Molecular Analysis of Protocadherins in Mammalian Cells

by

Gürkan Mollaoğlu

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

Gürkan Mollaoğlu

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examining committee have been made.

Committee Members:

_____

Nurhan Özlü, Ph. D. (Advisor)

_____

Gülayşe Ince Dunn, Ph. D.

_____

Seda Kızılel, Ph. D.

Date: _____

to my mother,

for enlightening my path

and warming my heart…

ABSTRACT

Cadherins are a big family of transmembrane, cell-cell adhesion proteins and protocadherins constitute the largest subgroup of cadherins. Although cadherins are extensively studied, less information is available about protocadherins. Protocadherins have been shown to function in cell adhesion and they are thought to be the molecular markers of neuronal identity. Also, recent studies revealed the involvement of protocadherins in cancer and neurodegenerative diseases. We aimed to characterize a protocadherin, which showed a cell cycle dependent subcellular localization. We generated various mutant versions of the protocadherin and analyzed subcellular localizations of wild type and mutated proteins throughout the cell cycle using microscopy. We also investigated the role of the cytoplasmic domain in regulation of cell cycle dependent localization of the protein. In order to identify the interaction partners of the cytoplasmic domain, we used two orthogonal approaches: 1. GST-fusion pull-down assay followed by mass spectrometry; 2. BioID method (proximity-dependent biotin identification combined with biotin-streptavidin affinity chromatography and mass spectrometry). These two experimental strategies helped us to discover interaction partner candidates of the protocadherin. Furthermore, we performed bead aggregation assay. We demonstrated that the protocadherin that we studied does not have homophilic adhesion activity. This research project produced novel knowledge about the protocadherins and showed the promise of protocadherins to be used in cancer diagnosis and treatment.

ÖZET

Cadherin proteinleri hücre-hücre adezyonunda görev alan büyük bir transmembran protein ailesidir ve protocadherinler bu ailenin en büyük alt grubudur. Cadherinler iyi çalışılmış olsa da protocadherinler hakkındaki bilgi kısıtlıdır. Protocadherinlerin hücre adezyonunda rol aldığı gösterilmiştir ve nöron tanımlanmasında moleküler işaretçi oldukları düşünülmektedir. Ayrıca, yakın zamanda yapılan çalışmalar protocadherinlerin kanser ve nörolojik hastalıklarla ilişkisini açığa çıkarmıştır. Bu çalışmada biz hücre döngüsüne dayalı hücresel yerleşim gösteren bir protocadherini karakterize etmeyi amaçladık. Araştırdığımız protocadherinin çeşitli mutant versiyonlarını ürettik ve fizyolojik ve mutant proteinlerin hücresel lokasyonlarını hücre döngüsü boyunca mikroskop ile takip ettik. Ayrıca, sitoplazmik domainin, protenin hücre döngüsüne bağlı yerleşiminin kontrolündeki rolünü araştırdık. Sitoplazmik domainin etkilşim partnerlerini tanımlamak için iki doğrudan yöntem kullandık: 1. GST-füzyon afinite kromatografisini takiben kütle spektroskopisi; 2. BioID metodu (yakınlık-bağımlı biyotin tanımlamanın biyotin-streptavidin afinite kromatografisi ve kütle spektroskopisiyle kombinasyonu). Bu iki deneysel strateji protocadherinin aday etkileşim partnerlerini keşfetmemizi sağladı. Ek olarak, boncuk öbekleşme deneyi gerçekleştirdik. Çalıştığımız spesifik protocadherinin homofilik adezyon aktivitesi olmadığını gösterdik. Bu araştırma projesi protocadherinler hakkında yeni bilgiler ortaya koydu ve protocadherinlerin kanser tanı ve tedavisinde kullanılabilme potansiyelini gösterdi.

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NOMENCLATURE

APC/C Anaphase-promoting complex

BCA Bicinchoninic acid

BioID Proximity-dependent biotin identification

BirA* Promiscuous *Escherichia coli* biotin protein ligase enzyme

BSA Bovine serum albumin

CAK CDK-activating kinase

CAKs Cell adhesion kinases

CAMs Cell adhesion molecules

CDK Cyclin dependent kinase

cDNA Complementary DNA

CIP Calf intestinal phosphatase

CKI CDK-inhibitory protein

CM Conserved cytoplasmic motif

CYT Cytoplasmic domain

DAPI 4',6-diamidino-2-phenylindole

DMEM Dulbecco's Modified Eagle Medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

dNTP Deoxyribonucleotide

DpnI Restriction enzyme which digests methylated DNA

DTT Dithiothreitol

EC Extracellular domain

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EGTA Ethylene glycol tetraacetic acid

ER Endoplasmic reticulum

FBS Fetal bovine serum

G_0 Quiscent state

G_1 Gap one

G_2 Gap two

GFP Green Fluorescent protein

GST Glutathione S-transferase

HEK 293T Human embriyonic kidney cell line

HeLa Human cervical cancer cell line

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IPTG Isopropyl β-D-1-thiogalactopyranoside

kDa Kilodalton

LB Lysogeny broth medium

LC-MS/MS Liquid chromatography coupled with tandem mass spectrometry

M Mitosis

MCS Multiple cloning site

mRNA Messanger ribonucleic acid

MS^- Mutant Pcdh7 which terminates at the begining of MS

MS Putative modification site

MS^+ Mutant Pcdh7 which terminates at the end of MS

MWCO Molecular weight cut off

OD Optic density

Opti-MEM Reduced Serum Media

PAPC Paraxial protocadherin

PBS Phosphate buffered saline

Pcdh7 Protocadherin 7

Pcdh7 *ΔCYT* Cytoplasmic domain deleted mutant of Pcdh7

PCR Polymerase chain reaction

PS Penicillin and streptomycin

RT-PCR Reverse transcriptase pcr

S Synthesis

SDS-PAGE Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SILAC Stable isotope labeling by amino acids in cell culture

SNP Single nucleotide polymorphism

SP Signal sequence

TM Transmembrane domain

U2OS Human osteosarcoma cell line

YTA pGex growth medium

Chapter 1

INTRODUCTION

As famous epigram "*Omnis cellula e cellula*" by François-Vincent Raspail states, a cell can only be produced from an already existing cell [1]. This crucial fact of life bases on a simple event: a cell grows and then divides into two new cells. Although cell division is the mean of reproduction for unicellular organisms, it becomes the way of development and renewal for multicellular organisms such as humans. For example, hematopoietic stem cells undergo mitosis to produce blood cells such as lymphocytes and macrophages or fibroblasts proliferate with mitosis in order to heal a wound. Therefore, it is vital for multicellular organisms to have some of their cells divide with mitosis. The very important point here is that mitosis must only happen when and where it is necessary. Otherwise, mitotic dysregulation results in several diseases, most notably cancer. The cell has evolved complex control mechanisms to ensure a correct cell cycle.

Cells become social units in multicellular organisms as they need to operate in synchrony. Cell surface is a cellular component where a cell makes connections with other surrounding cells and the extracellular matrix. Cell surface is also important to receive signals from other cells and the environment. For these operations, the cell has a set of different proteins on the cell membrane. A large group or these cell surface proteins is cell adhesion molecules (CAMs) which are responsible for cell-cell and cell-extracellular matrix adhesions. Cadherins are a superfamily of CAMs and these glycoproteins can form calcium-dependent homophilic adhesion between two neighboring cells. Protocadherins are

the largest subgroup of cadherin superfamily and they are predominantly expressed in the nervous system. The extraordinarily diverse protocadherins may be the basis of neuronal identity. They have been associated with several neurological diseases as well as cancer.

The cell is a very dynamic entity and its components are subject to change in terms of structure, function, localization and quantity. Intracellular changes through the different phases of the cell cycle have been extensively studied while our knowledge about alteration on the cell membrane is infant. With the development of high-throughput methods in cell biology, it has been possible to track complex global changes in the cell. One of these methods is mass spectrometry based proteomics, which has been useful to reveal such dynamic changes.

In a very recent study, Özlü *et al.* have compared protein composition of cell membrane between mitosis and interphase (unpublished data) [2]. They showed that the amount of some proteins on the cell surface changes dramatically between mitosis and interphase. Protocadherin 7 (Pcdh7) is a member of protocadherins and it has been shown in this study that quantity of Pcdh7 on the plasma membrane is increased a few folds during mitosis compared to interphase. This intriguing finding drove us to further investigate this protein, which is under studied. We aimed to test cell cycle dependent localization of Pcdh7 using orthogonal methods; determine its molecular mechanisms required for its cell cycle dependent surface localization; discover its intracellular binding partners and its physiological function.

In the Chapter 2, a more detailed introduction and review of the current literature related to the thesis topic can be found. The materials and methods used for this project are described in the Chapter 3. Results of the experiments are presented in the Chapter 4. The Chapter 5 covers a discussion of our findings.

Chapter 2

LITERATURE REVIEW

2.1 The Cell Cycle

A cell must duplicate itself to produce new cells. This central phenomenon of biology is valid for both unicellular organisms, such as bacteria and yeasts, and complex multicellular organisms, such as plants and mammals. This life cycle of a cell, which is basically duplicating its components and then dividing into two new cells, is termed as the cell cycle. In human body, majority of cells are "out-of-cycle" which means that either they are terminally differentiated to specialized cell types such as neurons or they are reversibly in quiescent state (G_0) such as hepatocytes. The remaining minority of cells proliferates usually in stem-transit amplifying compartments of tissues with self-renewing capacity to produce new cells to replace old cells.

The cell cycle is composed of two distinct parts: interphase and mitosis. Interphase part is divided into three sequential phases: gap one (G_1), synthesis (S) and gap two (G_2) phases (Figure 2.1). The cell senses its environment and inner state to decide between going on the cell cycle or stay in the quiescent state during G_1. Alternatively, the cell can go into differentiated or senescent states, both of which are irreversible out-of-cycle states. The cell duplicates its genome as well as its organelles during the next phase, S phase. In the G_2 phase, the cell prepares itself for the mitosis. Interphase is followed by M phase which is

composed of mitosis followed by cytokinesis. The cell is divided into two genetically equal daughter cells during M phase [3].

