of copper [148] enabling the streptavidin enrichment of the palmitoylated proteins. Palmitoylated proteins can then be identified by comparing the abundance of the proteins with an unlabeled control dataset.

I modified this method to reveal if the PCDH7 is palmitoylated (**Figure 13**). In order to scale up the PCDH7 amount in the samples, I utilized the PCDH7-BAC-GFP cell lines and enriched the protein with GFP affinity purification before performing the click-chemistry reaction.

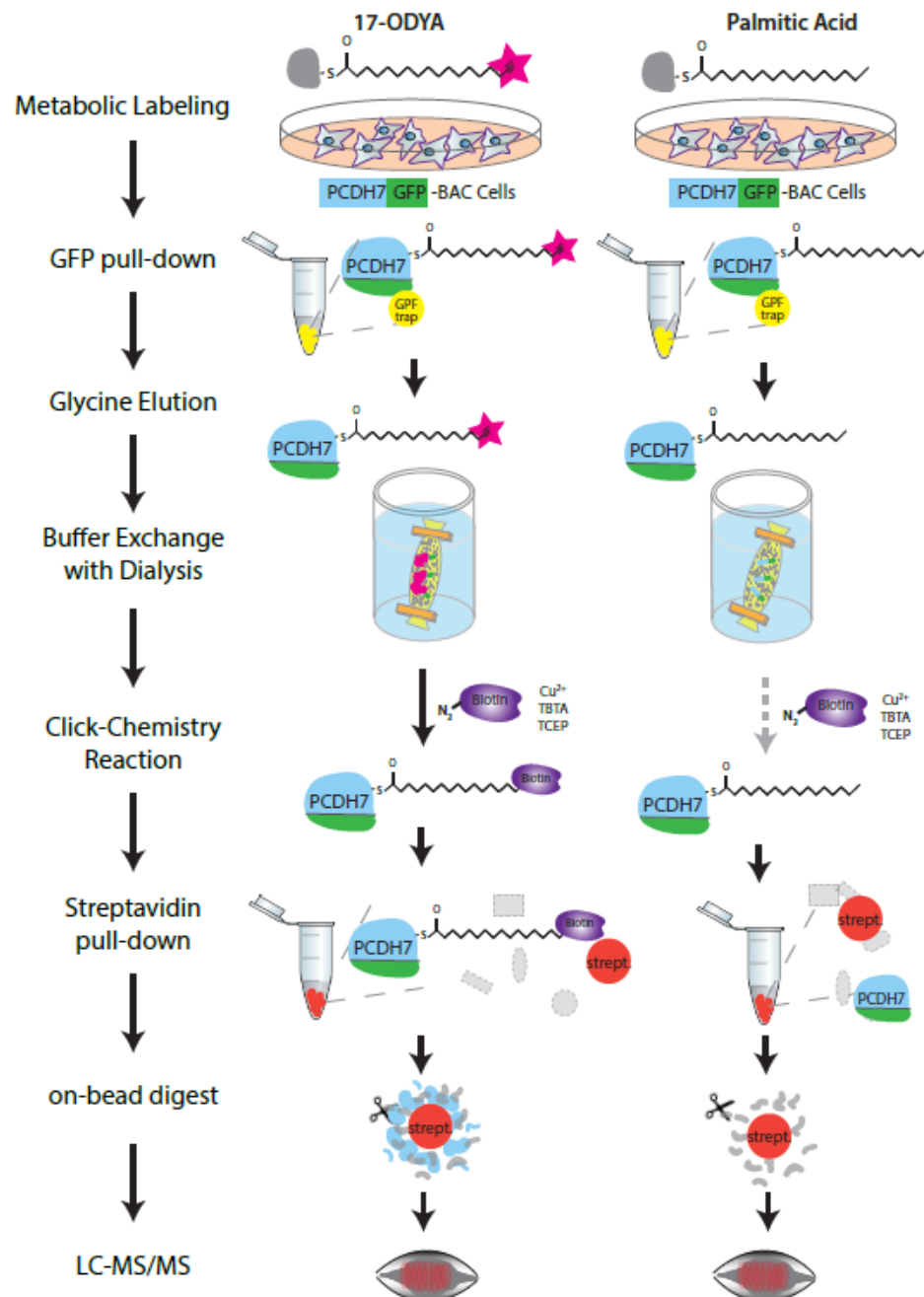


Figure 13. Overview of the optimized method for palmitoylated protein identification using MS.

Approximately 30×10^6 cells were used. PCDH7-BAC-GFP cells were incubated with labeling media that contains 1% Pen-Strep, 10% dialyzed FBS (26400044; Lifesciences) and 25 μ M 17-ODYA (90270.10; Cayman) or palmitic acid (as control) (B20322; Alfa Aesar) overnight and pelleted in ice-cold PBS and snap frozen in liquid nitrogen. 10 μ M STC was also added to labeling media to increase the mitotic cell percent. Chromotek GFP-Trap_A (GTA-20; Chromotek) beads were used for immunoprecipitation according to manufacturer's instructions. Bound proteins were recovered with glycine elution and dialysis was performed for buffer exchange using 7K SnakeSkin dialysis tubings (PI-68011; Pierce). Click-chemistry reaction was performed as described using 100 μ M biotin-azide (13040.5; Cayman), 1mM TCEP (BIT0122; Apollo Science), 1XTBTA (18816.50; Cayman) and 1 mM CuSO_4 (30826; Lach-Ner) [147, 148]. 1% SDS was also added into reaction to prevent over-precipitation of the proteins and reaction was incubated at room temperature for 1 hour. After the click reaction, proteins were extracted with phenol-chloroform extraction. Briefly, 4x volume of methanol, 1x volume of chloroform and 3x volume of ddH₂O were added to samples sequentially. After 2 minutes of spinning at 14,000g, upper layer that contains the methanol-water mixture was removed, 4x volume of methanol was added and proteins were pelleted by spinning at 14,000g for 3 minutes. Next, pellet was washed with methanol, spun at 14,000g for 3 minutes and methanol was evaporated in Speed-Vac. Remaining protein pellet was solubilized with 6M urea, 2% SDS in PBS and bound to streptavidin beads after diluting 10X with PBS. Unbound fraction was removed, and beads were extensively washed with 1 % SDS and PBS sequentially. Next, samples were reduced with 10 mM TCEP in 6M urea (neutralized with KOH) for 30 minutes and alkylated with 20 mM iodoacetamide in 6M urea for 30 minutes in dark. Trypsin digest was performed with sequence grade trypsin (25247; Thermo Scientific) in 2M urea, 1mM CaCl_2 in PBS at 37°C overnight. Digested peptides were collected, desalted and analyzed in LC-MS/MS.

3.3.10 Data Acquisition

Peptides were analyzed by online C18 nanoflow reversed-phase nLC (NanoLC-II, Thermo Scientific) or C18 nanoflow reversed-phase HPLC (Dionex Ultimate 3000, 3500 RSLC nano, Thermo Scientific) combined with orbitrap mass spectrometer (Q Exactive Orbitrap, Thermo Scientific). Samples were separated in an in-house packed 100 μm i.d. $\times$ 23 cm C18 column (Reprosil-Gold C18, 5 μm , 200 $\text{\AA}$, Dr. Maisch) using 100 minute linear gradients from 5-25%, 25-40%, 40-95% acetonitrile in 0.1% formic acid with 300 nL/min flow in 120 minutes total run time. The scan sequence began with an MS1 spectrum (Orbitrap analysis; resolution 70,000; mass range 300–1,500 m/z ; automatic gain control (AGC) target $1\text{e}6$; maximum injection time 32 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 $1\text{e}6$; normalized collision energy (NCE) 26; maximum injection time 85 ms). The isolation window for MS/MS was 2.0 m/z .

3.3.11 Data Processing

Raw files were processed with MaxQuant version 1.5.2.8 [44]. 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 20 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 2015) containing 21,039 entries using the Andromeda search engine integrated into the MaxQuant environment [45].

For the identification of the proximity interactors, spectral counts of proteins identified in 4 experiments against 4 control experiments are analyzed via non-paired QSPEC module of QPROT [149]. This method uses a Bayesian model for estimating the

protein abundances from the spectral counts of proteins and allows possible variability between mass spectrometry runs across the experiments. Interphase and mitosis specific interaction partners are selected from the QSPEC outputs where $FDR < 0.2$ and the fold change (FC) greater than two ($\ln(FC) > 0.7$).



3.4 RESULTS

3.4.1 Sub-cellular Localization of PCDH7

In order to understand how PCDH7 is enriched on the mitotic plasma membrane, we first analyzed its subcellular localization throughout the cell cycle. PCDH7-BAC-GFP cell line was used to visualize the cellular localization of PCDH7. GFP signal was amplified using GFP antibody. PCDH7 localizes to the cell to cell contacts during interphase (**Figure 14**-top panel), enriched in the mitotic plasma membrane (**Figure 14**-middle panel) and concentrated in the middle phase of the separating cells during cytokinesis (**Figure 14**-lower panel).

In order to distinguish whether cytokinesis specific localization corresponds to cleavage furrow or to freshly forming cell to cell contacts of the two cells, I utilized the monopolar cytokinesis approach. In monopolar cytokinesis, cells are induced for asymmetrical cell division using specific inhibitors. Chromosomes stay in one pole while plasma membrane forms a bud-like extension which will eventually result in formation of a chromosome-less daughter cell. In monopolar cytokinesis, area of the cleavage furrow is greatly expended and there isn't a true second cell to form cell to cell contacts [137, 138]. Thus, this synchronization method represent an useful tool to understand if PCDH7 localizes to either cell to cell contact or cleavage furrow during cytokinesis. Cells were synchronized to monopolar mitosis with kinesin inhibitor Stc (S-trityl-L-cysteine) and incubated with Cdk-1 inhibitor purvanol A for monopolar cytokinesis.

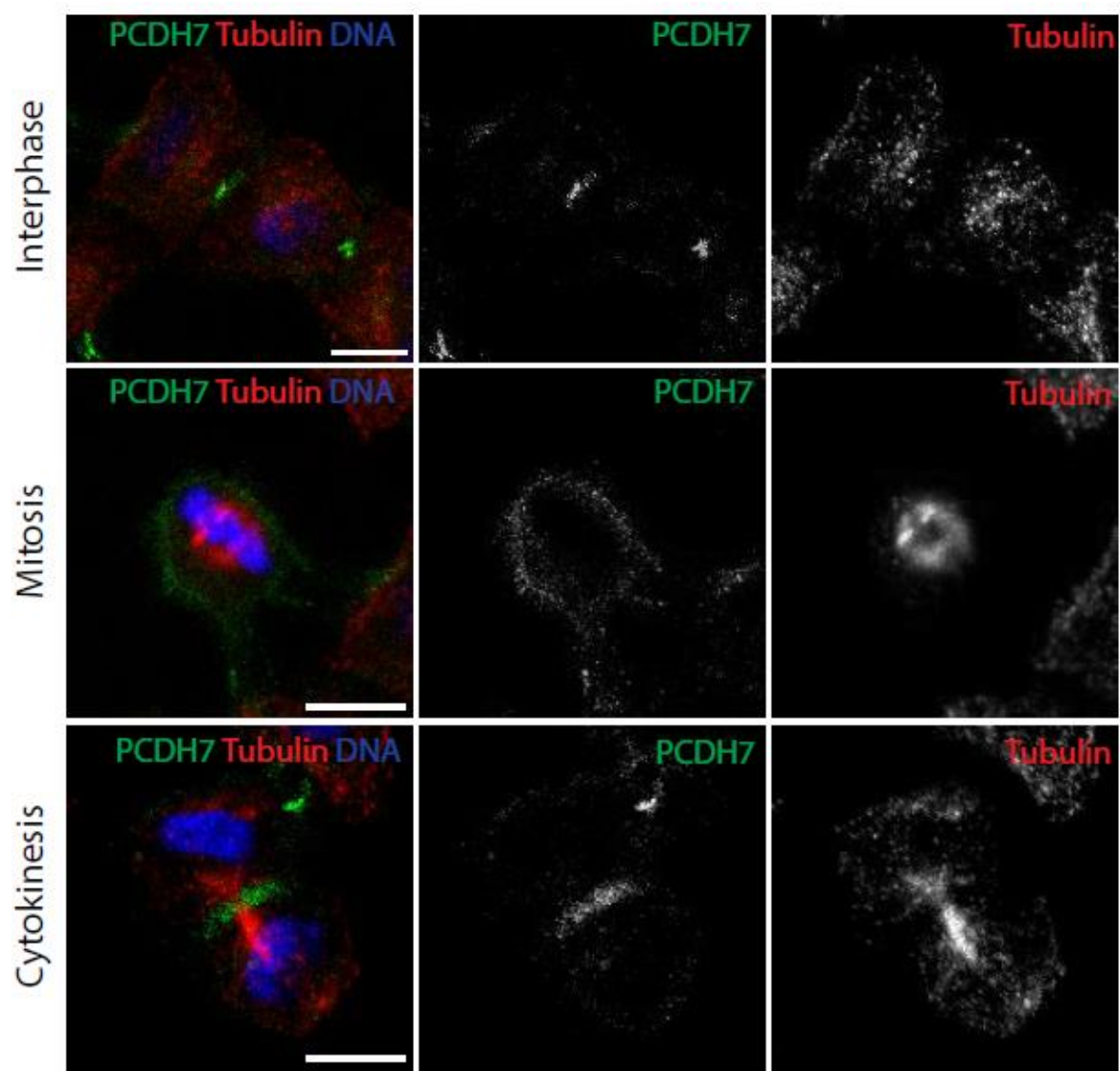


Figure 14: Cellular localization pattern of PCDH7 throughout the cell cycle phases. Interphase (top panel), mitosis (middle panel) and cytokinesis (lower panel). Scale bar, 10 μm .

According to immunofluorescence images, PCDH7 was enriched in the mitotic plasma membrane as expected (**Figure 15-top panel**) and concentrated on the budding site in the monopolar cytokinesis (**Figure 15-lower panel**). These results show that PCDH7 localizes to cell-to cell contacts in interphase cells, is enriched in the plasma membrane in mitosis and get concentrated to the cleavage furrow as cells proceed to cytokinesis.