The cell cycle is very sophisticated and it is a complex of vital cellular events. Therefore, it is under control of both intrinsic and external signals that the cell senses to govern the cycle. Mitogens, growth factors and survival factors are sensed by receptors and conveyed by signaling cascades into the cell. The cell also controls inner changes and states such as successful DNA replication and chromosomal segregation during the cell cycle. Overall sensation reacts via cyclin-dependent kinase (CDK) family of serine/threonine kinases and their regulators, the cyclin proteins. Different CDK-cyclin couples work at each cell cycle check points. Although amount of each CDK in a cell is almost constant, cyclin levels oscillate through the cell cycle. For example, cyclin B-CDK1 complex promotes the cycle through G_2 into mitosis. CDK1 amount is similar through every phase of the cycle whereas cyclin B is produced in S phase, acts in G_2 and degraded in M phase. CDK activity is also regulated by Cdk-activating kinase (CAK) and Cdk-inhibitory proteins (CKIs). There are three major cell cycle checkpoints: At the end of G_1, the cell goes through start checkpoint (G_1/S checkpoint) in the presence of necessary signals and enters the cell cycle and proceeds to S phase. At the end of G_2, the cell controls if all DNA is replicated successfully and the environment is favorable to continue the cycle at the G_2/M checkpoint. During metaphase-to-anaphase transition, the cell checks if all chromosomes are attached to the spindle for proper chromosome segregation. This checkpoint is regulated by anaphase-promoting complex (APC/C) instead of a CDK-cyclin couple [4].

The cell needs this complicated control mechanism on the cell cycle because improper, scarce or excessive cell duplication results in many diseases. Cancer is excessive cell proliferation which is caused by acquisition of mutations. Though a cancer cell has distinct mutations some of which are results of the genomic chaos of the disease itself, driving mutations are ones that affect components of the cell cycle. Genes are categorized into two

groups in this context: tumor-suppressor genes and oncogenes. If protein product of a gene functions as a brake in the cell cycle, it is called as a tumor-suppressor gene and loss-of-function mutations of this gene can cause cancer. On the other hand, protein products of oncogenes function as accelerators in the cell cycle and gain-of-function mutations of these genes can result in cancer [5].

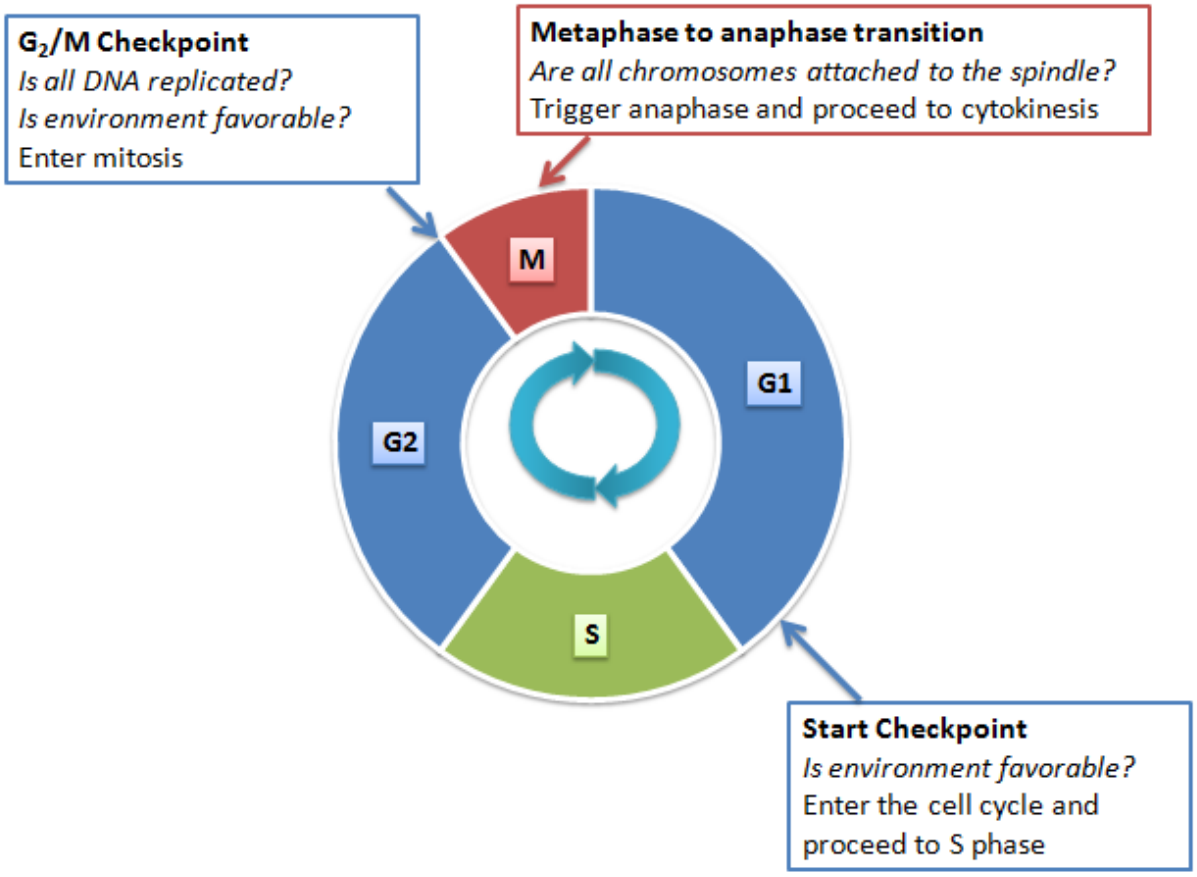

Figure 2.1: The cell cycle. The cell cycle is not a non-stop process. The cell combines information from signaling pathways which convey information from both inside and outside of the cell and then decide to proceed to next phase or stop the cycle temporarily or permanently. The first major cell cycle checkpoint is "Start Checkpoint" where the cell decides to continue the cycle; enter to quiescent state; or terminally differentiate. After the S and G2 phases, the cell controls if genome replication is successfully achieved and the environment is still favorable at "G2/M Checkpoint" and it halts the cycle if necessary or proceeds to mitosis. During "metaphase to anaphase transition", the cell controls proper chromosome – spindle organization to ensure correct separation of sister chromatids. (Adapted from Molecular Biology of the Cell, Fifth Edition 2007 [3])

2.2 The Cytoskeleton, Cell Adhesion, the Extracellular Matrix and Cell Migration

A cell is a biochemical construct which has its own physical dynamics. In multicellular organisms, cells become constituents of tissues and organs. Therefore, they have to organize themselves and interact with each other. This physical formation is enabled by special proteins which are intracellular, transmembrane and extracellular units that work in cohort.

The cytoskeleton is composed of three families of filament proteins: actin filaments (microfilaments), microtubules and intermediate filaments. These self-assembly and dynamic filaments facilitate spatiotemporal organization of cells. They function to shape a cell, give it its strength and ability to move. They are also necessary for intracellular organization of a cell such as organelle movement and chromosome segregation [3].

The cytoskeleton filaments cooperate with motor proteins such as kinesin, dynein and myosin for some of the dynamic actions. They also interact with several adaptor proteins and transmembrane proteins which indirectly link them to neighboring cells and extracellular matrix. These interactions build a social multicellular organization in which cells are organized, move and communicate to each other. These physical attachments can be categorized based on function: Anchoring junctions (cell-cell adhesion and cell-extracellular matrix adhesion) transmit stress with cooperation of the cytoskeleton filaments. They also trigger several signaling pathways. Occluding junctions form barriers in epithelial tissue by attaching cells as sheets. Channel-forming junctions (gap junctions and plasmodesmata) form channels between cytoplasms of adjacent cells. Signal-relaying junctions transmit signals from cell to cell such as those in neuronal synapses [3].

Transmembrane adhesion proteins have a pivotal role in all of these anchoring junctions. They interact with each other, the cytoskeleton filaments and extracellular matrix components. Basically, cadherin proteins mediate cell-cell junctions (adherens junctions and desmosome); integrin proteins mediate cell-matrix junctions (actin-linked-cell-matrix

adhesion and hemidesmosome); claudin and occludin forms tight junctions; connexins form gap junctions (Figure 2.2) [6].

Cells produce and secrete several biomolecules to form the extracellular matrix (ECM). ECM does not only provide a medium for cells but also influences cell shape, function, survival, proliferation and migration. ECM is composed of mesh fibrous proteins (collagen, elastin, fibronectin, laminin) and proteoglycans.

Cell migration is a critical process for several biological phenomena including embryogenesis and development of the nervous system, inflammatory response of the immune cells, wound healing, metastasis of tumor cells as well as tissue engineering applications. Quantitative and spatiotemporal regulation of molecular components such as ECM ligands, CAMs, and cytoskeleton-linking proteins as well as related cellular signaling is the basis of cell migration [7]. For example, in directed cell migration, a protrusion in the cell front which is termed as lamellipodium or pseudopodium forms and adheres to substratum. Simultaneously, the cell rear retracts with deadhesion from the substratum and the cell body translocation occurs through the migration direction [8]. During this movement, cytoskeleton elements, adhesion molecules and their interaction with extracellular matrix components are dynamically regulated.

Complex interactions among the cytoskeleton proteins, junction proteins and extracellular matrix components are dynamic and vital for survival and function of a cell. Thus, dysfunctions in this system contribute to several diseases including neurodegenerative diseases and cancer.

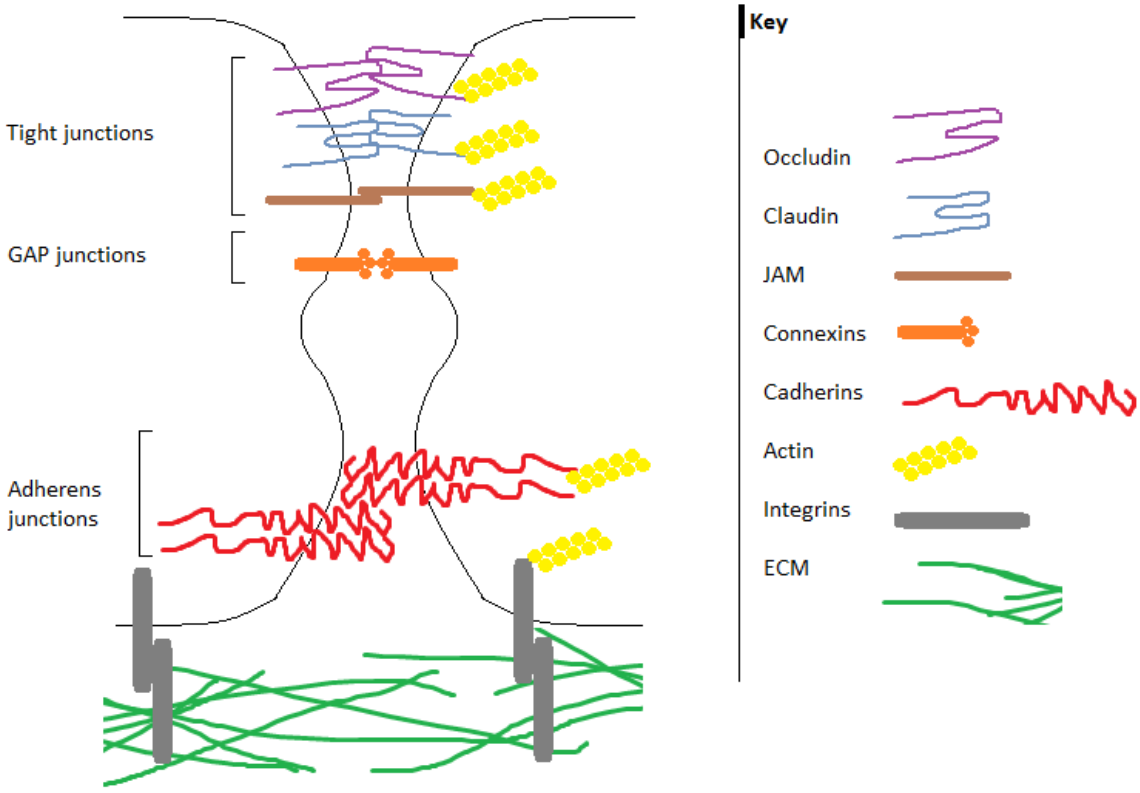

Figure 2.2: Cell junctions. Cells contact and adhere to each other and extracellular matrix with special junction proteins. These transmembrane proteins interact with cytoskeleton of the cells via several adaptor proteins which are not shown here for simplicity. (Adapted from Mehta, D. & Malik, A. B.2006 [6])

2.3 Dynamic Cell Morphology through the Cell Cycle

The cell morphology undergoes dramatic changes throughout the cell cycle [9]. Inside the cell, metabolic changes warrant sufficient biomass for cell growth and division [10]. Amount and modifications of thousands of proteins are affected by the cell cycle via transcriptional and translational regulations as well as phosphorylation-dephosphorylation of specific sites on the proteins [11] [12] [13].

Dynamic changes related to the cell cycle are not limited to cytoplasm of the cell. Cell membrane biochemistry is affected by the cell cycle [14] while it grows during the cell cycle [15] with endosomal recycling [16]. While key activities of Rap1 (a small GTPase) and moesin (an actin-binding protein) are essential for cell retraction [17] [18], the actomysoin cortex and hydrostatic pressure also drive mitotic cell rounding [19]. Cell-cell and cell-matrix junctions also alter during mitosis to enable cell rounding [20]. In addition, cell-ECM interaction which is necessary for cell survival and proliferation controls cell cycle by anchorage signaling [21]. Not surprisingly, cancer cells acquire ability to survive and proliferate in the absence of proper cell-cell and cell-ECM interactions [22].

2.4 The Protocadherin Subfamily of Cadherins

Cadherins are a big family of cell adhesion molecules with more than 100 different members. These calcium-dependent cell adhesion molecules also play important roles in cell recognition, cell signaling, during embryogenesis and the formation of neural circuits. Cadherins are transmembrane proteins with several copies of cadherin domains at the extracellular domain and distinct cytoplasmic domains which interact with actin cytoskeleton via adaptor protein, catenin. In the presence of calcium ions, cadherin proteins of neighboring cells usually form homophilic adhesion; and heterophilic interactions in some cases. Chappuis-Flament *et al.* postulated that an initial interaction between EC1

domains of *trans* cadherins enables interactions between EC2-5 domains which facilitate full adhesive transaction between two cadherin proteins [23]. There are many different types of cadherin proteins (e.g. E-cadherin, N-cadherin etc.) and homophilic adhesion of one type between cells enables selective cell-cell adhesion. Although, interaction between two cadherins is weak, the strength comes from interaction of many cadherin couples in proximity.

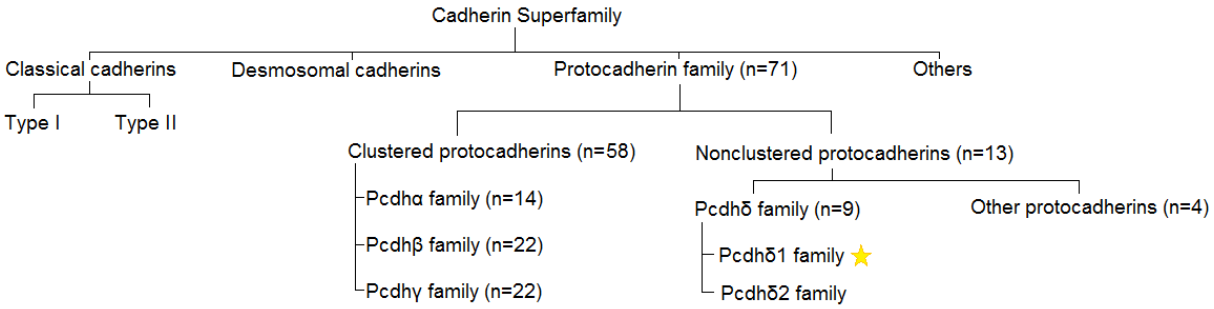

Figure 2.3: Classification of cadherin superfamily members. Protocadherin family is the largest subgroup of cadherin superfamily. Protocadherin family can be divided into two groups based on their genomic structures: the clustered, and nonclustered protocadherins. PCDH7 is a member of Pcdhδ1 family (marked with a star). (Adapted from Morishita and Yagi 2007 [24])

Cadherins are divided into four main subfamilies: classical cadherins, desmosomal cadherins, protocadherins and unconventional cadherins (Figure 2.3). In 1993, Sano *et al.* isolated and characterized cDNAs which are highly homologous to the extracellular domain of cadherins and they named the encoded proteins as protocadherins. They were also first to show that protocadherins localize to cell-cell contacts and have adhesive

activity [25]. Protocadherins are the largest subgroup of cadherins superfamily and they are predominantly expressed in the nervous system. More than 70 different protocadherin genes are divided into two groups based on their genomic organization: clustered protocadherins (Pcdhα, Pcdhβ and Pcdhγ) and nonclustered protocadherins (Pcdhδ and solitary protocadherins). Protocadherins differ from cadherins structurally as they lack homophilic adhesiveness features (conserved tryptophan and hydrophobic pocket in EC1) and their EC repeats are different than those of classical cadherins in term of sequence similarity (Figure 2.4). Also, they have characteristic disulfide-bonded loop in EC1 and they lack catenin binding site at cytoplasmic domain. Protocadherins have six or seven EC repeats, their cytoplasmic domains are divergent and they are single-pass transmembrane proteins [24].

Experiments with Pcdhα and Pcdhγ showed that protocadherins do not have trans-homophilic adhesion activity (compared to classical cadherins) and this is mostly due to difference of EC1 domain between protocadherins and classical cadherins [26] [27]. On the other hand, they can form *cis*-homodimer or *cis*-heterodimer [28] [29] [30]. So far, protocadherins are shown to have no homophilic adhesion activity or may mediate weak adhesion [26] [27] [31] [32] [33] [34] [35] [36]. However, an *in vitro* cell aggregation assay in HEK 293T cell line showed that there is heterophilic cell adhesion between Pcdha4 and the β1 integrin [27]. Emond *et al*. discovered that PCDH19 forms a complex with N-cadherin to exhibit adhesion in bead aggregation assay although PCDH19 alone has a weak homophilic adhesion activity. They proposed that activity of cadherins depends on complement of protocadherins [37]. Paraxial Protocadherin (PAPC) interacts with FLRT3 upon induction of TGFβ in Xenopus embryos. PAPC inhibits C-Cadherin mediated cell adhesion and cell sorting occurs [38] [39].

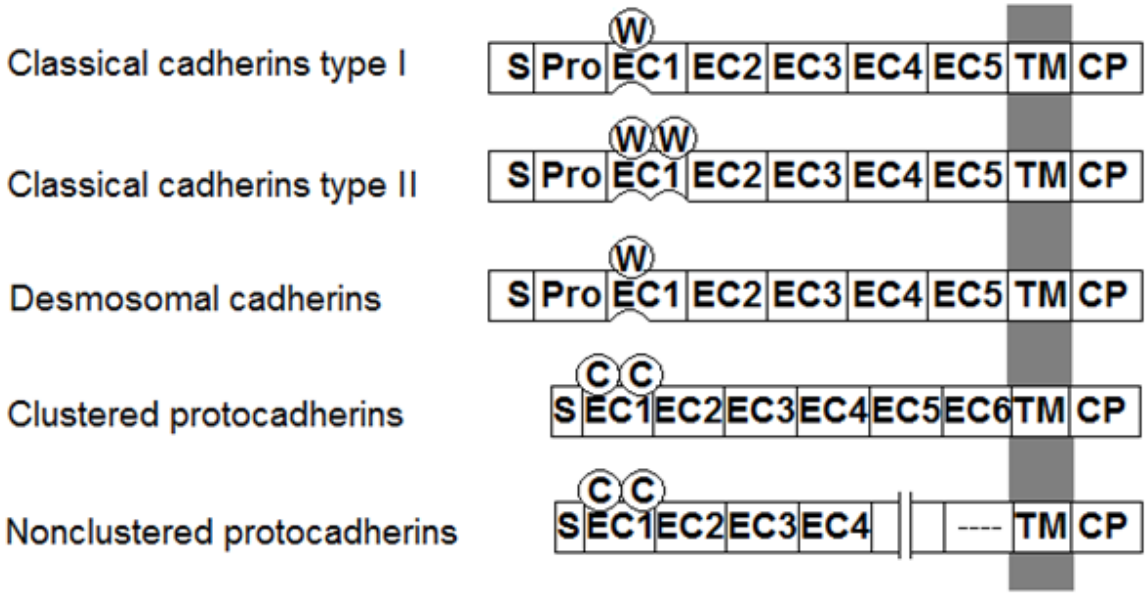

Figure 2.4: Structural comparison of cadherin subgroups. Classical cadherins have one (in type I) or two (in type II) conserved tryptophan (W2) in EC1 domain and one (in type I) or two (in type II) hydrophobic pockets to accommodate tryptophan of other cadherin to form homophilic adhesiveness. The cytoplasmic region of classical cadherins have catenin binding site for linking to actin cytoskeleton. Extracellular region of desmosomal cadherins are similar to type I classical cadherins but they do not have a catenin binding site in their cytoplasmic region. Protocadherins are different from other subgroups of the cadherin family because they do not have conserved tryptophan, hydrophobic pockets or catenin binding site but they have a characteristic disulfide-bonded loop in EC1 domain. (Adapted from Morishita and Yagi 2007 [24])

Pcdhα and Pcdhγ proteins are specifically cleaved by ADAM10 and presenilin and a cytoplasmic fragment of proteolysis products of these protocadherins can localize to nucleus and activate all Pcdhγ promoters [29] [34] [40] [41].