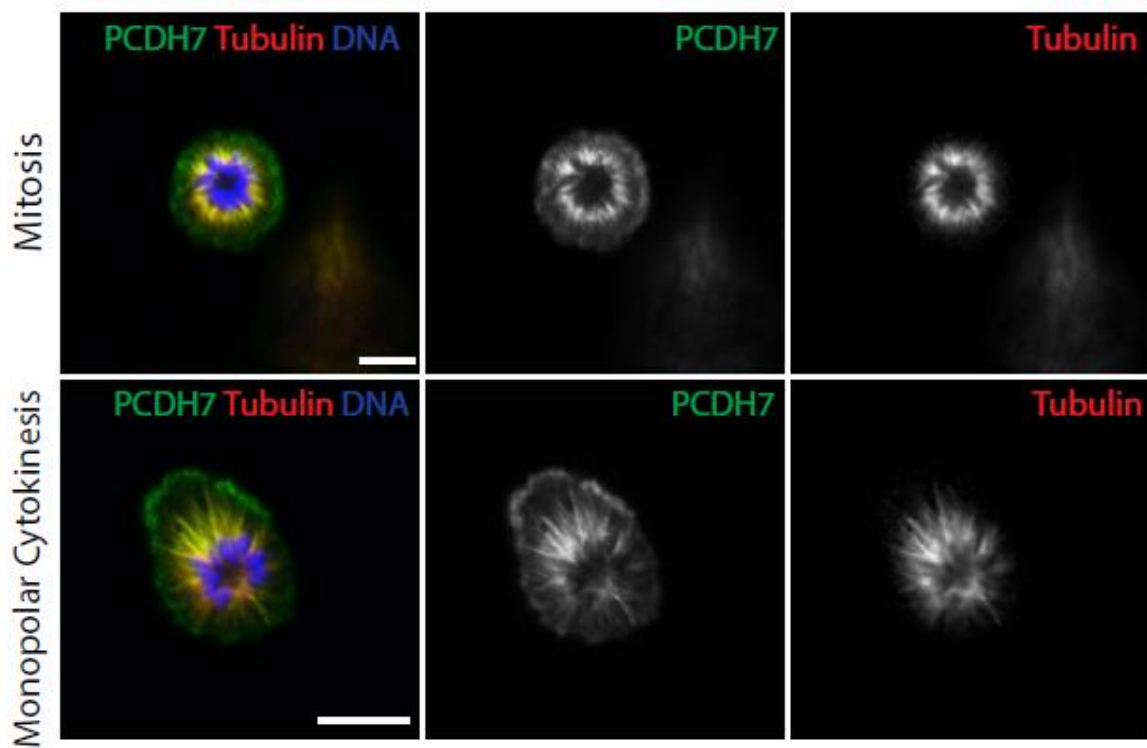


Figure 15: Cellular localization of PCDH7 in monopolar mitosis (upper panel) and monopolar cytokinesis (lower panel). Scale bar, 10 μ m.

3.4.2 Dynamics of PCDH7 Translocation During Cell Cycle

In order to understand the molecular mechanisms for the PCDH7's translocation from inside to the cell surface, I utilized the FRAP approach and compared the localization dynamics of the PCDH7 in a cell cycle dependent manner. In the FRAP approach, recovery of the fluorescence signal after photobleaching is parallel to the protein turnover in the bleached area. Thus, recovery rate of the fluorescence signal is an indicator of the mobility of the protein.

In order to reveal the any possible differences on the localization dynamics of the PCDH7 on the cell to cell contacts during interphase and on the plasma membrane during mitosis, I compared the recovery of the protein after photobleaching (**Figure 16A-B**). Recovery levels in mitotic plasma membrane and cell-cell contacts did not showed a significant difference (**Figure 16C**). However, PCDH7 recovered significantly faster in the mitotic plasma membrane, when compared to the cell to cell contacts in the interphase cells (**Figure 16D**). PCDH7 is much more dynamic and there is a high turnover in the mitotic PCDH7 localization in comparison to the cell-cell contact regions.

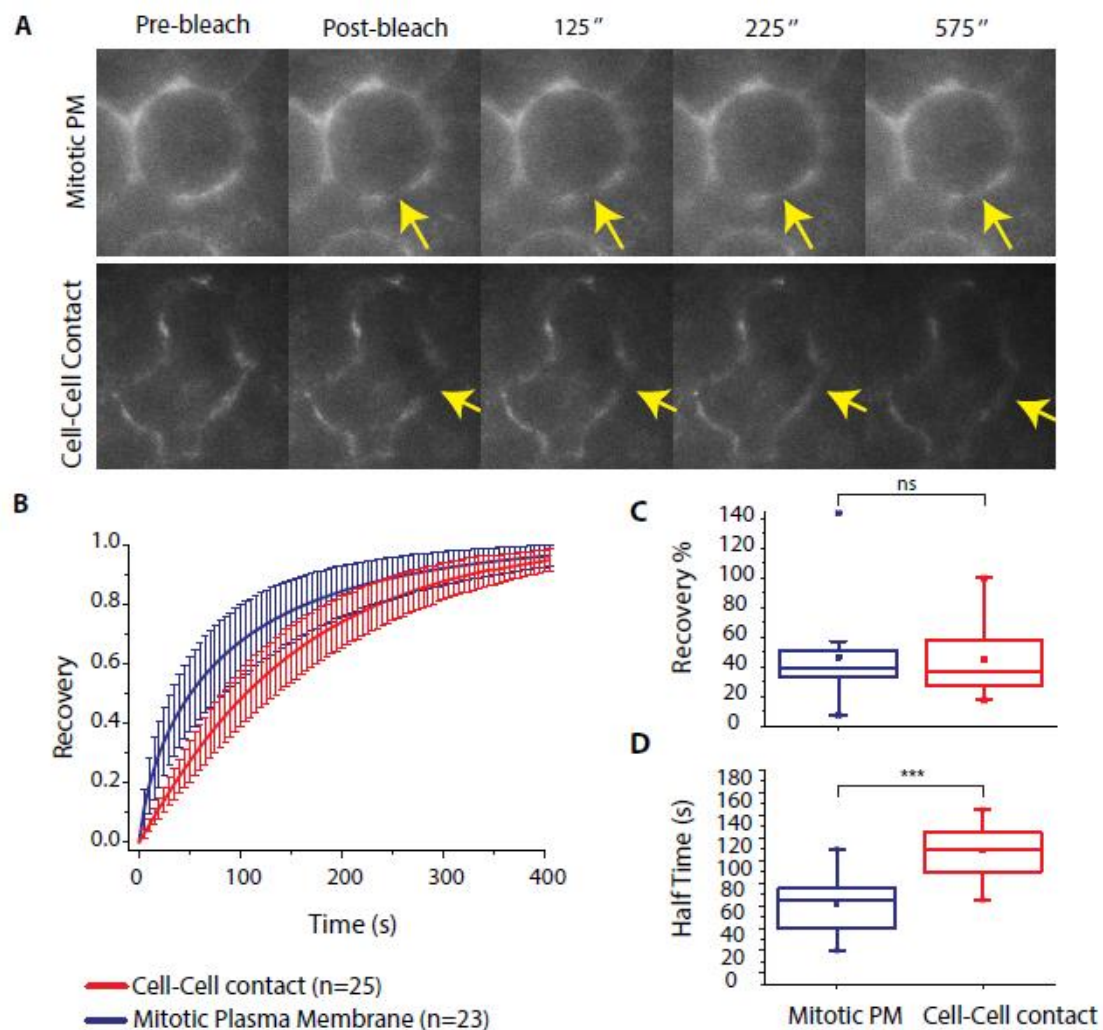


Figure 16: Turnover of the PCDH7 on plasma membrane. **A.** Representative images for mitotic plasma membrane (upper panel) and cell-cell contact (lower panel) localization of PCDH7 during FRAP analysis. Arrows indicate the bleached area. **B.** Recovery curve of PCDH7 in mitotic plasma membrane (blue) and cell-cell contacts (red) after photobleaching. **C.** Comparison of recovery levels in mitotic plasma membrane (blue) and cell-cell contacts (red). **D.** Comparison of recovery halftimes in mitotic plasma membrane (blue) and cell-cell contacts (red) (***: $p < 0.001$, ns: non-significant)

3.4.3 Cell Cycle Dependent Proximity Interactions of PCDH7

I took advantage of the BioID proximity dependent biotinylation method to uncover the mechanisms that govern the plasma membrane localization of PCDH7 during interphase and mitosis. BioID method utilizes a mutated form of the BirA biotin ligase which can biotinylate the proteins in close proximity to the bait enabling identification of weak and the transient interactors [150]. Thus, gives the possibility to identify interphase and mitosis specific interaction partners of PCDH7.

Before proceeding to the identification of the cell cycle dependent proximity partners of PCDH7, I first tested the localization and the functionality of the cloned BioID constructs. HeLa S3 cells were transfected with the BioID vectors and growth in media that supplemented with 50 μ M D-Biotin and 1 mM ATP. Immunofluorescence staining was performed subsequent to PFA fixation using the specific antibodies for either PCDH7 itself or for the HA which is present in the vector backbone. Biotinylation was verified using florescence-conjugated streptavidin.

As shown in **Figure 17**, each construct showed expected subcellular localization and proper biotinylation. Transfection with GFP-BirA-HA construct resulted with an unspecific biotinylation that covers the whole cells (**Figure 17-top panel**). On the other hand, transfection with both PCDH1-BirA-HA (**Figure 17-middle panel**) and PCDH7-BirA-HA (**Figure 17-lower panel**) constructs resulted specific biotinylation on the cell-to-cell contacts as expected.

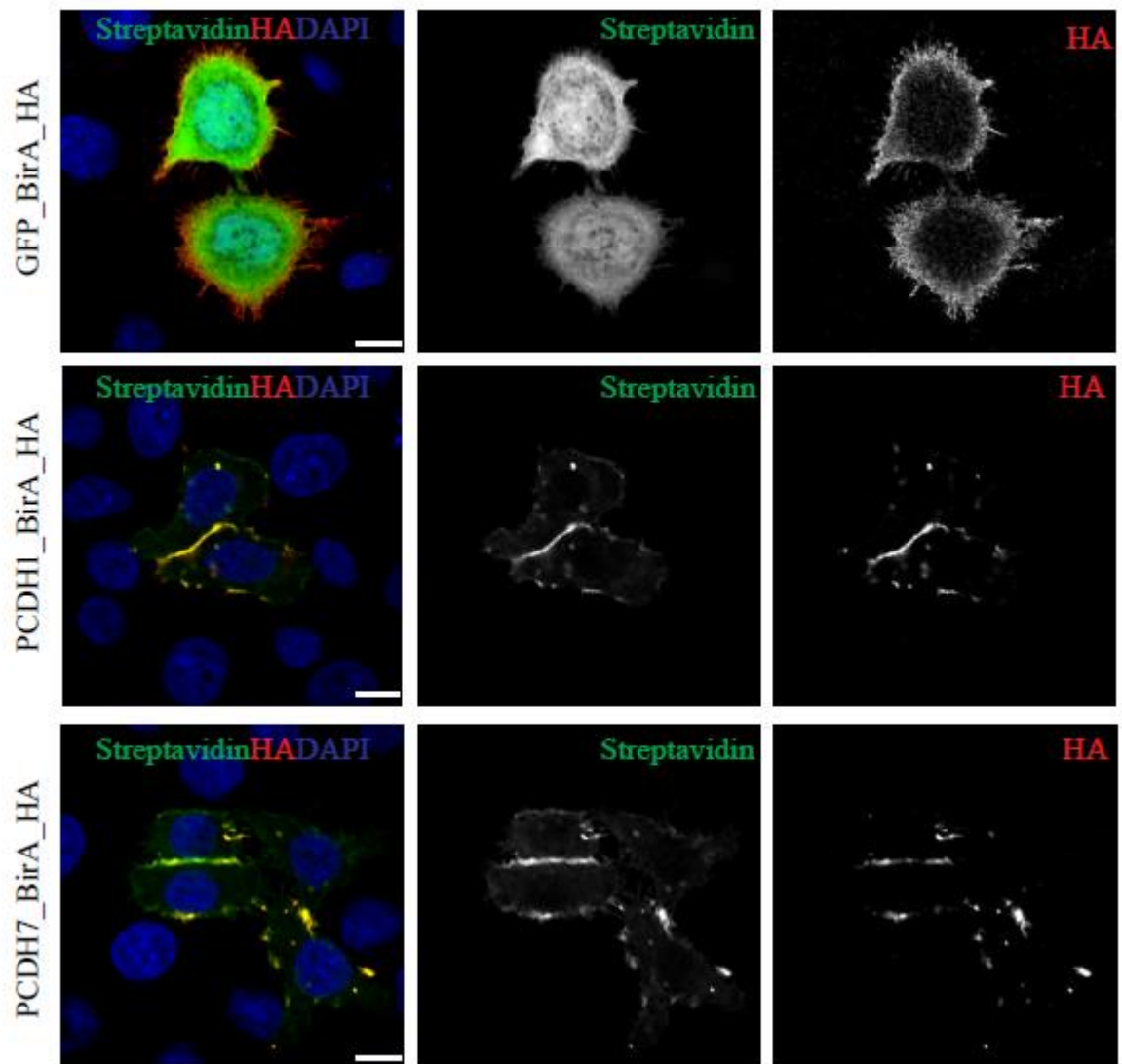


Figure 17: Subcellular localization of the BioID constructs. GFP-BirA-HA (top panel), PCDH1-BirA-HA (middle panel) and PCDH7-BirA-HA (lower panel). Scale bar, 10 μ m.

Next, I tested if the localization of PCDH7-BirA-HA and the biotinylation pattern mimics the cell cycle specific localization of PCDH7. As shown in the **Figure 18**, both localization and the biotinylation pattern resembled the localization of PCDH7 during mitosis (**Figure 18**-top panel) and interphase (**Figure 18**-lower panel).

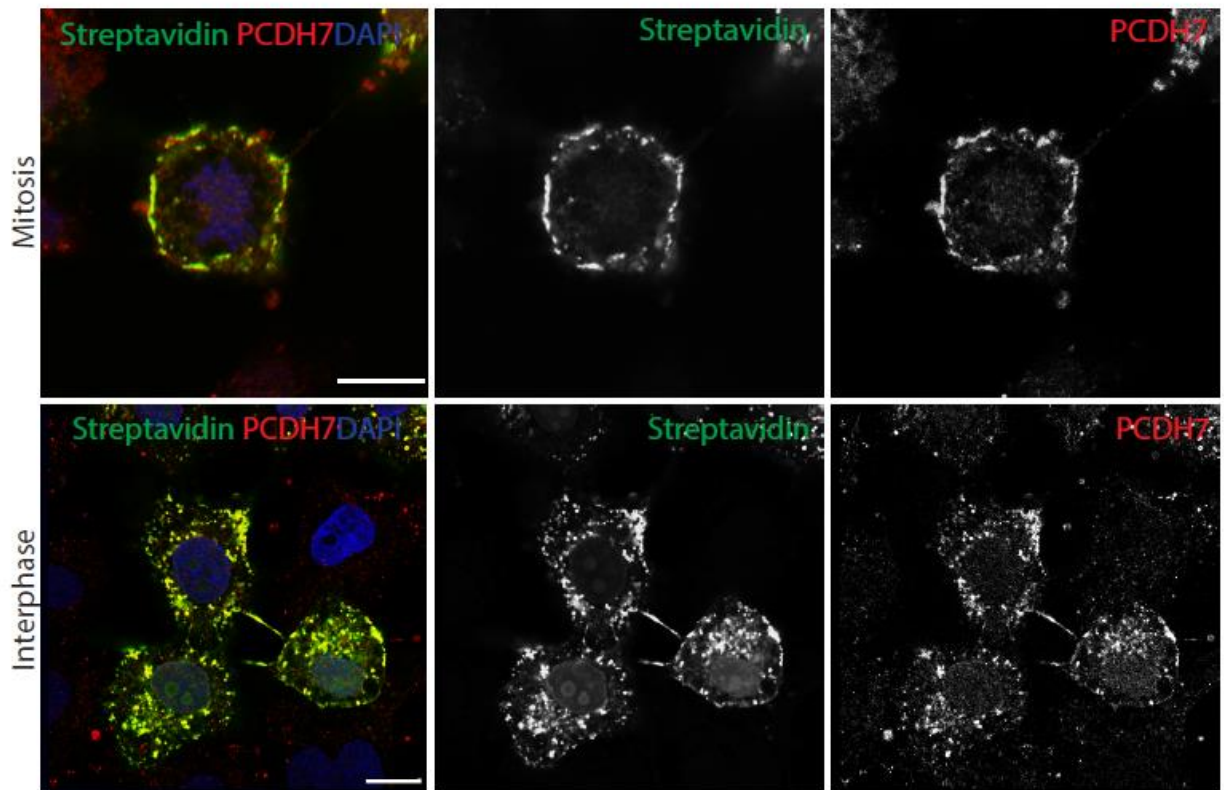


Figure 18: Cell cycle dependent localization of PCDH7-BirA-HA construct. Mitosis cells (upper panel) and Interphase cells (lower panel). Scale bar, 10 μ m.

In order to test the efficiency of the BioID constructs, I collected the cell pellets 48 hours after transfection, lysed and pulled down the biotinylated proteins using streptavidin beads and analyzed with Western blot using biotin antibody. Non transfected control cells and the C terminal empty vector (BirA-HA) transfected cells showed a basal level of biotinylation both in the presence (**Figure 19A-left**) and absence (**Figure 19A-right**) of the excess biotin whereas N terminal empty vector (mcy-BirA) showed unspecific biotinylation in the presence of excess biotin (**Figure 19A-left**, last lane). On the other hand, PCDH7-BirA-HA, PCDH7cytdel-BirA-HA and myrPalm-BirA-HA transfected cells showed specific biotinylation patterns that differ from each other in the presence of excess biotin (**Figure 19B-left**). In the absence of excess biotin, biotinylation patterns were reversed back to the basal levels (**Figure 19B-right**).

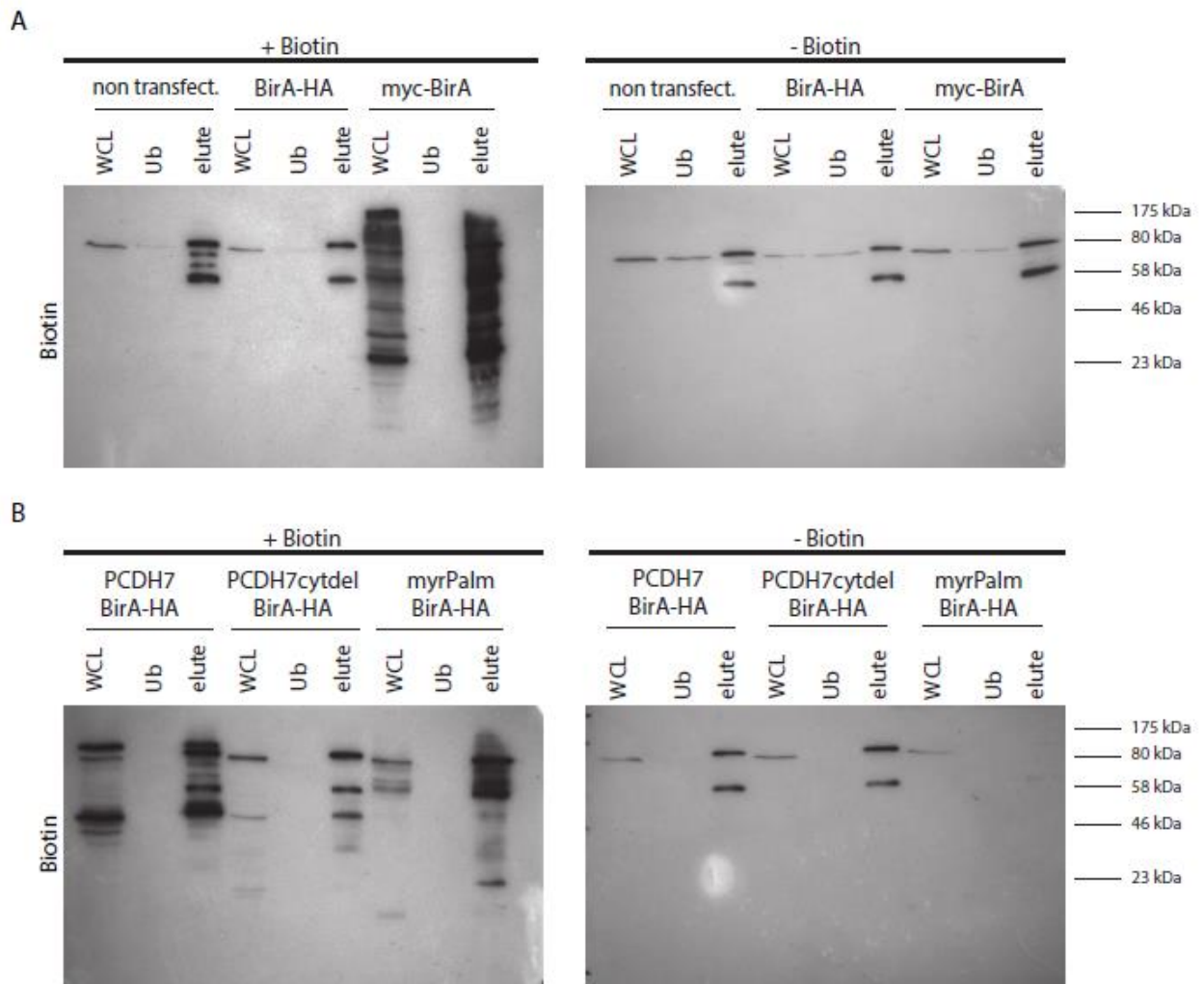


Figure 19: Biotinylation efficiency of BioID constructs. Western blots shows the biotinylated proteins. **A.** Controls; non transfected, empty vector transfected (BirA-HA and mycBirA). Left; supplied with biotin and ATP. Right; not supplied with biotin and ATP. **B.** Samples; PCDH7-BirA-HA, PCDH7cytdel-BirA-HA, myrPalm-BirA-HA (an unrelated construct as control). Left; supplied with biotin and ATP. Right; not supplied with biotin and ATP. WCL: whole cell lysate, Ub: unbound

As a next step, I tested the specificity of the biotinylation by the BioID constructs. Membranes that were blotted with the biotin antibody were stripped and incubated with the specific antibodies for the PCDH7 and the Actin. PCDH7 and PCDH7cytdel were present in the elute fractions only in the presence of biotinylation (**Figure 20-left**, lanes 3 and 6). Absence of PCDH7 in the elute fraction of myrPalm (**Figure 20-left**, last lane) further verified the specificity of the pull down.

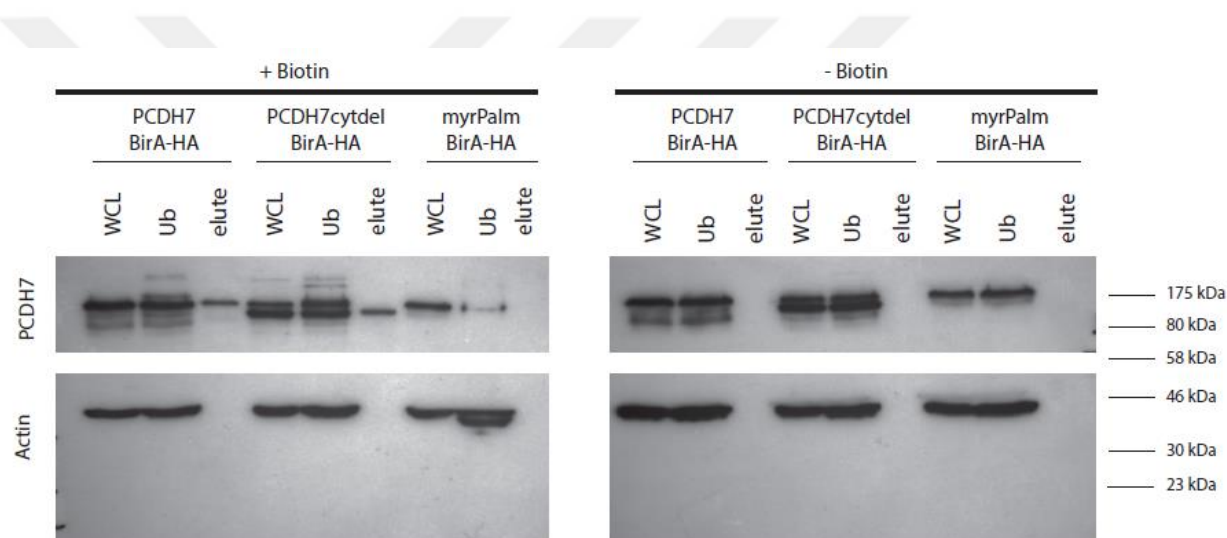


Figure 20: Biotinylation specificity of BioID constructs. Western blots shows presence of PCDH7 in different pull-downs. Left; transfected and supplied with biotin and ATP. Right; transfected but not supplied with biotin and ATP. WCL: whole cell lysate, Ub: unbound.

After confirmation the functionality and the specificity of the BioID-construct, I transfected cells with PCDH7-BirA-HA, synchronized to interphase and mitosis and performed streptavidin pull-down of the biotinylated proteins. Candidate interactors were then identified in LC-MS/MS following the on-bead digest of the streptavidin bound proteins. **Figure 21** represents the cell cycle dependent candidate interactions of PCDH7.

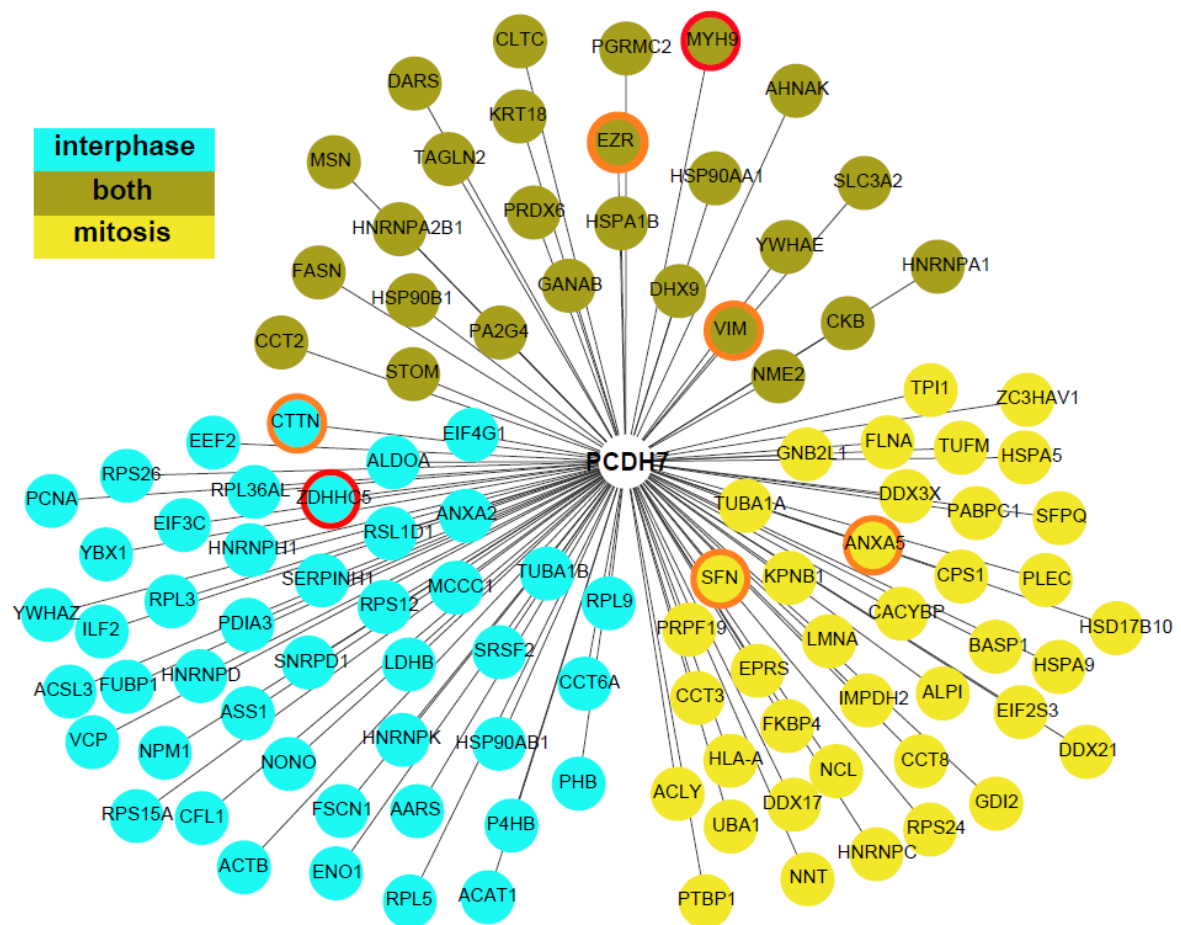


Figure 21: Cell cycle dependent proximity interaction map of PCDH7. Candidate interactors specific to interphase and mitosis are represented in cyan and yellow respectively. Common interactors are represented in green. Proteins that are discussed in the text are labeled with colored circles.

3.4.4 Effect of Palmitoylation on PCDH7's sub-cellular localization

According to BioID experiments, we identified a palmitoyltransferase enzyme as one the proximity interaction partners of the PCDH7. Palmitoylation is a reversible post translational modification that is known to effect the hydrophobicity, and the membrane domain interactions and the conformation of the transmembrane proteins [151]. Thus, I wanted to test the effect of palmitoylation on PCDH7's localization to plasma membrane in a cell cycle dependent manner.

HeLa S3 cells were synchronized to interphase or mitosis and incubated with 100 mM palmitoylation inhibitor 2BP overnight. Following 2 BP incubation, plasma membrane proteins were labelled with sulfo-NHS-SS-biotin for plasma membrane protein enrichment and subsequently captured with streptavidin beads for Western blot analysis. Cell surface protein EFGR was used to control the plasma membrane enrichment, while Tubulin was used to control cytoplasmic contamination. 2BP treatment did not result any decrease on the PCDH7's proportion in the membrane fraction (elute) for the interphase cells (**Figure 22A**-left panel). However, proportion of the PCDH7 in the membrane fraction strikingly decreased upon the 2BP treatment (**Figure 22A**-right panel, **Figure 22B**).