Clustered protocadherins (α, β and γ) are organized as three sequential gene clusters and they can produce more than 50 transcripts with monoallelic and combinatorial gene regulation [42]. On the other hand, nonclustered protocadherin genes produce alternative splicing variants with only small variations at cytoplasmic domain [43].

Pcdh gene cluster is located at 5q31 in humans and it contains many SNPs which support the notion that Pcdh genes determine characteristics of the human brain and may be related to some psychiatric diseases [44] [45] [46]. With extraordinary diversity, protocadherins may be the basis of neuronal identity [24]. Protocadherin-α genes have combinatorial gene expression of variable exons, similar to the immunoglobulin (Ig) genes. This mechanism enables production of diverse Pcdhα receptors in the central nervous system which is a potential explanation of neuronal identity [47]. Kim *et al.* demonstrated that non-clustered protocadherins (including PCDH7) have differential expression patterns in adult rat hippocampus and they are important for formation and maintenance of hippocampal circuitry [48]. Knock-out and knock-down experiments of Pcdhα and Pcdhγ gene clusters revealed that these genes regulate dendrite complexity. Pcdhα proteins inactivate membrane-associated CAKs (cell adhesion kinases) and activate small GTPase proteins Rac1 and RhoA [49]. Dietmar Schreiner and Joshua Weiner proposed a model for the mechanism of Pcdh-γ family members based on their experiments. According to this model, γ-Pcdh proteins form promiscuous *cis* tetramers while interaction of *trans* tetramers are very specific. This model, along with known role of γ-Pcdhs in synaptogenesis, implies a role for γ-Pcdhs in control of neuronal specificity [50].

Recent reports uncovered relation between protocadherins and cancer. For example, Ying *et al.* revealed that expression of PCDH10 is silenced in nasopharyngeal carcinoma (NPC)

via promoter methylation. When PCDH10 is ectopically expressed in this tumor cells, tumor growth, migration and invasion are strongly suppressed. This study classifies PCDH10 as a tumor suppresser gene [51]. In another study, Wang *et al.* showed that expression of PCDH9 is significantly downregulated in the primary cerebral glial tumor and they suggested PCDH9 as a tumor suppressor gene and possible biomarker for cerebral glial tumors [52].

2.5 Protocadherin 7

Protocadherin 7 (also known as PCDH7 and BH-protocadherin) is a member of Pcdhδ1 subgroup of nonclustered protocadherins. Although our knowledge of PCDH7 is in its infancy, available data indicates the importance of this protein.

Kenichi Yoshida *et al.* were the first to identify PCDH7. They showed that PCDH7 has the highest homology with PCDH1 (46-49%) and it is expressed in various tissues in human with the highest expression profiles in heart and brain. They found that there are 3 splice-variant isoforms of PCDH7 (namely isoforms a, b, and c) with polypeptide lengths of 1069, 1072, and 1200 amino acids, respectively. N-terminal extracellular domain of PCDH7 has 28 amino acid long, hydrophobic signal sequence which directs the protein to the cell membrane and seven cadherin repeats. The EC1 of PCDH7 lacks adhesion recognition sequences necessary for homophilic cell-cell adhesion and the EC2 domain is longer than the usual cadherin repeats (165 amino acids and 110 amino acids, respectively) and its middle region is unique in terms of homology to other protein sequences. The isoform c lacks the region from the middle of EC2 to the N-terminus of EC3. Extracellular domain of PCDH7 is followed by a 24 amino acid long, hydrophobic transmembrane domain. Cytoplasmic domains of the three isoforms differ in terms of length with 168, 171, and 346 amino acids, respectively. Importantly, the cytoplasmic domain of PCDH7 lacks catenin-

binding site which links classical cadherins to actin cytoskeleton via catenin proteins. Yoshida *et al.* also found that a 4.5 kb mRNA of PCDH7 is produced in various cancer cell lines, including HeLa S3, human lung carcinoma cell line A549, and human gastric cancer cell lines MKN28 and KATO-III while they detected only a 9.0 kb mRNA message of PCDH7 in normal tissues. They showed that PCDH7 is conserved among various vertebrate species. Also, they mapped PCDH7 to chromosome 4p15 which is a loss of heterozygosity region detected in some head and neck squamous cell carcinomas [53].

In a later study, Yoshida *et al.* used yeast two-hybrid system to identify interaction partners of the cytoplasmic domain of PCDH7 isoform c. They identified protein phosphatase type 1 isoform α (PP1α) as a candidate and further confirmed their result with pull-down and co-immunoprecipitation experiments. PP1α is known to associate with focal adhesion and have a role in long-term synaptic plasticity. They also employed yeast two-hybrid system to examine PCDH7a cytoplasmic domain but they could not find any candidate interaction partner [54].

PCDH7 has three conserved cytoplasmic motifs (CM1, CM2, and CM3). All three isoforms have CM1 which interacts with TAF1 (template-activating factor 1) which may regulate activity of PCDH7. Only isoform c has CM3 which interacts with PP1α (protein phosphatase type 1 isoform α). PCDH7 does not have extracellular RGD motif which is essential for integrin-dependent cell adhesion activity. Some other non-clustered protocadherins have RGD motif and they interact with integrins [55].

In an early study, PCDH7 isoforms a and b but not c are shown to increase cell adhesion in the presence of Ca^{+2} in cell aggregation assay with PCDH7 transfected mouse fibroblast L cells. In the same study, intracellular localization of PCDH7 is shown to be in endoplasmic reticulum [56].

Dysregulation of cadherin family members are associated with many cancer types either as tumor-suppressor or proto-oncogenic proteins [57] and protocadherins are not an exception.

Methyl-CpG-binding protein 2 (MeCP2), which is an epigenetic regulator protein, downregulates PCDH7 expression in human neuroblastoma cells and brain tissue and mutations of MeCP2 cause Rett syndrome, which is a neurodevelopmental and autistic disease [58]. One of the top five SNPs (risk alleles) associated with survival in Early-stage Non-Small-Cell Lung Cancer is located in PCDH7 [59]. In a recent study, gene expression analysis of brain metastatic breast cancer cells identified PCDH7 as an enhancer of metastatic activity [60].

NF-protocadherin (NFPC), the *Xenopus* homolog of human PCDH7 is shown to regulate ectodermal cell sorting [61], neural tube closure [62], and retinal axon initiation and elongation [63]. These findings affirm the importance of PCDH7 homologs among different species.

Chapter 3

MATERIALS AND METHODS

3.1 Cloning of human PCDH7 Isoform b to pEGFP Vectors

Human PCDH7 (Protocadherin-7; GenBank: AB006756.1) was cloned to pEGFP-N1 (Clontech #6085-1) and pEGFP-C1 (Clontech Catalog #6084-1) vectors. cDNA of PCDH7 was a gift from Prof. Sumio Sugano, Laboratory of Functional Genomics, Department of Medical Genome Sciences, the University of Tokyo. Insert DNA was amplified from gifted cDNA with PCR (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#1 or P#3 10 µM 1.0 µl; Reverse primer P#2 or P#4 10 µM 1.0 µl; dH_2O 11.6 µl; template DNA 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.4 µl); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 55 °C – 30 sec; Extension 72 °C – 2 min; Final extension 72 °C – 10 min; 35 cycles). Eppendorf Mastercycler PCR machine was used. PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. Then, PCR products were purified with Sigma GenElute PCR Clean-Up Kit #NA1020; according to the manufacturer's instructions. PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl; DNA 5.0 µl; HindIII NEB #R3104S 1.0 µl; BamHI NEB #R3136S 1.0 µl; dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour). Digestions products were also

purified with PCR Clean-Up Kit. pEGFP vector plasmids were also digested with the same RE couple with the same reaction conditions above. Digested vectors were run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vectors were dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 13.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested inserts and plasmids were used in the ligation reaction with vector: insert ratio of 1:3. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer and ligation calculation was done with "Ligation Calculator" online program (see Appendix for the link). Ligation reaction was performed (10X T4 Buffer 1.0 µl; T4 Ligase NEB #M0202S 1.0 µl; Vector 1.0 µl; Insert 3 µl; dH_2O 4 µl) – negative control reaction had no insert – ; reaction conditions (4 °C overnight). Ligation products were used to transform chemical competent *E. coli* DH5α bacteria (gift from Dr. Cory Dunn, Koç University, Department of Molecular Biology and Genetics, Istanbul, Turkey) according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pEGFP vectors had kanamycin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#1 or P#3 10 µM 1.0 µl; Reverse primer P#2 or P#4 10 µM 1.0 µl; dH_2O 11.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl); PCR

conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 55 °C – 40 sec; Extension 72 °C – 3 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (1 ml of 50 mg/ml kanamycin stock into 1 L LB agar) and 5 ml LB (50 µg/ml kanamycin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with pEGFP forward and reverse sequencing primers (P#5 and P#6) by Macrogen Company. After confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (50 µg/ml kanamycin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT.