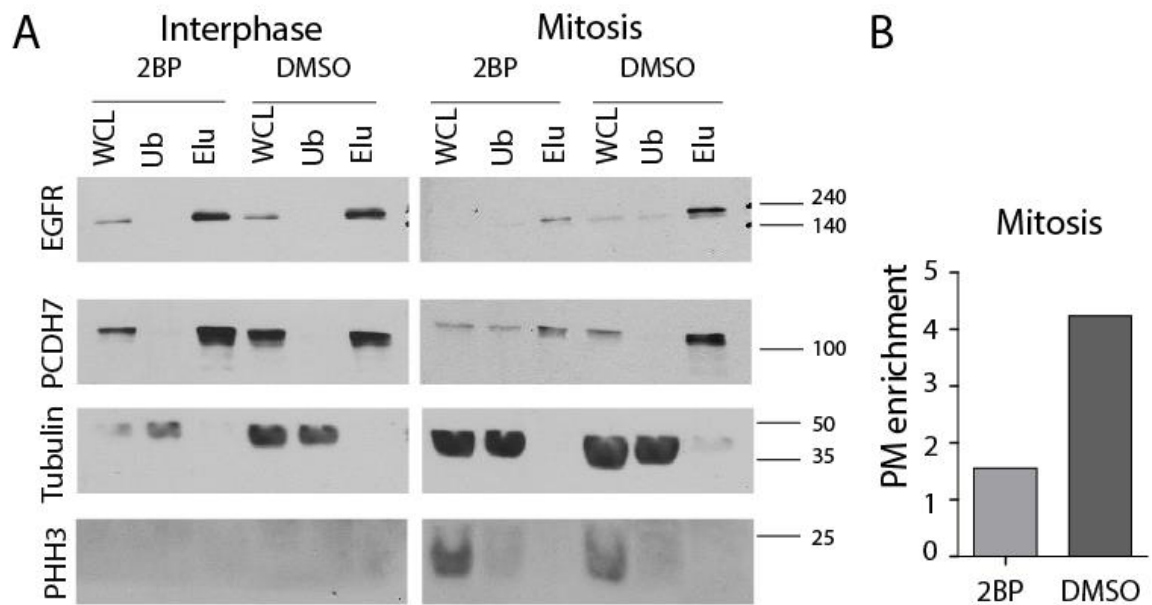


Figure 22: Effect of palmitoylation inhibition on PCDH7's localization. **A.** Western Blot analysis to show the effect of 2BP on PCDH7's plasma membrane localization. Plasma membrane proteins were labeled with amine reactive biotin and pulled down with streptavidin. Plasma membrane fraction is indicated with EGFR. Cytoplasmic fraction is indicated with Tubulin. Presence of PHH3 is an indicator of mitosis. WCL: Whole cell lysate, Ub: Unbound, Elu: Elute **B.** Comparison of the plasma membrane enrichment (elute/WCL) of PCDH7 in the presence and absence of 2BP during mitosis.

In order to visualize the effect of palmitoylation inhibition I used PCDH7-BAC-GFP cells that stably express PCDH7-GFP near physiological levels and used GFP antibody to amplify the GFP signal was. Briefly, cells were synchronized to interphase and mitosis and incubated with 100 mM 2BP containing media overnight. Inhibition of palmitoylation doesn't seem to effect the localization of PCDH7 to cell-to-cell contacts (**Figure 23A**). However, in the mitosis cells, inhibition of palmitoylation has a significant effect on the plasma membrane localization of the PCDH7 (**Figure 23B-C**).

Next, I further visualize the effect of 2PB in mitotic cells by performed live imaging with PCDH7-BAC-GFP cells using Zeiss Cell Observer SD spinning disk microscope. **Figure 24** shows the snapshots from the live imaging experiment. Left panel shows the control cells and the right panel shows the 2BP treated cells. Imaging was performed following the overnight incubation of the cells with 2BP.

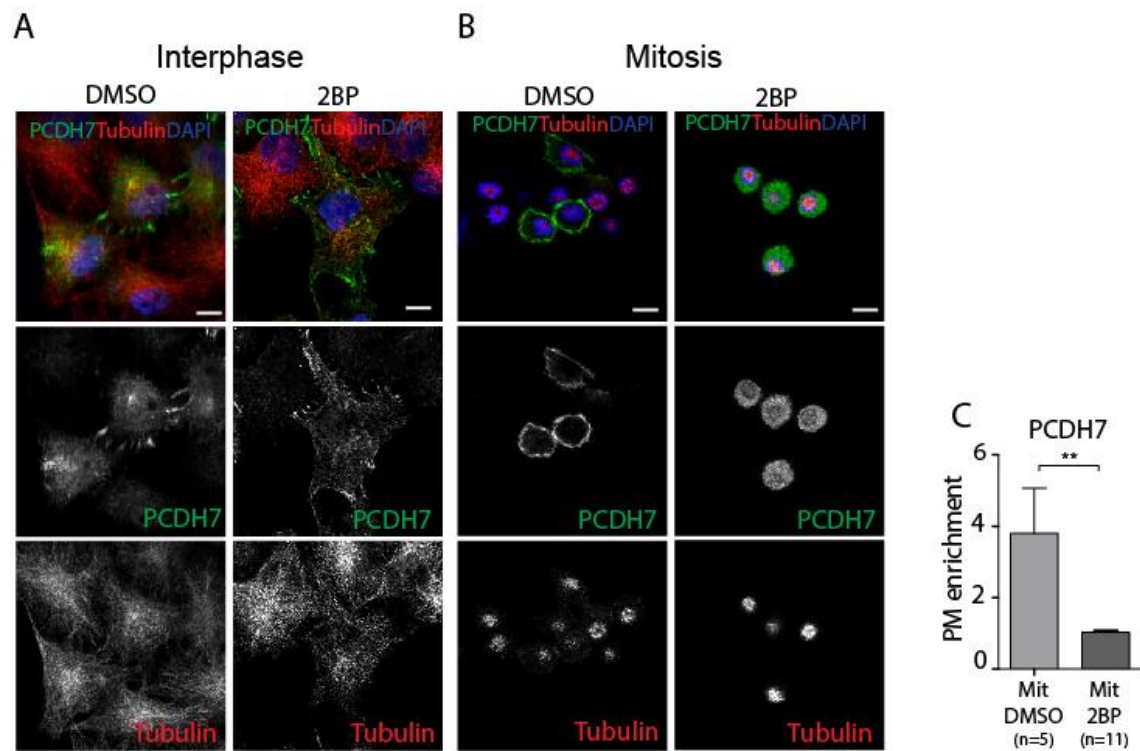


Figure 23: Effect of palmitoylation on PCDH7's cell cycle dependent plasma membrane localization. A. Effect of 2BP on PCDH7's localization on interphase cells. **B.** Effect of 2BP on PCDH7's localization on mitosis cells. **C.** Quantification of the plasma membrane enrichment of PCDH7 during mitosis in the presence/absence of 2BP. Scale bar, 10 μ m

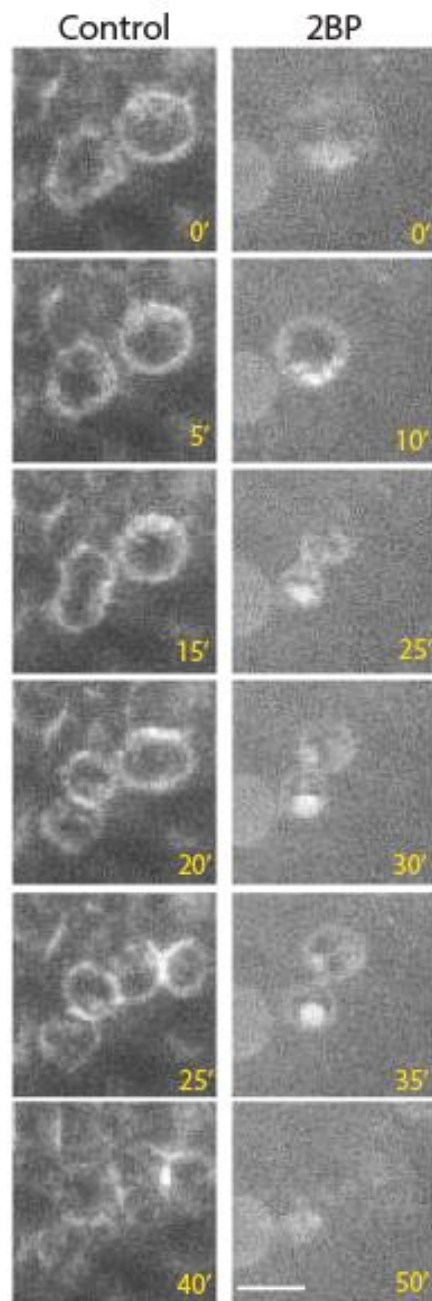


Figure 24: Live imaging snapshots of the dividing cells in the absence (left panel) and presence (right panel) of 2BP. Scale bar, 10 μ m

In order to assess the effect of palmitoylation inhibition of PCDH7's localization dynamics, I compared the recovery of the protein on the plasma membrane with and without the palmitoylation inhibitor 2BP in interphase and mitosis cells. As expected, inhibition of the palmitoylation did not have a significant effect on the turnover of the PCDH7 on the cell to cell contacts during interphase (**Figure 25A**-red lines). In contrast, inhibition of the palmitoylation dramatically delayed the recovery of the PCDH7 during mitosis (**Figure 25A**-blue lines, **Figure 25B**-right).

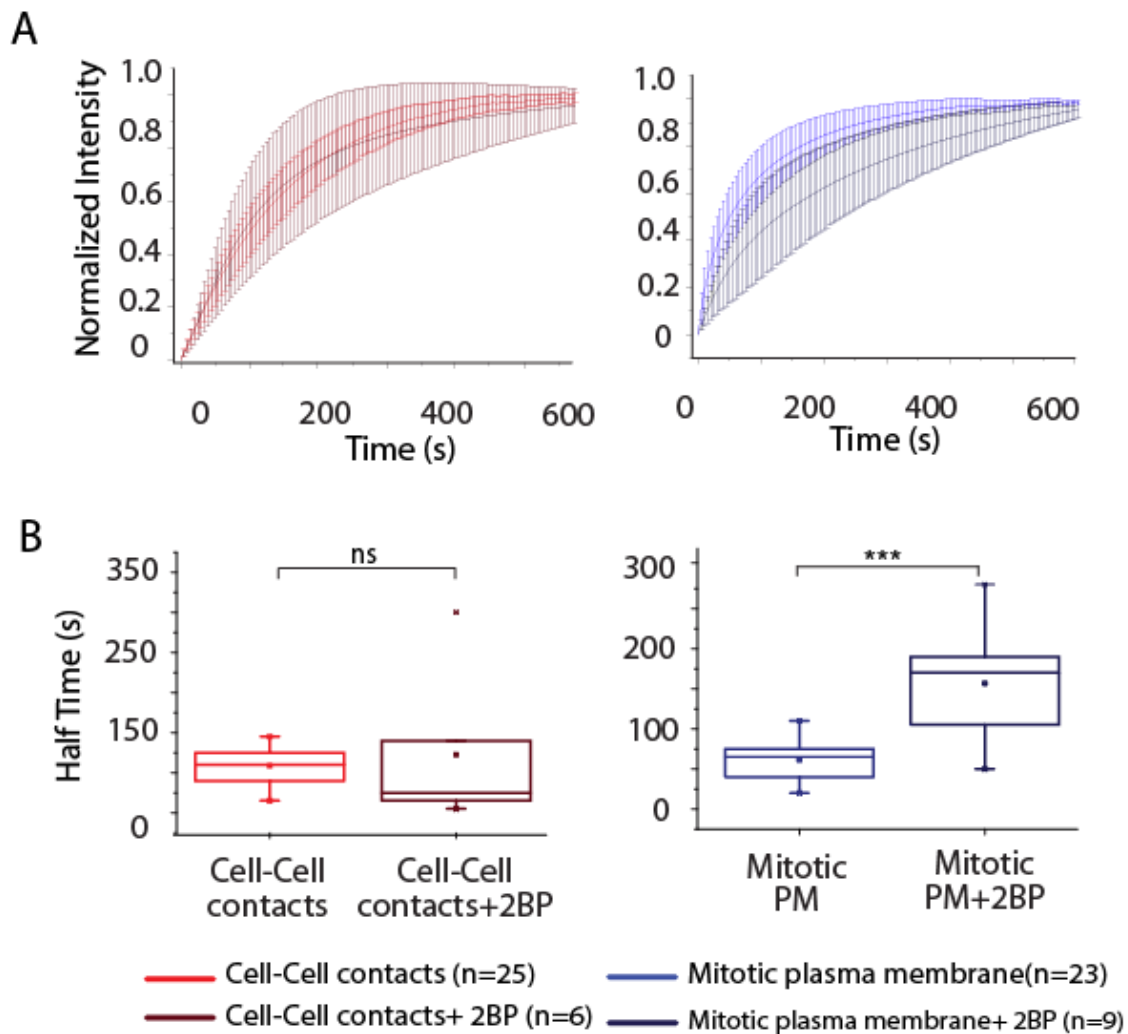


Figure 25: Effect of palmitoylation on mobility of PCDH7 turnover. **A.** Effect of palmitoylation inhibitor 2BP on the recovery pattern of PCDH7 in cell-cell contact (left) and mitotic plasma membrane (right). Darker colors represent the inhibitor-treated cells. **B.** Effect of the palmitoylation inhibitor 2BP on the half-life of the PCDH7 in cell-cell contacts (left) and mitotic plasma membrane (right). Scale bar; 10 μ m **: $p < 0.001$; ns: non-significant

A more direct method to determine the effect of palmitoylation on a protein's localization is to mutate the corresponding cysteine residues on the protein. However, this may also be challenging due to lack of a defined consensus sequence to predict palmitoylated cysteines. Still, it is possible to make educated guesses using specified algorithms. I used CSS-Palmitoylation Site Predictor [152] tool to determine the candidate palmitoylation sites on PCDH7 and site directed mutagenesis strategy [153] to mutate these candidate palmitoylation sites on PCDH7-eGFP vector. In order to visualize the effect of mutations on PCDH7's localization, HeLa S3 cells were transfected with mutated PCDH7-eGFP constructs and imaged with confocal microscopy following by the PFA fixation and immunofluorescence staining. No detectable difference was observed for any of the mutants tested neither for interphase nor for the mitosis cells. As a possible explanation, simultaneous mutations on multiple cysteines might be needed to mimic the effect of 2BP.

Mass spectrometry based identification is another alternative approach to determine whether a protein is palmitoylated. I've adapted an existing method that combines the metabolic labeling and click chemistry for the mass spectrometry based identification of the palmitoylated proteins [146, 147]. According to this method, if a protein is palmitoylated it should appear in the metabolically labeled dataset (17-ODYA) but not in control dataset (Palmitic Acid). PCDH7 was identified in 4 of 6 technical replicates for the first biological replicate and 3 of 3 technical replicates for the second biological replicate of the 17-ODYA dataset. On the other hand, no PCDH7 was identified in any replicates of the control (Palmitic acid) dataset. In order to reveal any possible difference in the protein abundance between the ODYA and the control samples, I compared the coverages of the identified proteins in different replicates. **Figure 26** represents the coverage of some of the commonly identified proteins in all replicates. Even though there are slight fluctuations in the coverages between the replicates, values are consistent. Coverage of the PCDH7 is relatively lower than the other proteins. However, all other proteins are highly abundant

common contaminant proteins such as ribosomal subunits and GAPDH thus, observed high coverages might be expected. These results supports our previous observations obtained using the palmitoylation inhibitor 2BP thus, we can suggest that PCDH7 is palmitoylated.