3.2 Isothermal Assembly of Truncated PCDH7

A short nucleotide sequence was introduced into the missing site of the truncated PCDH7 construct to facilitate in-frame transcription of the gene. Isothermal Assembly (or Gibson Assembly) method was performed for this purpose. First, I prepared 100 µl 5X reaction buffer (1 M Tris-HCl pH 7.5 50 µl; $MgCl_2$ 1 M 5 µl; dNTP mix 10 mM 10 µl; DTT 1M 5 µl; PEG-8000 0.025 g; NAD 100 mM 5 µl) – stored at -20 °C. Then, I prepared 180 µl Assembly Master Mixture (5X reaction buffer 48 µl; T5 Exonuclease Epicentre #T5E4111K 0.1 µl; Phusion HF DNA Polymerase NEB #F-530S 3 µl; T4 DNA ligase NEB #M0202S 24 µl; dH_2O 105 µl) – stored at -20 °C as 15 µl aliquots. Then, PCR for

two fragments to be amplified was performed (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#7/P#9/P#11/P#13 10 µM 1.0 µl; Reverse primer P#8/P#10/P#12/P#14 10 µM 1.0 µl; dH_2O 11.6 µl; template DNA [30 ng/µl PCDH7-pEGFP-N1] 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.4 µl) – primer pairs per each reaction (First fragment for pEGFP-N1: P#7 and P#8; Second fragment for pEGFP-N1: P#9 and P#10; First fragment for pEGFP-C1: P#11 and P#12; Second fragment for pEGFP-C1: P#13 and P#14); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 55 °C/65 °C [55 °C for the second fragment's PCR / 65 °C for the first fragment's PCR] – 30 sec; Extension 72 °C – 2 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis and ~100 bp (first fragment) and ~2400 bp (second fragment) bands were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. pEGFP-N1 and pEGFP-C1 vector plasmids were digested with restriction endonucleases (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl; DNA 5.0 µl; HindIII NEB #R3104S 1.0 µl; BamHI NEB #R3136S 1.0 µl; dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour). Digested vectors were run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vectors were dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 13.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer. Mole calculations of the DNA fragments were calculated with the formula: pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons). DNA fragments were added to the Assembly Master Mixture (15 µl aliquots) with accordance to their calculated moles (For pEGFP-N1: pEGFP-N1 4 µl [0.008 pmol; 28.4 ng]; First fragment [~100 bp] 1.2 µl [0.168 pmol; 11.16 ng]; Second fragment [~2400 bp]

0.6 µl [0.024 pmol; 38.82 ng] / For pEGFP-C1: pEGFP-C1 4 µl [0.016 pmol; 42.4 ng]; First fragment [~100 bp] 1.4 µl [0.24 pmol; 14.98 ng]; Second fragment [~2400 bp] 1.2 µl [0.048 pmol; 65.4 ng]); reaction condition (50 °C – 1 hour). As negative control, reaction mixtures were prepared as duplicates with control tubes lacking first fragment in the mix. Isothermal reaction products were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pEGFP vectors had kanamycin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#9/P#13 10 µM 1.0 µl; Reverse primer P#10/P#14 10 µM 1.0 µl; dH_2O 15.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl); PCR conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 55 °C – 40 sec; Extension 72 °C – 3 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (1 ml of 50 mg/ml kanamycin stock into 1 L LB agar) and 5 ml LB (50 µg/ml kanamycin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with pEGFP forward and reverse sequencing primers (P#5 and P#6 for pEGFP-N1 / P#5 and P#15 for pEGFP-C1) by Macrogen Company. After

confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (50 μg/ml kanamycin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT.

3.3 Cloning of PCDH7Δ*CYT*, PCDH7 MS$^-$ and PCDH7 MS$^+$ to pEGFP Vectors

Three mutant versions of Isothermal PCDH7 construct were produced in order to examine functional role of the protein's cytoplasmic domain. PCDH7Δ*CYT* lacks the entire cytoplasmic domain (except for a short juxtamembrane domain); PCDH7 MS$^-$ lacks the hypothetical modification site (MS: 974 – 1011 amino acids); PCDH7 MS$^+$ terminates just after the MS (at amino acid 1015). Insert DNAs were amplified from Isothermal PCDH7 in pEGFP-N1 (30 ng/μl) with PCR (10X Phusion HF Buffer: 4.0 μl; DMSO 100% 0.6 μl; dNTP mix 10 mM 0.4 μl; Forward primer P#16/P#18/P#20/P#22/P#24/P#26 10 μM 1.0 μl; Reverse primer P#17/P#19/P#21/P#23/P#25/P#27 10 μM 1.0 μl; dH_2O 11.6 μl; template DNA 1.0 μl; Phusion HF DNA Polymerase NEB #F-530S 0.4 μl) – primer pairs per each reaction (PCDH7Δ*CYT* for pEGFP-N1: P#16 and P#17; PCDH7Δ*CYT* for pEGFP-C1: P#18 and P#19; PCDH7 MS$^-$ for pEGFP-N1: P#20 and P#21; PCDH7 MS$^-$ for pEGFP-C1: P#22 and P#23; PCDH7 MS$^+$ for pEGFP-N1: P#24 and P#25; PCDH7 MS$^+$ for pEGFP-C1: P#26 and P#27); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 60 °C / 59 °C / 53 °C [60 °C for PCDH7Δ*CYT* PCRs; 59 °C for pEGFP-N1 versions of both PCDH7 MS$^-$ and PCDH7 MS$^+$; 53 °C for pEGFP-C1 versions of both PCDH7 MS$^-$ and PCDH7 MS$^+$] – 30 sec; Extension 72 °C – 1 min 30 sec; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1%

agarose gel electrophoresis to control the success of PCR reaction. Then, PCR products were purified with Sigma GenElute PCR Clean-Up Kit #NA1020; according to the manufacturer's instructions. PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl; DNA 5.0 µl; HindIII NEB #R3104S 1.0 µl; BamHI NEB #R3136S 1.0 µl; dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour). Digestions products were also purified with PCR Clean-Up Kit. pEGFP vector plasmids were also digested with the same RE couple with the same reaction conditions above. Digested vectors were run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vectors were dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 13.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested inserts and plasmids were used in the ligation reaction with vector: insert ratio of 1:3. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer and ligation calculation was done with "Ligation Calculator" online program. Ligation reaction was performed (10X T4 Buffer 1.0 µl; T4 Ligase NEB #M0202S 1.0 µl; Vector 1.0 µl; Insert n µl; dH_2O 7-n µl) – insert amounts (n) were different for all inserts, ranging from 1 µl to 3 µl; negative control reaction had no insert – ; reaction conditions (4 °C overnight). Ligation products were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pEGFP vectors had kanamycin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies

from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#16/P#18/P#20/P#22/P#24/P#26 10 µM 1.0 µl; Reverse primer P#17/P#19/P#21/P#23/P#25/P#27 10 µM 1.0 µl; dH_2O 15.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl) – primer pairs per each reaction (PCDH7Δ*CYT* for pEGFP-N1: P#16 and P#17; PCDH7Δ*CYT* for pEGFP-C1: P#18 and P#19; PCDH7 MS^- for pEGFP-N1: P#20 and P#21; PCDH7 MS^- for pEGFP-C1: P#22 and P#23; PCDH7 MS^+ for pEGFP-N1: P#24 and P#25; PCDH7 MS^+ for pEGFP-C1: P#26 and P#27); PCR conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 60 °C / 59 °C / 53 °C [60 °C for PCDH7Δ*CYT* PCRs; 59 °C for pEGFP-N1 versions of both PCDH7 MS^- and PCDH7 MS^+; 53 °C for pEGFP-C1 versions of both PCDH7 MS^- and PCDH7 MS^+] – 40 sec; Extension 72 °C – 3 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (1 ml of 50 mg/ml kanamycin stock into 1 L LB agar) and 5 ml LB (50 µg/ml kanamycin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with pEGFP forward and reverse sequencing primers (P#5 and P#6 for pEGFP-N1 / P#5 and P#15 for pEGFP-C1) by Macrogen Company. After confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (50 µg/ml kanamycin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT.

3.4 Cloning of PCDH7 Domains to pGEX Vector

Extracellular domain (EC: 241 – 862 aa), extracellular and transmembrane domains (ECTM: 241 – 900 aa), transmembrane and cytoplasmic domains (TMCYT: 880 – 1072 aa) and cytoplasmic domain (CYT: 901 – 1072 aa) of PCDH7 were produced and cloned to pGEX-6P-1 GE Healthcare #28-9546-48 vector for the aim of peptide expression. Insert DNAs were amplified from Isothermal PCDH7 in pEGFP-N1 (30 ng/µl) with PCR (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#28/P#30/P#32/P#34 10 µM 1.0 µl; Reverse primer P#29/P#31/P#33/P#35 10 µM 1.0 µl; dH_2O 11.6 µl; template DNA 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.4 µl) – primer pairs per each reaction (EC: P#28 and P#29; ECTM: P#30 and P#31; TMCYT: P#32 and P#33; CYT: P#34 and P#35); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 50 °C – 30 sec; Extension 72 °C – 1 min 30 sec; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. Then, PCR products were purified with Sigma GenElute PCR Clean-Up Kit #NA1020; according to the manufacturer's instructions. PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (10X NEB4 Buffer 2.5 µl; DNA 5.0 µl; BamHI NEB #R3136S 1.0 µl; EcoRI NEB #R3101S 1.0 µl; dH_2O 15.5 µl); reaction conditions (37 °C – 30 min). Digestions products were also purified with PCR Clean-Up Kit. pGEX-6P-1 vector plasmid was also digested with the same RE couple (10X NEB4 Buffer 5.0 µl; DNA 15.0 µl; BamHI NEB #R3136S 1.0 µl; EcoRI NEB #R3101S 1.0 µl; dH_2O 28.0 µl); reaction conditions (37 °C – 30 min). Digested vectors were run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12.