Gene Name	Coverage																	
	17-ODYA									Palmitic Acid (Control)								
	BR1						BR2			BR1						BR2		
	TR1	TR2	TR3	TR4	TR5	TR6	TR1	TR2	TR3	TR1	TR2	TR3	TR4	TR5	TR6	TR1	TR2	TR3
GAPDH	12,5	11,6	22	16,7	16,7	12,5	16,8	16,8	12,5	9,9	17	11,3	23	14,9	17	23	14,9	17
60S RP-L19	8,7	8,7	8,7	13,3	8,7	8,7	10,5	10,5	10,5	4,6	13,3	8,7	8,7	13,3	8,7	6,2	6,2	10,5
60S RP-L8	10,5	9,7	6,2	10,5	10,5	10,5	13,3	8,8	8,8	6,2	6,2	10,5	6,2	6,2	10,5	8,8	13,5	
PCDH7		1,5		2,6	1,1	1,1	2,6	1,1	1,1									

Figure 26. Comparison of the coverages of the commonly identified proteins in 17-ODYA and Palmitic Acid (control) dataset. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, RPL8: 60S ribosomal protein L8, RPL19: 60S ribosomal protein L19. TR: Technical Replicate BR: Biological replicate

3.4.5 Role of Membrane Trafficking Pathway on PCDH7's sub-cellular localization

From our earlier study we know that cytosolic pool of the PCDH7 localizes to the ER during interphase [1]. Since the vesicular trafficking pathway is a well-known transport pathway from ER to plasma membrane, we tested the role of endo-vesicular trafficking on PCDH7's translocation to the plasma membrane during mitosis. Inhibition of the vesicular trafficking with dynamin inhibitor Dynasore did not have an effect on the localization of PCDH7 to cell-cell contacts in the interphase cells (**Figure 27A**-left panel) however; it dramatically decreased the plasma membrane enrichment of PCDH7 during mitosis (**Figure 27B**-right panel, **Figure 27C**).

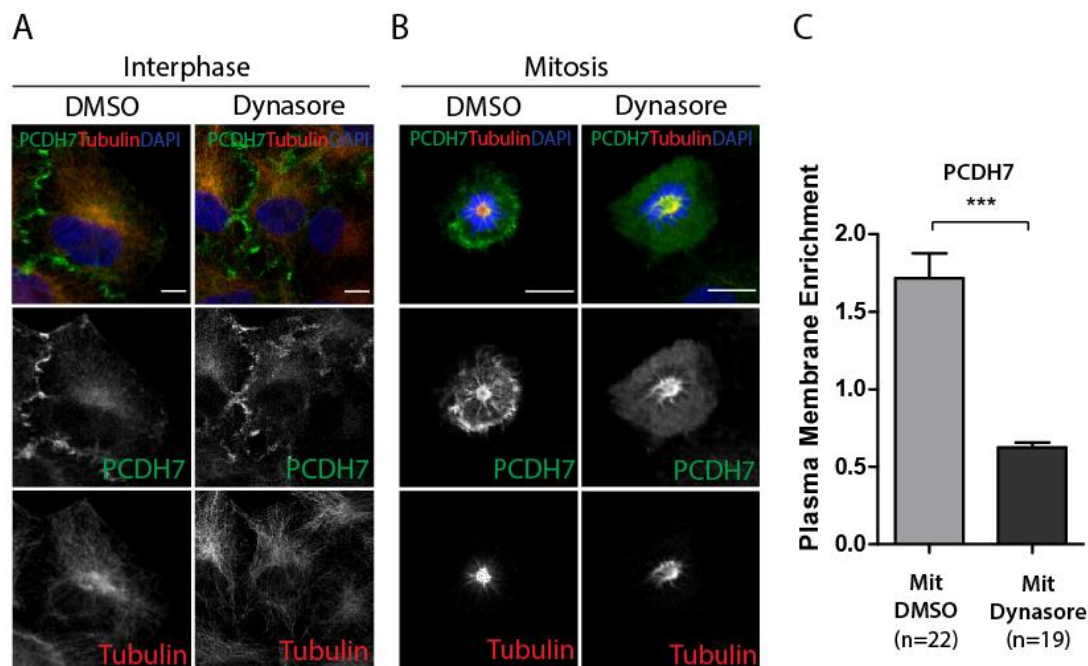


Figure 27: Role of membrane trafficking on PCDH7's localization. **A.** Cellular localization of the PCDH7 in the cell-cell contacts during interphase (left panel) and mitotic plasma membrane (right panel) in the presence of the vesicular trafficking inhibitor Dynasore. **B.** Quantification of the effect of Dynasore on PCDH7's plasma membrane enrichment in mitotic cells. Scale bar; 10 μ m **: $p < 0.001$

Next we tested whether PCDH7 co-localizes with the different vesicular markers. Early endosomal marker EEA1 did not show any co-localization pattern with the PCDH7 while late endosomal marker CD63 showed a moderate co-localization pattern (**Figure 28A-B**). On the other hand, late endo-lysosomal marker LAMP1 significantly co-localized with the PCDH7 when compared with the other markers (**Figure 28C-D**).

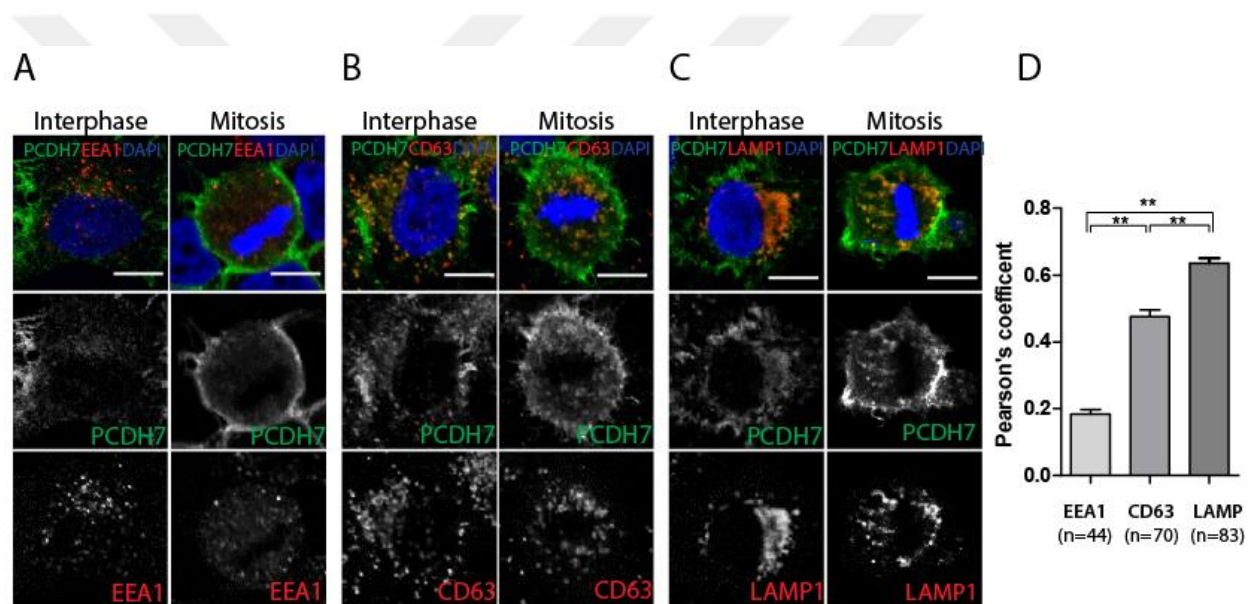


Figure 28: Co-localization pattern of the PCDH7 with the different vesicular markers.

A. Localization pattern of the early endosomal marker EEA1 and the PCDH7 in cell-cell contacts during interphase (left panel) and mitosis (right panel) cells. **B.** Localization pattern of the late endosomal marker CD63 and the PCDH7 in cell-cell contacts during interphase (left panel) and mitosis (right panel) cells. **C.** Localization pattern of the lysosomal marker LAMP1 and the PCDH7 in cell-cell contacts during interphase (left panel) and mitosis (right panel) cells. **D.** Pearson's coefficients for the co-localization of the PCDH7 with each vesicular marker.

We next analyzed the effect of palmitoylation inhibition on PCDH7's co-localization pattern with the LAMP1. Cellular localization pattern of PCDH7 and LAMP1 is represented in **Figure 29** both for interphase (**Figure 29A**) and mitosis cells (**Figure 29B**) in the presence and absence of palmitoylation inhibitor 2BP. To quantify the effect of palmitoylation inhibition, we calculated the Mander's coefficients M2, which represents the fraction of PCDH7 overlapping with LAMP1; in the presence of 2BP. Overlap of PCDH7 with LAMP1 is significantly higher in the interphase cells when compared to mitotic cells (**Figure 29C**, Bars 1-3). Inhibition of the palmitoylation doesn't have a significant effect on the PCDH7's overlap with the LAMP1 in interphase (**Figure 29C**, Bars 1-2). On the other hand, inhibition of the palmitoylation significantly increases the overlap of the PCDH7 with LAMP1 in mitosis (**Figure 29C**, Bars 3-4).

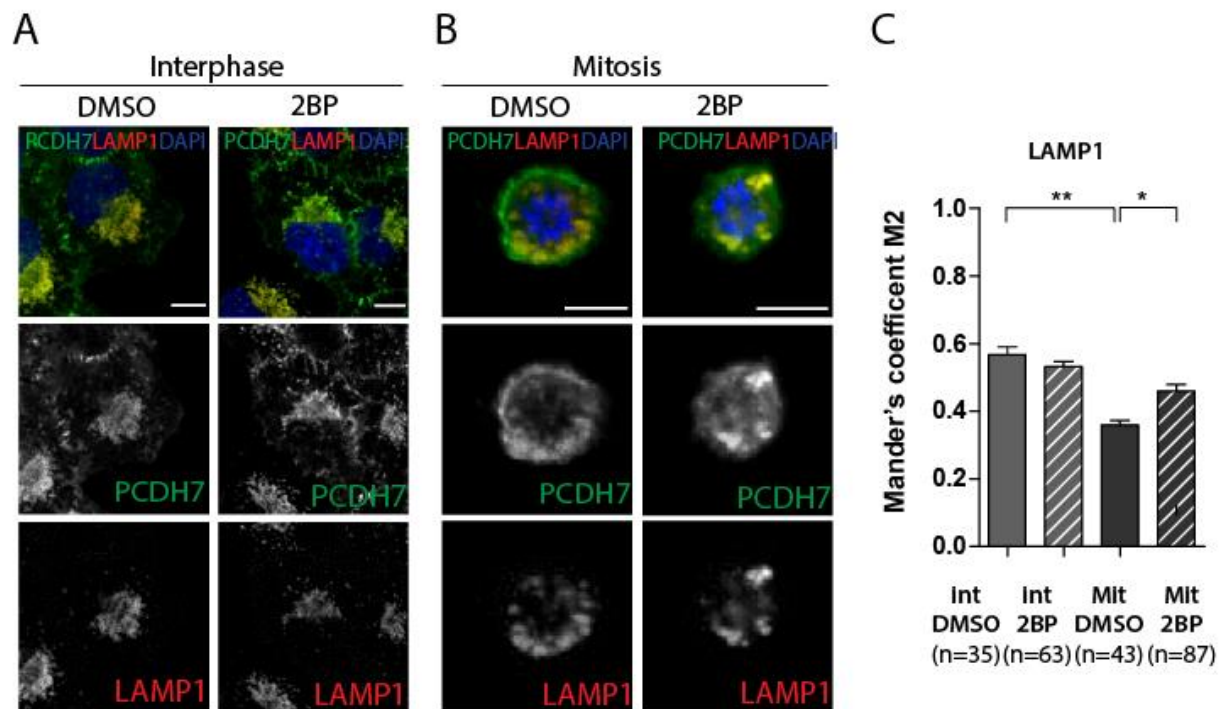


Figure 29: Effect of the palmitoylation on PCDH7's vesicular localization. **A.** Cellular localization of PCDH7 and LAMP1 during interphase in the presence of 2BP. **B.** Cellular localization of PCDH7 and LAMP1 during mitosis in the presence of 2BP. **C.** Mander's coefficient M2; fraction of green (PCDH7) signal overlapping with red (LAMP1) signal in mitotic cells in the presence and absence of palmitoylation inhibitor 2BP. Scale bar: μm 10; *: $p < 0.005$; **: $p < 0.001$

These results suggest that vesicular trafficking is important for the PCDH7's dynamic plasma membrane localization in mitosis. PCDH7 does not co-localize with the early endosomes but shows significant co-localization with late endo-lysosomal vesicles during interphase. Moreover, inhibition of the vesicular transport pathway prevents the plasma membrane enrichment of the PCDH7 during mitosis. Thus, PCDH7 might be stored in the late endo-lysosomal vesicular pool and recycled back to the plasma membrane on the onset of mitosis. Furthermore, inhibition of the palmitoylation prevents the plasma membrane enrichment of the PCDH7 during mitosis. As a result of the palmitoylation inhibition, the increased cytoplasmic pool of PCDH7 seems to be sequestered to the late endo-lysosomal vesicles. These results suggest the necessity of the palmitoylation for the recycling of the PCDH7 from late endo-lysosomal vesicles back to plasma membrane. In conclusion, at the onset of mitosis, PCDH7 translocates to the plasma membrane via endo-vesicular pathway, particularly late endo-lysosomal vesicles and palmitoylation is required for the transfer of the PCDH7 from the vesicles to the plasma membrane.

3.4.6 Role of PCDH7 During Cell Division

In order to understand the role of PCDH7 during mitosis, we used CRISPR-Cas9 genome editing system and knocked-out the endogenous PCDH7 from HeLa cells. PCDH7 sequence was targeted using different sgRNAs (Appendix B) and isolated single colonies were tested for the PCDH7 protein levels using Western blotting. PCDH7sg1 Colony 2, which was generated by the transient transfection of PCDH7-CRISPR vector, (**Figure 30-lane5**) was selected for the further characterization.

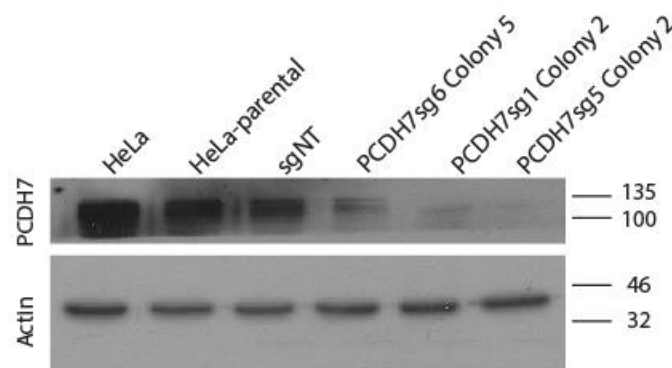


Figure 30: PCDH7 levels in the PCDH7 CRISPR cells. Western blot shows the PCDH7 levels in the different single colonies for PCDH7 CRISPR, non-treated HeLa cells, and non-targeting sgRNA (sgNT) treated cells. Actin was used as the loading control.

After confirming the depletion of the PCDH7, I tested the capability to restore the PCDH7 levels by expression of PCDH7-GFP which is crucial for the rescue experiments. PCDH7-eGFP plasmid was introduced to CRISPR colonies by lentiviral transduction to generate the rescue cell lines. **Figure 31** represent the PCDH7 levels of the colonies and the control cells before (left) and after (right) introduction of the PCDH7 expression.

PCDH7-GFP expression successfully restored the PCDH7 levels while empty-GFP expression did not.

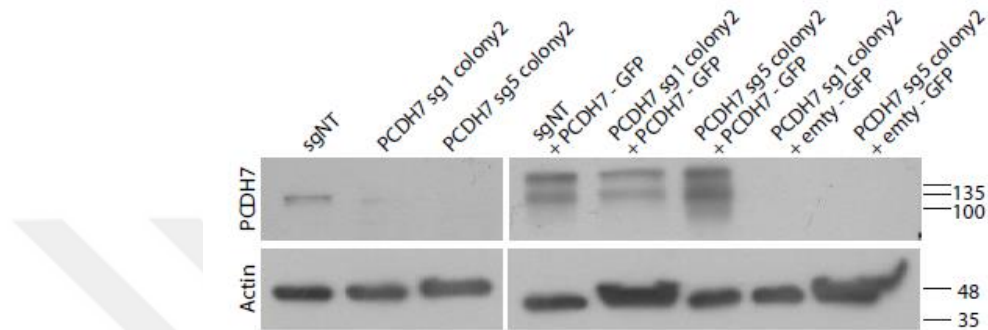


Figure 31: PCDH7 levels in CRISPR colonies and rescue cell lines. Western blot shows the PCDH7 levels of the PCDH7-CRISPR single colonies before (left) and after (right) viral transduction of PCDH7-eGFP or empty-GFP. Upper band corresponds to fusion protein (PCDH7-GFP, 116+27) while lower band corresponds to PCDH7 (116). Actin was used as the loading control.

The first phenotype that we observed in the PCDH7-CRISPR cells was the increased number of the multinucleated cells. **Figure 32** shows representative fluorescence images from the PCDH7 sg1colony2 and (right) and the non-targeting sgRNA treated control cells (left).

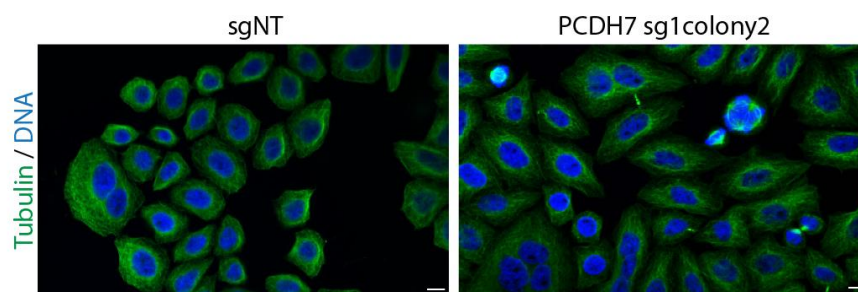


Figure 32: Representative image of control (left) and PCDH7 (right) CRISPR cells. Cells were fixed with PFA and stained for tubulin for visualization of the cell shape (green) and the DAPI for the visualization of the DNA (blue). Scale bar; 10 μ m.

I counted the number of multinucleated cells in each group for quantification. As shown in the **Figure 33**, ratio of the multinucleated cells in the PCDH7-CRISPR group is significantly higher than the control group and this effect can be reversed by restoring the expression of the PCDH7.

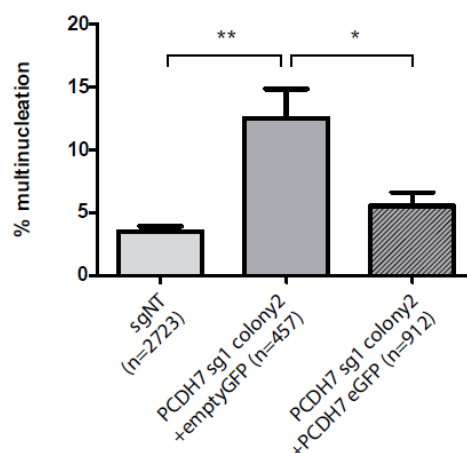


Figure 33: Comparison of the multinucleation ratio in PCDH7-CRISPR, control and rescue cells. (*: $p < 0.005$; **: $p < 0.001$)

Then I wanted to assess the effects of PCDH7 knock out on mitosis in real time and performed live imaging using Zeiss Cell Observer SD spinning disk microscope. There was a noteworthy increase in the number of multipolar cell divisions in the PCDH7 knock out cells (**Figure 34A**). As shown in **Figure 34B**, ratio of the multipolar divisions in the PCDH7 knock out cells (2nd bar) is remarkably higher than the control cells (1st bar). In addition, restoring the PCDH7 levels by PCDH7-GFP expression reversed this phenotype (3rd bar) while expression of empty-GFP did not caused such an effect. These results indicate that observed multipolar division phenotype is specific for the PCDH7 knock out.

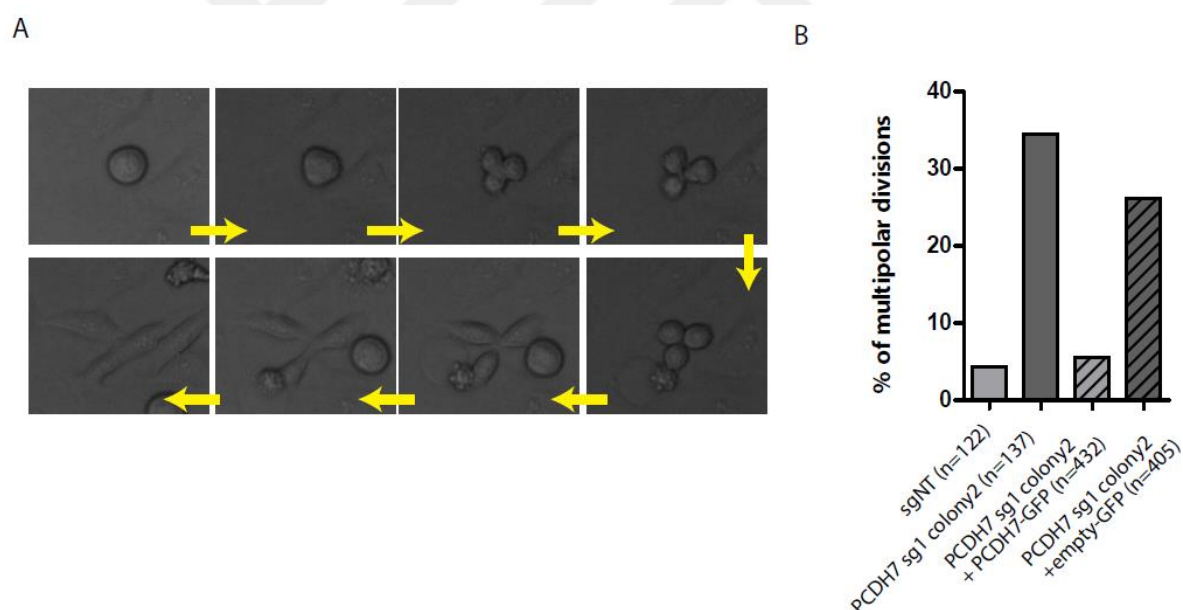


Figure 34: PCDH7 knock-out phenotype, multipolar division. **A.** Representative time-lapse images for the multipolar division phenotype observed upon PCDH7 knock-out. Images were taken in 5 minutes intervals. **B.** Comparison of the multipolar division percentages in the control, PCDH7 knock out and rescue cell lines.

The first results strongly suggested that knock out of PCDH7 has an effect on mitotic. Thus, I wanted to examine the mitotic processes in details and performed another set of the live imaging experiment, this time shortening the imaging interval to 1 minute to be able to discriminate the different phases of mitosis. Starting from the first retraction of the cell, I measured the time frames between the fully rounded state, formation of metaphase plate, start of anaphase until the end of the cytokinesis. In addition, I measured the distance between the two opposite poles of the dividing cells to see any possible difference on the segregation of the daughters. Representative time lapse images for the different phases of the cell division is presented in the **Figure 35A**. Segregation pattern of the PCDH7 knock out cells were significantly different than the control cells (**Figure 35B**). In the absence of PCDH7, daughter cells are more separated from each other.

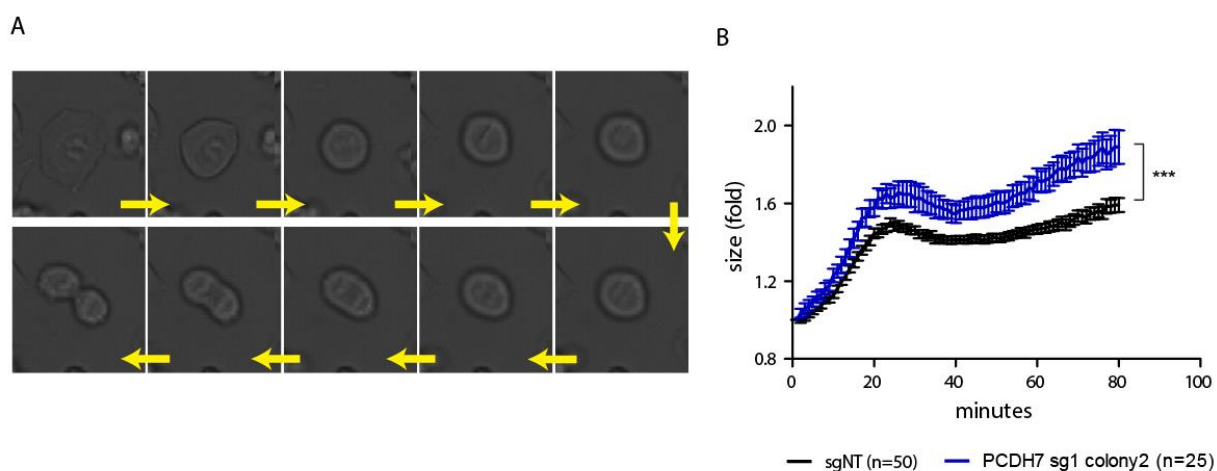


Figure 35: PCDH7 knock-out phenotype, separation of the daughter cells. A. Representative time-lapse images for the different phases of cell division in control cells. Images were taken in 1 minute intervals. **B.** Comparison of the distance between the two opposite poles of the dividing cell through the cell division. Size is normalized to the initial

diameter of each rounded cell. Black lines: control; blue lines: PCDH7 knock-out. (***: $p < 0.001$)

Since the segregation of the daughter cells are affected, I also measured the total distance between the opposite poles of the daughter cells when they are fully separated and spreaded. In accordance with the segregation data, PCDH7 knock-out daughter cells reached to significantly greater distances (**Figure 36-left**). Similarly, maximum distance between the two daughters was significantly longer in the absence of PCDH7 (**Figure 36-right**).

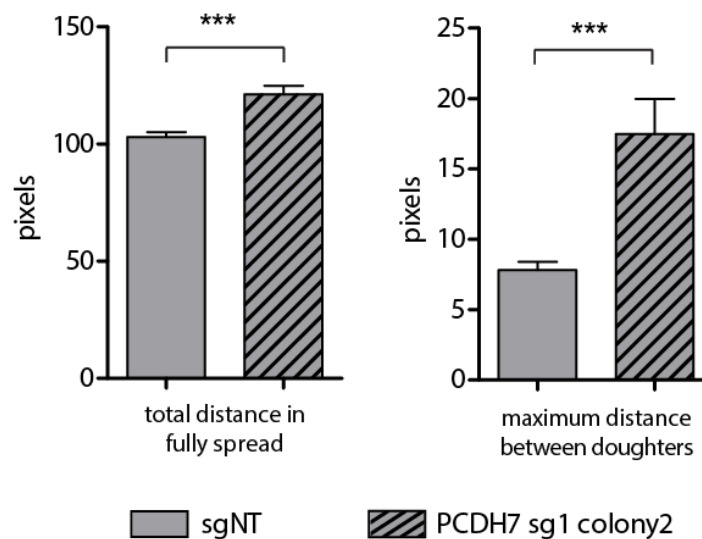


Figure 36: PCDH7 knock-out phenotype, distance between the daughters. (*: $p < 0.001$)**

Next, I compared the time spent in the different phases of the cell division in PCDH7 knock-out and control cells. Rounding takes significantly longer time in the absence of PCDH7 (**Figure 37**). There was no significant difference observed for either chromosomal or spindle alignment and cytokinesis. However, time spent to align the mitotic spindle was slightly shorter for the PCDH7 knock outs while cytokinesis took slightly longer (**Figure 37**).

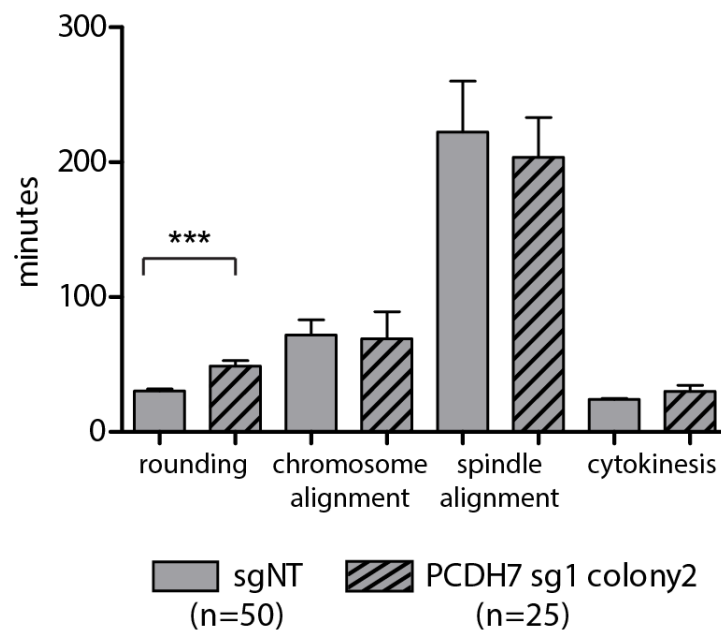


Figure 37: CDH7 knock-out phenotype, analysis of cell division timing. (***:p<0.001)

3.5 DISCUSSION AND CONCLUSION

In this study I aimed to reveal the mechanism of cell cycle dependent localization of the PCDH7 and understand its role in cell division.

In order to visualize and track the subcellular localization of the PCDH7, I utilized the PCDH7-BAC-GFP cell line that expresses the fluorescence tagged protein from its own promoter. Using a BAC-GFP cell line provides an expression profile near the physiological levels, eliminating the possible artifacts of overexpression or antibody staining. In accordance with our previous study [1], PCDH7 localized to the cell-to cell contacts during interphase, enriched in the mitotic plasma membrane and concentrated in the cleavage furrow during cytokinesis. When I used the FRAP approach to test the dynamicity of the PCDH7, I found that PCDH7 at the mitotic cell surface is much more dynamic than the at the cell-to-cell contact sites suggesting the involvement of different mechanism for the plasma membrane localization of PCDH7 during interphase and mitosis.

In order to reveal mechanisms that govern the cell cycle specific surface localization of PCDH7, I used proximity dependent interaction mapping [150] in a cell cycle dependent manner and identified a candidate group of cell cycle dependent proximity interactors.