Digested vectors were dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 13.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested inserts and plasmids were used in the ligation reaction with vector: insert ratio of 1:5. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer and ligation calculation was done with "Ligation Calculator" online program. Ligation reaction was performed (2X T4 Buffer 8.0 µl; T4 Ligase Promega #M1804 1.0 µl; Vector 3.0 µl; Insert n µl; dH_2O n-7 µl) – insert amounts (n) were different for all inserts, ranging from 1 µl to 3 µl; negative control reaction had no insert – ; reaction conditions (at room temperature for 1 hour). Ligation products were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pGEX vector had ampicillin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#28/P#30/P#32/P#34 10 µM 1.0 µl; Reverse primer P#29/P#31/P#33/P#35 10 µM 1.0 µl; dH_2O 15.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl) – primer pairs per each reaction (EC: P#28 and P#29; ECTM: P#30 and P#31; TMCYT: P#32 and P#33; CYT: P#34 and P#35); PCR conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 50 °C – 40 sec; Extension 72 °C – 2 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were

run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (10 ml of 10 mg/ml ampicillin stock into 1 L LB agar) and 5 ml LB (100 µg/ml ampicillin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with pGEX5 forward and pGEX3 reverse sequencing primers (P#36 and P#37) by Macrogen Company. After confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (100 µg/ml ampicillin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT.

3.5 Cloning of PCDH7 and PCDH7Δ*CYT* to BioID Vectors

Isothermal PCDH7 and PCDH7Δ*CYT* constructs were cloned to BioID-N (Addgene Plasmid 35700: pcDNA3.1 mycBioID) and BioID-C (Plasmid 36047: pcDNA3.1 MCS-BirA(R118G)-HA) vectors which were gifts from Dr. Mark Petronczki, Cell Division and Aneuploidy Laboratory, Cancer Research UK London Research Institute, UK. Two of the inserts were amplified from Isothermal PCDH7 in pEGFP-N1 (30 ng/µl) with different primer pairs in PCR (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#38/P#40/P#42/P#44 10 µM 1.0 µl; Reverse primer P#39/P#41/P#43/P#45 10 µM 1.0 µl; dH_2O 11.7 µl; template DNA 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.3 µl) – primer pairs per each reaction (PCDH7 for BioID-N: P#38 and P#39; PCDH7 for BioID-C: P#40 and P#41; PCDH7Δ*CYT* for BioID-

N: P#42 and P#43; PCDH7Δ*CYT* for BioID-C: P#44 and P#45); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 53 °C / 56 °C / 60 °C [53 °C for PCDH7 – BioID-N; 56 °C for PCDH7 – BioID-C; 60 °C for PCDH7Δ*CYT* versions of both BioID-N and BioID-C] – 30 sec; Extension 72 °C – 1 min 30 sec; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. Then, PCR products were purified with Sigma GenElute PCR Clean-Up Kit #NA1020; according to the manufacturer's instructions. PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl; DNA 5.0 µl; [EcoRI NEB #R3101S 1.0 µl; BamHI NEB #R3136S 1.0 µl] for BioID-N / [NheI NEB #R3131S 1.0 µl; BamHI NEB #R3136S 1.0 µl] for BioID-C; dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour). Digestions products were also purified with PCR Clean-Up Kit. BioID vector plasmids were also digested with the same RE couples with the same reaction conditions above. Digested vectors were run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vectors were dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 13.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested inserts and plasmids were used in the ligation reaction with vector: insert ratio of 1:3. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer and ligation calculation was done with “Ligation Calculator” online program. Ligation reaction was performed (10X T4 Buffer 1.0 µl; T4 Ligase NEB #M0202S 1.0 µl; Vector 1.0 µl; Insert n µl; dH_2O n-7 µl) – insert amounts (n) were different for all inserts, ranging from 2.5 µl to 4 µl; negative control reactions had no insert – ; reaction conditions (4 °C overnight). Ligation products were used to transform chemical competent *E. coli* DH5α

bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – BioID vectors had ampicillin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#38/P#40/P#42/P#44 10 µM 1.0 µl; Reverse primer P#39/P#41/P#43/P#45 10 µM 1.0 µl; dH_2O 15.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl) – primer pairs per each reaction (PCDH7 for BioID-N: P#38 and P#39; PCDH7 for BioID-C: P#40 and P#41; PCDH7Δ*CYT* for BioID-N: P#42 and P#43; PCDH7Δ*CYT* for BioID-C: P#44 and P#45); PCR conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 53 °C / 56 °C / 60 °C [53 °C for PCDH7 – BioID-N; 56 °C for PCDH7 – BioID-C; 60 °C for PCDH7Δ*CYT* versions of both BioID-N and BioID-C] – 30 sec; Extension 72 °C – 2 min 30 sec; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (10 ml of 10 mg/ml ampicillin stock into 1 L LB agar) and 5 ml LB (100 µg/ml ampicillin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with BGH-R reverse primer (P#46) for BioID-N constructs and CMV-F forward primer (P#47) for BioID-C constructs by Macrogen Company. After confirmation by sequencing, stored selected colonies were

inoculated into 40 ml LB liquid (100 µg/ml ampicillin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT.

3.6 Protein Expression and Purification

Extracellular domain (EC: 241 – 862 aa) and cytoplasmic domain (CYT: 901 – 1072 aa) of PCDH7 and GFP (Green Fluorescent Protein GenBank: M62653.1) were expressed as GST (Glutathione S-transferase) tagged fusion proteins in *E. coli* BL21 bacteria and purified with affinity chromatography with glutathione beads. pGEX-6P-1 vectors (see above for details of cloning) which had the inserts (PCDH7 domains and GFP) were used to transform E. coli BL21 bacteria (gift from Dr. Cory Dunn, Koç University, Department of Molecular Biology and Genetics, Istanbul, Turkey) according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pGEX vector had ampicillin resistance. Plates were incubated at 37 °C overnight. Next day, single colony (per each sample) was inoculated into 40 ml 2X YTA media (with 100 µg/ml ampicillin) and incubated at 37 °C overnight with 170 rpm rotational shaking. 3 flasks of 400 ml each 2X YTA media (with 100 µg/ml ampicillin) were prepared and 4 ml from the overnight bacteria culture was added into each of these 400 ml fresh media. Newly started liquid cultures were incubated at 37 °C with 170 rpm rotational shaking for about 3 hours until OD (optical density) at A600 reached between 0.5

– 2.0. At this step, a few ml of non-induced culture control was taken and centrifuged at 8000 rpm for 10 min to store bacterial pellet at -20 °C for preparation of lysate at the later steps along with induced culture. 4 ml of IPTG (isopropylthio-β-galactoside) 100 mM was added into each of the 400 ml cultures (final IPTG concentration was 1mM). Induced cultures were incubated at 21 °C for 20 hours with 170 rpm rotational shaking. Then, induced cultures were centrifuged at 5000 rpm for 10 minutes at 4 °C (total of 1200 ml induced culture was split to 4 tubes of 300 ml in big centrifuge tubes). Supernatants were discarded and bacterial pellets were resuspended 10 ml cold 1X PBS (for each centrifuge tubes) and then suspensions were pooled in one 50 ml Falcon tube. Pooled suspension was centrifuged at 5000 rpm for 10 minutes at 4 °C. 40 ml cold lysis buffer (recipe for 20 ml lysis buffer: 2.86 ml 7X protease inhibitor Roche #11-836-153-001; 1 ml 20% Triton-X; 16.14 ml 1X PBS) was added onto bacterial pellet to resuspend the pellet. Then, suspension was split equally into 2 of 50 ml Falcon tubes for ease of the next steps. 10 seconds of pulse sonication was applied 3 times with 30 seconds intervals to each of the bacterial lysates. Sonicated samples were incubated at 4 °C (cold-room) with 10 rpm – 180 degrees rotation for 30 minutes to help solubilization of proteins. Lysates were centrifuged at 12000 rpm for 15 minutes at 4 °C. Supernatants were transferred to new tubes. At this step, 1 ml of induced control was saved and stored at -20 °C for SDS-PAGE. 1 ml of glutathione beads (BioWorlds #20182003-2) was prepared as follows: 1 ml of beads was centrifuged briefly at bench-top centrifuge machine to pellet beads. Supernatant was discarded and 1 ml of cold 1X PBS was added onto beads. The tube was rotated up-and-down at 4 °C for 5 minutes. Centrifuge and washing steps were repeated 3 times and finally beads which were suspended in PBS were ready to use. Prepared beads were added into lysate and this suspension was incubated at 4 °C (cold-room) with 10 rpm – 180 degrees rotation overnight for bead – GST-fused-protein binding. Poly prep column (BioRad #731-1550EDU) was wetted with 5 ml cold 1X PBS. Then, bead-lysate suspension was applied

to the column when the cap was closed. After settlement of beads, the cap was opened to let unbound proteins pass through the column. Collected flow-through was re-applied to the column to increase yield. This process was done in cold-room. At this step, 1 ml of flow-through control was saved and stored at -20 °C for SDS-PAGE. 5 ml of cold, high-salt-concentrated 1X PBS (300 mM NaCl) was applied to the column two times to wash the beads. Wash step was repeated one more time with cold, standard-salt-concentrated 1X PBS. 600 µl elution buffer (0.03 g reduced L-glutathione Sigma #G4251-5G was dissolved in 10 ml Tris-HCl pH 7.2 and pH was adjusted to 8.0 with NaOH. Add 1 ml 50% glycerol to 9 ml L-glutathione solution to prepare the elution buffer with 5% final concentration of glycerol. Storage temperature is -20 °C) was applied to column when the cap was closed. After 10 minutes incubation at cold, the cap was opened to collect the elution. Elution step was repeated one more time to increase the yield. At this step, about 50 µl of elution was saved and stored at -20 °C for SDS-PAGE. Eluted protein was dialyzed with Slide-A-Lyzer G2 Dialysis Cassettes 7K MWCO 3 ml Pierce #PI-87728 to dialysis buffer (500 ml PBS pH 7.4 with 10% glycerol) at 4 °C overnight. Dialyzed protein was split into 100 µl aliquots which were snap-freeze in liquid nitrogen and then stored at -80 °C. Pierce #23225 BCA Protein Assay Kit was used according to the manufacturer's instructions to measure protein concentration. SDS-PAGE (12% separation gel) was employed to run and compare non-induced, induced and flow-through controls with elution sample. Visualization of protein bands was done with Coomassie brilliant blue dye.