One of the interphase specific candidate interactor of PCDH7 was cortactin (CTTN) which is an actin binding adaptor protein that was proposed to be a critical regulator of the actin assembly [154-156]. It can interact with Arp2/3, activate actin nucleation and stabilize the cortical actin network by inhibiting the disassembly of Arp3/3 generated filaments [157, 158]. Cortactin localizes to the cell-cell adhesive contacts and was shown to be essential for the Arp2/3 mediated actin assembly on those sites and formation of E-cadherin mediated contact zones [159]. In addition to E-cadherin, cortactin was also shown to interact with other adhesion molecules such as ICAM-1 (Intercellular adhesion

molecule-1) in endothelial cells, mediating its interaction with the actin cytoskeleton [160]. Considering these relations with the other adhesion proteins, interaction of cortactin with PCDH7 might be important for the cell-to cell contact localization of the PCDH7 during interphase. In addition, cortactin was also shown to function in the focal adhesion (FA) turnover and migration [161] which might be related with the suggested migratory role of the PCDH7 [55].

An interesting mitosis specific candidate interactor is 14-3-3 sigma (SFN) which have diverse roles in signaling and was shown to control the mitotic transition and facilitate cytokinesis [162, 163]. Annexin A5 (ANXA5) is another mitosis specific candidate interactor which belongs to a Ca^{2+} regulated protein family. Ca^{2+} bound forms of annexins can bind to negatively charged membranes thereby participate membrane-linked events such as vesicular transport, membrane-cytoskeleton linkage [164]. Besides, one of the Annexins (Annexin 11) was previously shown to localize to spindle midzone during cytokinesis and function on the formation of the midbody. [165] Considering their known functions, these candidate interactors might be functionally interacting with PCDH7 during mitosis yet further validation is needed.

Ezrin (EZR) is another candidate interactor and belongs to the ERM (Ezrin-Radixin-Moesin) family of proteins that is known to crosslink the actin cytoskeleton with the plasma membrane [166]. During mitosis, ERM proteins have been shown to function on mitotic cell rounding and also interaction of the mitotic spindle with the actin cortex [167]. Thus, Ezrin-PCDH7 might be in relation during the process of mitotic cell rounding.

Next candidate interactor is the intermediate filament vimentin (VIM). Vimentin is known to regulate the vesicular trafficking pathway in multiple levels [168]. It has been shown to interact with the AP-3 adaptor complex and function in the sorting of late endo-lysosomal cargo proteins including LAMP-1 and LAMP-2 [169]. Since we have shown that

PCDH7 co-localizes with the LAMP1 positive late endo-lysosomal vesicles, we can suggest a possible role of vimentin for the vesicular localization of PCDH7.

Another candidate interactor is Myosin-9 (MYH9) which is a myosin II heavy chain protein. Myosin II is the main motor protein that regulates the contraction during cytokinesis [170, 171]. In addition to its role in the cytokinesis, Myosin II in the cortex of rounded mitotic cells was shown to be required for the spindle positioning [172]. Thus, it is possible that PCDH7 and Myosin II might be in relation during cell rounding and cytokinesis. Our data with the PCDH7 knock-out cells further supported this hypothesis (discussed below).

Among all these possible interactors we have chosen one specific candidate that can provide a mechanistic explanation to PCDH7's cell cycle dependent surface localization. ZDHHC5 is a palmitoyltransferase enzyme that catalyzes palmitoylation reaction which is the transfers the palmitic acid groups to the cysteine residues of proteins. Palmitoylation is a reversible post translation modification and known to effect the localization of the proteins. Palmitoylation of the soluble proteins like Ras can regulate the membrane association and plasma membrane localization of the protein. On the other hand, palmitoylation of the membrane proteins might have a more diverse effect. Palmitoylation may initiate the conformational changes, regulate complex formation or specific membrane domain association and interact with other post translational modifications [151, 173]. Thus, I hypothesized that palmitoylation might have a role in cell cycle specific plasma membrane localization of PCDH7 and used palmitoylation inhibitor 2BP to test this hypothesis. Indeed, both biochemical approaches and imaging showed that mitotic surface localization of PCFH7 is dependent on the palmitoylation whereas, localization to cell-to-cell contacts during interphase is not effected by the palmitoylation inhibition. In addition, dynamicity of the PCDH7 during mitosis is also dependent on the palmitoylation since recovery of the protein is significantly decelerated upon palmitoylation inhibition.

These results indicate that palmitoylation is required for the mitotic surface localization of PCDH7 however do not prove that PCDH7 itself is palmitoylated. In order to test that, I took a mass spectrometry based approach that combines the metabolic labeling with the click chemistry to identify the palmitoylated proteins [146] [147] and adapted it for PCDH7. In this method, a clickable analog of palmitate (17-ODYA) is metabolically incorporated to the proteins. After “clicking” this palmitate analog with a biotin reagent, palmitoylated proteins can be enriched using biotin/streptavidin affinity purification. Comparison of these proteins with the unlabeled dataset enables the identification of the palmitoylated protein. If PCDH7 is palmitoylated, it should be identified in the metabolically labeled dataset but not in the control dataset. According to two different biological replicates and in total of nine technical replicates, this was the case, strongly suggesting that PCDH7 is palmitoylated. Next, I aimed to identify the palmitoylation sites on the PCDH7 and used site directed mutagenesis strategy [153] to mutate the candidate cysteines. Since there is no consensus sequence for the palmitoylation, searching for the palmitoylated cysteines is no different than a lottery. Using the prediction algorithms [152] did not help much and none of the tested mutations had significant effect of the plasma membrane localization of the PCDH7. A cumulative effect of the palmitoylation on the multiple cysteine residues might be involved thus; mutating just one or two residues might not be enough to mimic the effect of the inhibitor. Interestingly, one of the conserved cysteine residues among the all δ -Pcdhs was suggested to be palmitoylated in rat Pcdh1, together with the several other δ -Pcdhs [108, 174]. Moreover, according to the authors’ unpublished observations, mutating this cysteine residue did not affect the membrane localization of PCDH1 and they suggested that the contribution of the other residues might be important [108]. This result is in agreement with our data, suggesting an accumulative role of palmitoylation in different residues. It

will be important to uncover the effect of palmitoylation on PCDH1's cell cycle dependent membrane localization in the future.

Palmitoylation could be mechanism for the mitosis specific plasma membrane localization but what is the mechanism of PCDH7 transport to the cell surface? Our earlier study showed that PCDH7 co-localizes with the ER markers during interphase [1]. The most common way to shuffle a protein between ER and plasma membrane is to use the vesicular trafficking pathways. Transmembrane proteins are co-translationally translocated to the ER, matured through the ER-Golgi pathway and reach to transport vesicles in which they will be carried to the plasma membrane in a regulated manner [175]. However, journey of these proteins usually do not end here, they can be endocytosed back from the plasma membrane and shuffle between these membranous compartments according to regulatory signals [176]. Therefore, vesicular trafficking pathway could be the mechanism for the shuffling of PCDH7 between plasma membrane and the interior membranes. In addition, palmitoylation has been previously reported to effect the vesicular association of the protein which would fit to our hypothesis.

Thus, I tested whether the vesicular trafficking has role in PCDH7's plasma membrane localization and if PCDH7 co-localizes with the different vesicular markers. Inhibition of the vesicular trafficking significantly decreased the plasma membrane enrichment of PCDH7 during mitosis while had no effect on the cell-to-cell contact localization during interphase. In addition, PCDH7 co-localized with the late endo-lysosomal vesicles, especially during interphase. Beside, inhibition of the palmitoylation increased the co-localization of the PCDH7 in these vesicles during mitosis, meaning that the protein is trapped in those vesicles when the palmitoylation is inhibited.

Based on these data, **Figure 38** represents our predicted model for the mechanism of the plasma membrane localization of PCDH7. During interphase, a relatively stable pool of PCDH7 (dark green) localizes to the cell-to-cell contacts and this localization is not

dependent on the palmitoylation. Rest of the PCDH7 is stored in the interior membranes, especially late endo-lysosomal vesicles (**Figure 38A**). As cell enters to mitosis and start to get round, vesicular pool of the PCDH7 is transferred to the plasma membrane (light green) and this transfer is dependent on the palmitoylation (**Figure 38B**).

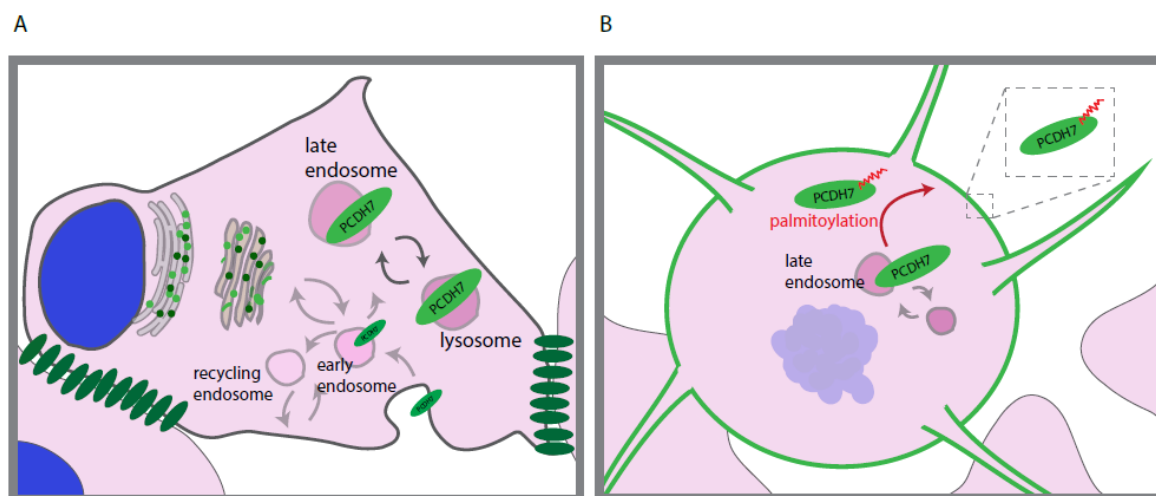


Figure 38: Model of plasma membrane localization of PCDH7. A. Interphase. Dark Green; a less dynamic pool of PCDH7 that localizes to the cell-cell contacts. Rest of the protein (light green) is stored in the vesicular pool-mainly in the late endo-lysosomal vesicles. **B.** Mitosis. Vesicular pool of PCDH7 (light green) is translocated to the cell surface in a palmitoylation dependent manner and decorates mitotic plasma membrane and retraction fibers.

Having this possible mechanism for the cell cycle dependent surface localization of PCDH7, we next wanted to assess the role of PCDH7 in cell division. From our earlier studies, we know that plasma membrane localization of PCDH7 is important for the mitotic rounding [1]. In order to understand the role of PCDH7, we knock out the protein using CRISPR-Cas9 system [144]. Absence of PCDH7 caused a significant delay in the mitotic rounding indicating the importance of PCDH7 on rounding process. This result is in line

with our previous study, in which the depletion of the PCDH7 using RNAi caused a significant decrease in the rounding pressure in mitotic cells resembling the effect of Myosin II depletion [1].

Mitotic rounding is an essential process for the fidelity of the cell division. Rounding of the cells provides the optimal geometry for the formation and the correct positioning of the mitotic spindle. Spherical shape keeps the chromosomes in a limited area in which they could be found by the spindle microtubules enabling the correct chromosomal capture [52]. Prevention of the mitotic cell rounding was shown to result in chromosome segregation errors [71] [52].

Accordingly, knock out of PCDH7 significantly increased the number of the multinucleated cells and this effect was reversed upon restoring the PCDH7 levels.

Another interesting effect of the PCDH7 knock out was the increased number of the multipolar cell division, which could be reversed by the re-expression of PCDH7.

Spherical shape was shown to be essential to maintain the bipolar spindle since prevention of the mitotic rounding caused multipolar cell division [71]. This is particularly important for the cancer cells that have increased number of chromosomes and centrosomes. Cancer cells usually have aberrant chromosome and centrosome numbers which likely to cause formation of multipolar spindles. However, multipolar cell divisions often result with the cell death whereas cancer cells have invented different mechanisms to maintain the bipolar spindle in the presence of multiple centrosomes and chromosomes [52, 177-179] . It has been suggested that mitotic rounding facilitate the formation of the bipolar spindles in those cells bearing extra centrosomes [52]. This hypothesis overlaps with our multipolar cell division phenotype that we observed in the absence of PCDH7. HeLa cells are cancer cells with a number of genetic abnormalities including increased number of chromosomes [180]. Mitotic rounding is negatively effected in the PCDH7 knock out cells thus; it is likely that they lack the adapted mechanism which would prevent the formation of multipolar spindles

in the HeLa cells. Besides, cells spend less time to align their spindle in the absence of PCDH7, supporting the idea that multi-chromosomal/multi-centrosomal cells would need more time to form bipolar spindle.

We can conclude that PCDH7 localization to the plasma membrane during mitosis is required for the proper mitotic rounding and fidelity of cell division, especially for the cancer cells with increased number of chromosomes. It has been suggested that rounding ability is particularly important for the cancer cells that need to divide in crowded tumor micro-environment and stiffness provided by the mitotic rounding enable cancer cells to divide under extreme physical pressure and in the metastatic sites that different from their origins [52, 181]. Thus, we can argue that the upregulation of PCDH7 in cancer cells may contribute their survival by contributing to the cell rounding, increasing the cortex stiffness and ensuring the bipolar cell division.

In addition to get enriched in the mitotic plasma membrane, PCDH7 localizes to cleavage furrow as cells proceed to cytokinesis. Thus, I wanted to dissect any possible effect of PCDH7 knock-out on the subsequent phases of mitosis and I examined the whole process in details. PCDH7 knock-out affected the separation of the daughter cells from each other. In the absence of PCDH7, daughter cells were separated more, reaching further away from each other and also spreading to greater distance suggesting a possible role of PCDH7 in cytokinesis.

Stiffness of the cortex and the membrane mechanics are crucial also for the cytokinesis. During the mitotic rounding, tension on the substrate is gradually decreased as cells detach, while cortical tension and membrane tension are increased [182]. Cortical tension remains uniformly distributed across the mitotic plasma membrane until anaphase and resists the deforming forces. On the onset of cytokinesis, cortical tension affects the ingression dynamics and help to push out the cytoplasm from the bridge region [66, 183].

Changes in the cortical tension and mechanics of the cytokinesis are explained with two different models which are proposed to work in cooperation [184]. The first model is the equatorial contractility model, and according to this model, myosin II generated forces drives the cleavage. The second model is the polar relaxation model, in which the expansive forces in the polar cortex increase the viscoelasticity of the cleavage furrow cortex and drive separation. This Myosin II independent, adhesion dependent model of cytokinesis was first described in the slime mold *Dictyostelium discoideum* mutants that lack the myosin II and further demonstrated in the mammalian cells [185, 186]. It is likely that in normal cells, balance of the forces generated by these two different mechanisms collectively control the cytokinesis dynamic. However, when one of these mechanisms is not functioning properly, as in the case of the myosin II mutants, remaining mechanisms may dominate to successfully complete the cytokinesis [184].

In PCDH7 mutants, pole to pole distance of the daughter cells significantly increased, which is a phenotype that resemble the cytokinesis in myosin II mutants [184]. In addition, distance between the daughter cells is also increased together with the spreading of the daughters and these might be expected in the action of adhesion dependent cytokinesis.