3.7 GST Pull-Down Assay

GST-tagged cytoplasmic domain of PCDH7 (see above for cloning and protein expression procedures) was incubated with whole cell lysate and then purified with glutathione affinity chromatography to identify intracellular interaction partners of PCDH7 (which are purified as bound to PCDH7-CYT) with Mass Spectrometry analysis. Two jumbo-size (145 mm) cell culture dishes of HeLa S3 PC cells were cultured in DMEM High Glucose with L-glutamine + 10% FBS + 1% PS (DMEM: Dulbecco's Modified Eagle Medium [Lonza #BE12-604F]; FBS: Fetal Bovine Serum [Sigma #F9665]; PS: Penicillin, Streptomycin antibiotics [Lonza #DE17-60E]) to full confluence. After media is sucked, each dish was washed with 10 ml 1X PBS and incubated with 3 ml of trypsin (Lonza #BE17-161E) in cell culture incubator for 5 minutes. 5 ml of DMEM is added onto each dish and scrapped cells from each dish were pipeted into 15 ml Falcon tubes. After centrifuge at 1200 rpm at 4 °C for 5 minutes, supernatants were discarded. 1 ml of lysis buffer (lysis buffer: 1mM EGTA; 1mM $MgCl_2$; 50 mM HEPES pH 7.4; 100 mM KCl; 10% glycerol; 0.05% NP-40; 1mM DTT; protease inhibitor with a final concentration of 1X) was added into each tube and cell pellets were suspended with pipetting. Cell suspensions in the lysis buffer were incubated on ice for 10 minutes. Lysates were transferred to Eppendorf tubes. To aid lysis, lysate was pulled in and pushed out through a syringe. Then, the lysates were centrifuged at 10000 rpm for 10 minutes at 4 °C and supernatants were transferred to new tubes. At this step, about 20 µl of supernatant can be saved and stored at -20 °C for later western blot analysis. 90 µl of glutathione beads (BioWorlds #20182003-2) were prepared as follows: 90 µl of beads was centrifuged briefly at bench-top centrifuge machine to pellet beads. Supernatant was discarded and 100 µl of cold 1X PBS was added onto beads. The tube was rotated up-and-down at 4 °C for 5 minutes. Centrifuge and washing steps were repeated 3 times and

finally beads which were suspended in PBS were ready to use. 45 µl of beads were added into each of lysate samples and they were incubated with up-and-down rotation at 4 °C for 1 hour. Beads (and any non-specific binding protein) were removed by bench-top centrifuge. 60 µg GST fused PCDH7-CYT protein was added to one of the collected supernatants and 60 µg GST-GFP fusion protein was added to the other one. Samples were incubated with up-and-down rotation at 4 °C overnight. Next day, 60 µl glutathione beads were prepared as explained above and added to each of the tubes. The tubes were incubated with up-and-down rotation at 4 °C for 1 hour. Beads were pelletted with spin-down in bench-top centrifuge. At this step, about 20 µl of supernatant can be saved and stored at -20 °C for later western blot analysis. Supernatants were discarded and beads in the each tube were washed with 200 µl lysis buffer for 4 times (incubation with lysis buffer at 4 °C with up-and-down rotation for 5 minutes > spin-down beads). After the final wash, 70 µl of Laemmli sample buffer with DTT were added to each sample and they were incubated at 90 °C (in water bath or block heater) for 5 minutes. Denatured samples were either stored at -20 °C or used directly in SDS-PAGE for Mass Spectrometry analysis.

3.8 HeLa cDNA Production

We produced cDNA from total HeLa mRNA in order to use it as template DNA in some of the cloning experiments. Both HeLa total mRNA and Roche #05-081-955-001 Transcriptor High Fidelity cDNA Synthesis Kit were kind gifts from Dr. Gulayse Ince Dunn, Koç University, Department of Molecular Biology and Genetics, Istanbul, Turkey. We followed "Standard Procedure for Qualitative RT-PCR" protocol in the kit's manual. Template-Primer Mix (3 µl [510 ng] total mRNA; 1 µl Anchored-oligo$(dT)_{18}$ Primer / 2 µl Random

Hexamer Primer; 7.4 µl / 6.4 µl dH_2O) was prepared for each primer type and incubated at 65 °C for 10 minutes. Then, the tubes were chilled on ice and remaining components of the reaction was added to each of the tubes (Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer 5X 4µl; Protector RNase Inhibitor 40 U/µl 0.5 µl; Deoxynucleotide Mix 10 mM 2 µl; DTT 1 µl; Transcriptor High Fidelity Reverse Transcriptase 1.1 µl). The reaction was performed at 55 °C for 30 minutes and then, 85 °C for 5 minutes. Tubes were chilled on ice and then stored at -20 °C. Note that, we used cDNA which had been produced with oligo(dT) primer as template in gene cloning PCRs.

3.9 Preparation of Lentiviral Transduction Constructs

We aimed to generate stably cell lines which express selected genes with tags in cells. For this aim, we needed to make cloning of selected genes (with their tags) to mammalian expression vector pLenti. First, we digested gene of interests with their tags (including Myc-BirA* or GFP) from previously constructed vectors (BioID or pEGFP vectors): (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl; DNA 5.0 µl; [NheI NEB #R3131S 1.0 µl; HindIII NEB #R3104S 1.0 µl] for inserts in BioID-N vector (Myc-BirA*; MyrPalm-Myc-BirA*; CD44-Myc-BirA*; PCDH7-Myc-BirA*; PCDH7Δ*CYT*-Myc-BirA*) / [NheI NEB #R3131S 1.0 µl; AflII NEB #R0520S 1.0 µl] for inserts in pEGFP-N1 vector (PCDH7-GFP; PCDH7Δ*CYT*-GFP); dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour; heat inactivation at 80 °C – 25 min). Digested inserts were blunt-ended with Quick Blunting Kit NEB #E1201S (10X Blunting Buffer 2.5 µl; 1mM dNTP Mix 2.5 µl; Blunt Enzyme Mix 1.0 µl; Digest reaction product 19.0 µl); reaction conditions (21 °C – 20 min; heat inactivation at 70 °C – 10 min). Reaction products were purified with PCR Clean-Up Kit. pENTR1A vector (Addgene

Plasmid 17398: pENTR1A no ccDB) was digested with EcoRV enzyme (10X NEB4 Buffer 2.5 µl; DNA 5.0 µl; EcoRV NEB #R3195S 1.0 µl; dH_2O 16.5 µl); reaction conditions (37 °C – 1 h; heat inactivation at 80 °C – 25 min). Digested vector was dephosphorylated with alkaline phosphatase enzyme (10X NEB4 Buffer 5.0 µl; Digest reaction product 25.0 µl; dH_2O 18.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Digested and CIP-applied vector was run in 1% agarose gel electrophoresis and then purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer. Prepared inserts and plasmids were used in the ligation reaction with vector: insert ratio of 1:6. Ligation reaction was performed (10X T4 Buffer 1.0 µl; T4 Ligase NEB #M0202S 1.0 µl; Vector 1.0 µl; Insert n µl; dH_2O n-7 µl) – insert amounts (n) were different for all inserts, ranging from 0.7 µl to 2.3 µl; negative control reaction had no insert – ; reaction conditions (4 °C overnight). Ligation products were used to transform chemical competent *E. coli* DH5α bacteria with standard transformation protocol (explained above) and transformed bacteria were incubated on agar plates with kanamycin antibiotic selection. Since this was cloning of blunt-ended inserts, inserts' ligation orientations could be in both directions. To choose the correctly oriented ones, I chose 4 colonies for each sample and grew them in liquid culture and then made their minipreps as explained above. Then, I performed digestion with EcoRI (for Myc-BirA* fusions) or BamHI (for GFP fusions) and ran digest products on agarose gel electrophoresis. These enzymes had one recognition site in insert and another one in pENTR1A vector which resulted in bands with different sizes (for right and wrong orientations) on the gel. This helped us to differentiate correctly oriented ligation products of each sample and we continued the next step of the cloning with them. In the next step, inserts (fused with their tags) were transferred from pENTR1A vector to pLenti vector (Addgene Plasmid 17454: pLenti CMV Hygro DEST) by Gateway Cloning Technology

(Invitrogen Gateway LR Clonase Enzyme Mix #11791-019): (Entry clone (in pENTR1A) 300 ng; Destination vector (pLenti: 150 ng/µl) 2 µl; 5X Reaction Buffer 4 µl; TE Buffer, pH 8.0 up to 16 µl; LR Clonase enzyme mix 4 µl); reaction conditions (25 °C – 2 h); inhibition with addition of 2 µl Proteinase K and incubation at 37°C for 10 minutes. Transformation was done with 2 µl reaction mix and 25 µl bacteria (One Shot Stbl3 Chemically Competent *E. coli*, Invitrogen #C7373-03) and bacteria were grown on agar plates with ampicillin antibiotic selection. DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with CMV-F forward primer (P#47) by Macrogen Company.

3.10 Lentiviral Transduction

HEK 293 T cells (gift from Dr. Gulayse Ince Dunn, Koç University, Department of Molecular Biology and Genetics, Istanbul, Turkey) were grown in 10 cm dishes for each sample with 2.5 x 10^6 density in day 0. In day 1, transfection was performed: First, media of HEK cells (DMEM high glucose with 10% FBS and 1%PS) was changed to serum-free DMEM. Then, lipofectamine and DNA mixes were prepared for each sample: 20 µl Lipofectamine (Lipofectamine 2000 reagent, Invitrogen #11668-019) + 180 µl Opti-MEM (Opti-MEM Reduced Serum Medium, Invitrogen #31985047) mix prepared and incubated at room temperature for 5 minutes. DNA mixes for each sample were prepared in 200 µl Opti-MEM [pMD2G (Addgene Plasmid 12259; Insert: VSV G) 225 ng + pMDLg/pRRE (Addgene Plasmid 12251; Insert: HIV-1 GAG/POL) 2250 ng + pRSV-rev (Addgene Plasmid 12253; Insert: Rev) 2000 ng + pLenti vector with cloned gene (see above) 2500 ng]

and incubated at room temperature for 5 minutes (Note 1: Plasmids that carry essential lentiviral genes were gifted by Dr. Nathan Lack, Koç University, Department of Molecular Biology and Genetics, Istanbul, Turkey; Note 2: pLenti-GFP vector was used as indicative control as an additional sample). For each sample, DNA mix and lipofectamine mix were pooled and gently fixed. After 25 minutes of incubation at room temperature, DNA + lipofectamine mix was added onto HEK cells. In day 2, cell culture media was changed to DMEM high glucose (and thereby lipofectamine was washed off). In day 3, culture media was collected (it includes viral particles) and stored at 4 °C while new media was added to the dishes. In day 4, culture media was collected again and it was pooled with one from the previous day. Then, viral supernatants of each sample were syringe filtered by 0.45 μm regenerated cellulose filters. They were divided to 1 ml aliquots and stored at -80 °C.