Mitotic plasma membrane localization of PCDH7 is important for the stiffening of the mitotic cortex and is likely contribute to generation of the cortical tension. As cells proceed to cytokinesis, cortical tension acts on the cleave furrow which is also the place that PCDH7 is concentrated. Thus, role of PCDH7 during the cytokinesis might be related with the sustaining the cortical pressure in the cleavage furrow, likely through generating a tension between the membranes of the daughters and keeping them detached from the substrate, thus supporting equatorial stimulated cytokinesis.

We identified a Myosin II heavy chain protein (MYH9) as one of the candidate interactors of PCDH7. When combined with PCDH7 knock out phenotypes, we can

speculate that, PCDH7 functions in close relation with the Myosin II both for in mitotic cell rounding and cytokinesis. PCDH7 localizes to the mitotic cortex of the rounded cells and gets concentrated in the cleavage furrow as cell proceeds to Myosin II dependent cytokinesis (**Figure 39-left**)).

Absence of PCDH7 resembles the Myosin II mutants in which the contractile ring stimulated cytokinesis is not functioning properly. Thus it is possible that adhesion dependent cytokinesis dominates in the absence of PCDH7 which would explain the increased pole-to-pole distance and spreading in PCDH7-null cells (**Figure 39-right**)).

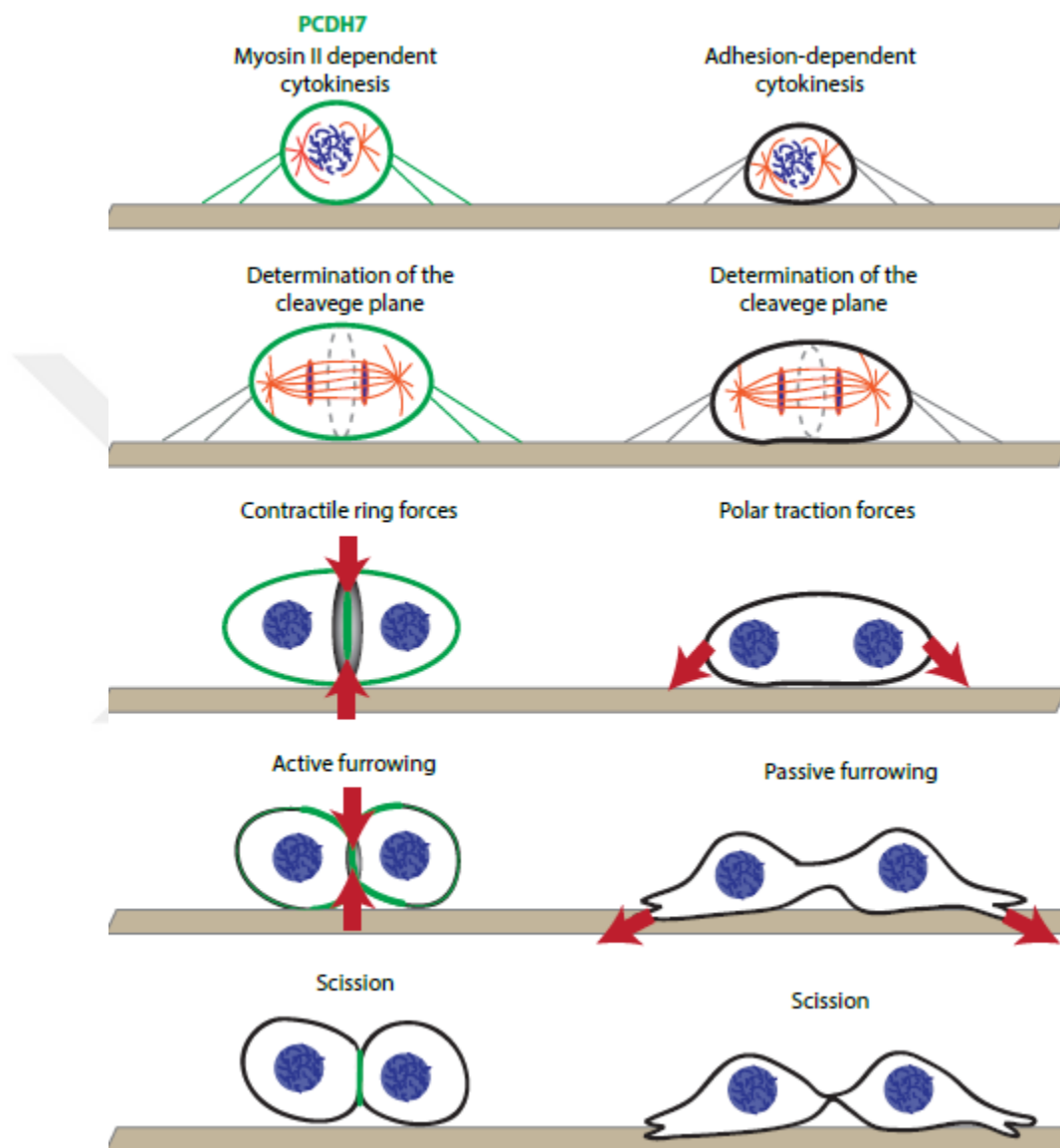


Figure 39: Proposed model for the role of PCDH7 in cytokinesis. PCDH7 (green) localizes to the cleavage furrow. In absence of the PCDH7 myosin II dependent cytokinesis is compromised. (Adapted from [187])

In summary, according to our proposed model, PCDH7 localizes to the mitotic plasma membrane via vesicular transport in a palmitoylation dependent manner. This localization is important for the mitotic rounding and generation of the cortical tension which are crucial to maintain the bipolar mitotic spindle and successful segregation of the chromosomes. Function of the PCDH7 during cytokinesis is possibly supplementary for the Myosin II dependent furrowing mechanism.



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Vita

Nazlı Ezgi Özkan Küçük was born in Antalya, Turkey on April 11, 1989. She graduated from Manavgat Anatolian High School in 2007 and then received her B.Sc. degree in Molecular Biology and Genetics from Istanbul University, Istanbul, in 2011. She received her M.Sc degree in Molecular Medicine with the thesis title “Investigation of possible relations between TCF7L2 gene and 8q24 gene desert variants, and their possible effects on c-myc gene expression and colorectal cancer risk.” from Istanbul University Experimental Medicine Research Institute, Istanbul, in 2013 under the supervision of Prof.Dr.İlhan Yaylım.

From December 2013 to August 2018, she has worked as a teaching and research assistant in Koç University, Istanbul and performed her PhD studies in the Cell Biology and Proteomics group under the supervision of Dr. Nurhan Özlü. Her PhD research was on “Analysis of Cell Surface Reorganization During Cell Division”.

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Appendix A

Site Directed Mutagenesis Primers		
1	PCDH7_C904A_F	GCCATTTTTATTTTTGGACCTGGCGTACCTTGCCATCACTAC AATT
2	PCDH7_C904A_R	AATTGTAGTGATGGCAAGGTACGCCAGGTCCAAAAATAAAA ATGGC
3	PCDH7_C99A_F	GTCGAAGATCATCTGAGCCTGGGGCAGCTTCTCG
4	PCDH7_C99A_R	CGAGAAGCTGCCCCAGGCTCAGATGATCTTCGAC
5	PCDH7_C108A_F	CTCGAAGTCCAGGAAGGCCTCGTTCTCGTCGAAG
6	PCDH7_C108A_R	CTTCGACGAGAACGAGGCCTTCCTGGACTTCGAG
7	PCDH7_C141718A_F	GGCAGGAGGAGGGCGGCGCCCAAGGCCAGCCGCGCGC
8	PCDH7_C141718A_R	GCGCGCGGCTGGGCCTTGGGCGCCGCCCTCCTCCTGCC
9	PCDH7_C14A_F	CAGCAGCCCAAGGCCAGCCGCGCGC
10	PCDH7_C14A_R	GCGCGCGGCTGGGCCTTGGGCTGCTG
11	PCDH7_C17A_F	CAGGAGGAGGCAGGCGCCCAAGCACCAG
12	PCDH7_C17A_R	CTGGTGCTTGGGCGCCTGCCTCCTCCTG
13	PCDH7_C18A_F	GTGCTTGGGCTGCGCCCTCCTCCTGCCG
14	PCDH7_C18A_R	CGGCAGGAGGAGGGCGCAGCCCAAGCAC
Cloning Primers		
15	NheI_PCDH7_F	GTCAGCTAGCACCATGCTGAGGATGCGGACC
26	EcoRI_PCDH7_R	GCTAGAATTCGCCCTCCCTGGGATATTTAAATATATTTG
27	AgeI_PCDH1_F	GTCAACCGGTACCATGGAC AGC GGG GCG GGC
28	EcoRI_PCDH1_R	GCTAGAATTCCTCCACACCTCGGTCCAGATG
29	NheI_GFP_F	GTCAGCTAGCACCATGGTGAGCAAGGGCGAGGAG
30	EcoRI_GFP_R	GCTAGAATTCCTTGTACAGCTCGTCCATGCC

Appendix B

CRISPR Guide RNA and Related Olinucleotide Sequences		
	sgRNA Sequence (5' to 3')	Oligonucleotide Sequences
PCDH7_sg1*	CGACGTCCGCATCGGCAACG	5'-CACCGCGACGTCCGCATCGGCAACG-3'
		5'-AAACCGTTGCCGATGCGGACGTGCG-3'
PCDH7_sg5*	CATCGTGACCGGATCGGGTG	5'-CACCGCATCGTGACCGGATCGGGTG-3'
		5'-AAACCACCCGATCCGGTCACGATG-3'
PCDH7_sg6	CGGGCTTCTCTTTGGCGCGC	5'-CACCGCGGGCTTCTCTTTGGCGCGC-3'
		5'-AAACGCGCGCCAAAGAGAAGCCCGC-3'
PCDH7_sg7	AATCCCCCGGCACGTGCTT'	5'-CACCGAATCCCCCGGCACGTGCTT-3'
		5'-AAACAAGCGACGTGCCGGGGGATTG-3'
PCDH7_sg8	CCGTGCCCCGAAGCGACGTGC'	5'-CACCGCCGTGCCCGAAGCGACGTGC-3'
		5'-AAACGCACGTGCTTCGGGCACGGC-3'
PCDH7_sg9	GTGCCCCGAAGCGACGTGCCG'	5'-CACCGTGCCCCGAAGCGACGTGCCG-3'
		5'-AAACCGGCACGTGCTTCGGGCAC-3'
PCDH7_sg10	TAGTCCCCGCTCTGTGCGAA	5'-CACCGTAGTCCCCGCTCTGTGCGAA-3'
		5'-AAACTTCCGACAGAGCGGGGACTAC-3'
PCDH1_sg1	GGCGTCTTCTTCGACTGGAC	5'-CACCGGCGTCTTCTTCGACTGGAC-3'
		5'-AAACGTCCAGTCGAAGAAGACGCC-3'
PCDH1_sg2	GACCTTCTCGTAGTCTAGCG	5'-CACCGACCTTCTCGTAGTCTAGCG-3'
		5'-AAACCGCTAGACTACGAGAAGGTC-3'
PCDH1_sg3	CACCCCGCTAGACTACGAGA	5'-CACCGCACCCCGCTAGACTACGAGA-3'
		5'-AAACTCTCGTAGTCTAGCGGGGTGC-3'
PCDH1_sg4	CCACCTCGCTCAGCTTACGT	5'-CACCGCCACCTCGCTCAGCTTACGT-3'
		5'-AAACACGTAAGCTGAGCGAGGTGGC-3'
PCDH1_sg5	CGCGGCAAGCCCCCACGCTA	5'-CACCGCGCGGCAAGCCCCCACGCTA-3'
		5'-AAACTAGCGTGGGGGCTTGCCGCGC-3'
*Designed and cloned by Mohammed Haroon Qureshi		

Appendix C

Generation of PCDH1 Knock-out Cell Lines

I used CRISPR-Cas9 genome editing system and knock out the endogenous PCDH1 from HeLa cells. PCDH1 sequence was targeted using different sgRNAs (Appendix B) depletion of PCDH1 protein levels were tested using Western blotting. PCDH1sg1 and PCDH1sg4 showed complete knockout and will be used for the further characterization experiments (Figure 1).

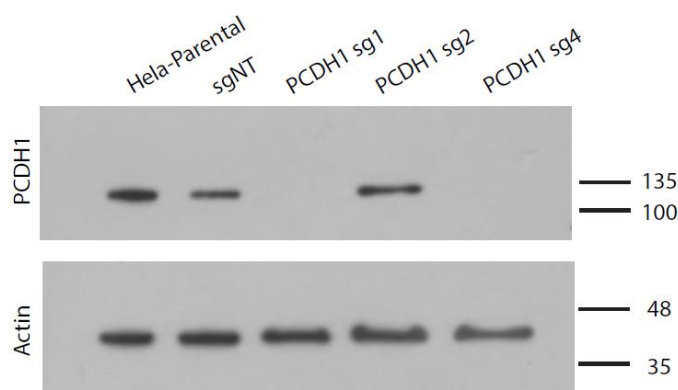


Figure 1: Western blot for the PCDH1 CRISPR cells. PCDH1 levels in the different sgRNA treated cells for PCDH1 CRISPR are compared with the non-treated HeLa cells, and non-targeting sgRNA (sgNT) treated cells. Actin was used as the loading control.

Next I tested if these sgRNAs can be used to generate the double knock out of the PCDH7 and PCDH1. I used PCDH7 knockout cells as background and introduced the PCDH1 sgRNAs by viral transduction. Depletion in the PCDH7 and PCDH1 protein levels were tested using Western blotting. One of the cell lines showed a promising decrease in the PCDH1 levels indicating the feasibility of the method (Figure 2). These results can be further improved with multiple tries and the single colony selection.

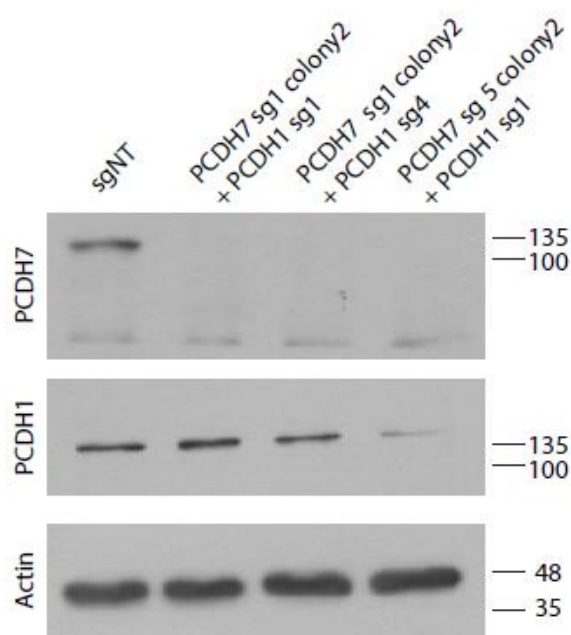


Figure 2: Western blot for the PCDH7-PCDH1 double CRISPR cells. PCDH7 and PCDH1 levels in the different sgRNA treated cells for PCDH1 in the PCDH7 knock-out background are compared with the non-targeting sgRNA (sgNT) treated cells. Actin was used as the loading control.