HeLa S3 cells were grown in 6-well plate with cell number of 5×10^4 per well. One day later, culture medium in each well was replaced with 1 ml DMEM high glucose which contained 16 μg/ml protamine sulfate (Sigma #P4505) and then 1 ml viral supernatants of each sample was added to labeled wells (Hence, final concentration of protamine sulfate was 8 μg/ml). After 2 days of incubation, culture medium was replaced with DMEM high glucose which had 150 μg/ml hygromycin (Sigma #H3274). Selection of successfully transduced cells with hygromycin took a few days and cell passaging. Control was HeLa cells which had not been transduced and selection process was continued until all control cells died. Hygromycin concentration was increased up to 300 μg/ml when it was necessary. After the selection procedure, the stable cell lines were grown in 10 cm dishes and one backup of each sample was frozen (Freezing medium: DMEM high glucose with 10% FBS and 1% PS 25 ml; additional FBS 15 ml; DMSO 10 ml) and stored at liquid nitrogen tank.

3.11 Cloning and Stable Cell Line Production of Signal-Myc-Pcdh7

Myc tag (Nt: GAACAAAAACTCATCTCAGAAGAGGATCTC; Aa: EQKLISEEDL) was inserted between signaling sequence (1-31 aa) of Pcdh7 and rest of it by using isothermal assembly method. At the same time, this engineered sequence was ligated to pENTR1A vector in the same isothermal reaction. First, PCR reactions were made to produce signal sequence and the rest of the gene each of which had overlapping flanks for ligation to vector and to each other. The flank region between the two sequences was Myc tag. (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#80 or P#82 10 µM 1.0 µl; Reverse primer P#81 or P#83 10 µM 1.0 µl; dH_2O 11.7 µl; template DNA 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.3 µl) – primer pairs per each reaction (Signal sequence: P#80 and P#81; Rest of the sequence: P#82 and P#83); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 73 °C / 62 °C [73 °C for Signal; 62 °C for rest of the sequence] – 30 sec; Extension 72 °C – 45 sec; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction and then they were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. pENTR1A vector was digested: (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl; DNA 5.0 µl; SalI NEB #R3138S 1.0 µl; BamHI NEB #R3136S 1.0 µl; dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour). Digest product was run in 1% agarose gel electrophoresis and purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vector was dephosphorylated with alkaline phosphatase enzyme (10X NEB4 Buffer 5.0 µl; Digest reaction product 25.0 µl; dH_2O 18.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Concentration measurements of prepared DNA molecules (PCR products and digested

vector) were made with Thermo Scientific Nanodrop Spectrophotometer. For isothermal assembly, mole calculations of the DNA fragments were calculated with the formula: pmols = (weight in ng) x 1,000 / (base pairs x 650 daltons). DNA fragments were added to the Assembly Master Mixture (15 µl aliquots) with accordance to their calculated moles (pENTR1A 5.5 µl [0.033 pmol; 50 ng]; First fragment [152 bp] 1.5 µl [0.165 pmol; 15.75 ng]; Second fragment [~2581 bp] 2.9 µl [0.099 pmol; 162.69 ng]); reaction condition (50 °C – 1 hour). Negative control reaction had water instead of the first fragment. Isothermal reaction products were used to transform chemical competent *E. coli* DH5α with the standard protocol and bacteria were grown on agar plate with kanamycin resistance. Next day, single colonies from sample plate were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#82 10 µM 1.0 µl; Reverse primer P#83 10 µM 1.0 µl; dH_2O 15.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl); PCR conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 57 °C – 30 sec; Extension 72 °C – 3 min 30 sec; Final extension 72 °C – 10 min; 30 cycles). The PCR products were run in agarose gel and miniprep of two selected colonies which passed the Colony PCR was made. Signal-Myc-Pcdh7 construct was transferred from pENTR1A vector to pLenti vector by Gateway Cloning Technology as described in Title 3.9. Then, amplified plasmids were sequenced with CMV-F forward primer (P#47) by Macrogen Company. Finally, stable HeLa cell line of this construct was prepared as described in Title 3.10.

3.12 Bead Aggregation Assay

Extracellular domain of Pcdh7 was cloned to pGEX vector (see Title 3.4) and then the fusion protein was expressed and purified (see Title 3.6). Overnight HeLa cell culture medium (DMEM high glucose + 10% FBS + %1 PS) was collected and filtered through 0.45 μm regenerated cellulose filters to produce conditioned medium. Glutathione beads (BioWorlds #20182003-2) was prepared by washing with 1X PBS three times. 30 μl beads and 120 μg purified protein (GST-PCDH7ec as sample and GST-GFP as negative control and no-protein as negative control) were incubated in 500 μl conditioned media at 4 °C for 2 hours. After the incubation, beads were washed with cold PBS (with 2% BSA) solution. For each sample and control, two tubes of 250 μl bead suspension (in PBS-BSA 2%) were prepared and either $CaCl_2$ or EDTA was added with 2 mM final concentration. Prepared bead solutions were incubated on depression glass slides at 37 °C for 1 hour. Images of the beads were taken before and after the incubation with JuLi Smart Cell Imager. Numbers of beads in each image area were counted by ImageJ software (Plugins > Analyze > Cell Counter) (ImageJ 1.46r by Wayne Rasband, National Institutes of Health, USA).

Chapter 4

RESULTS

4.1 Cell Cycle Dependent Localization of Protocadherin 7

4.1.1 Cloning of Pcdh7

Comparison of cell surface proteins in mitosis to those in interphase with SILAC (Stable Isotope Labeling by Amino acids in Cell culture) labeling and biotinylation selection followed by mass spectrometry analyses identified several proteins whose quantity on cell membrane differs when cells divide [2]. Protocadherin 7 is among the proteins which have increased localization to cell membrane during mitosis compared to interphase. We aimed to show this dynamic change of Pcdh7 localization in the cell. For this purpose, we started with cloning the gene to pEGFP vectors to produce GFP fused Pcdh7 which can be visualized by fluorescent microscopy. Although we started doing PCR (polymerase chain reaction) with HeLa cDNA as template, we could not amplify the gene of interest. This may be due to expression profile of the protein in HeLa cell line. We used a previous clone which is a gift from Prof. Sumio Sugano, Laboratory of Functional Genomics, Department of Medical Genome Sciences, The University of Tokyo. Human Pcdh7 has three isoforms (a, b, and c) and we designed primer pairs for all the isoforms. However, we were able to amplify only isoform b of Pcdh7 (Figure 4.1). Note that, PCR product was shorter than expected band size on agarose gel. PCR product was purified and cloned to pEGFP-N1 and

pEGFP-C1 vectors with use of selected digest enzymes (restriction endonucleases) and ligation (See Materials and Methods chapter for details). We had the construct sequenced by Macrogen Europe Inc. Sequence analysis (with ClustalW of European Bioinformatics Institute) showed that the cloned gene was a truncated version of Pcdh7 Isoform b as it lacked a region close to 5-prime of the gene. Absent sequence was corresponding to amino acids 30-240 which is EC1 and a part of EC2 (EC: extracellular conserved domain) (Figure 4.2). Since we repeated the PCR several times but had the same product, we decided to continue with this truncated version of Pcdh7-b. The major problem with this mutant clone was that the deletion caused a frame-shift. We tried to employ site-directed mutagenesis to insert a few nucleotides in the mutation site to rescue frame-shift. However, we did not have satisfactory results with this method. Next, we used isothermal assembly (Gibson assembly) method which assembles DNA fragments with overlapping complementary flanks in a single reaction (Figure 4.3). The two parts of the gene which are intersected by the mutation point were amplified by PCR in which special primers with overlapping flanks were used. The flank region between the two parts had additional nucleotides to overcome the frame-shift problem. The amplified fragments and digested vector (pEGFP-N1) were ligated with Gibson assembly method. The engineered construct was amplified with transformation followed by plasmid midi-preparation and sequenced to verify the success of the cloning.

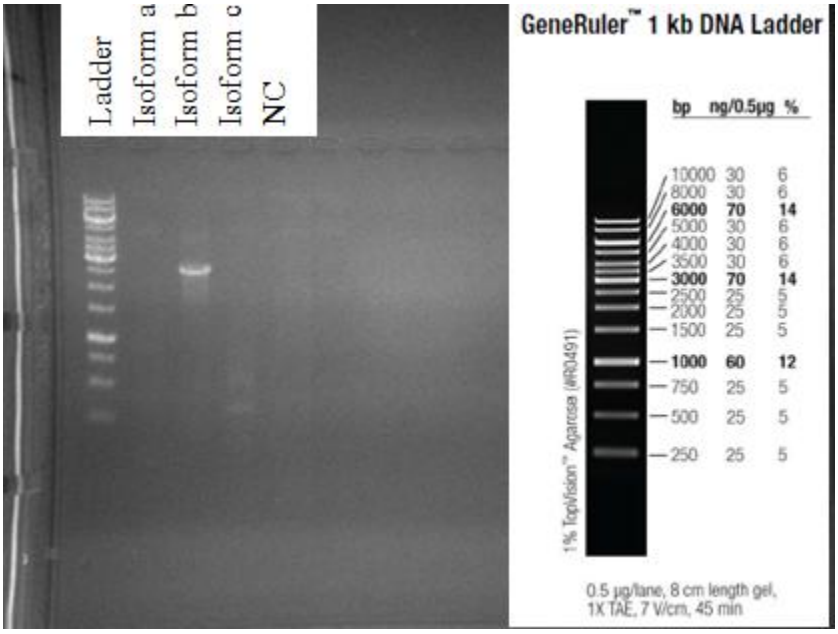

Figure 4.1: Cloning of Pcdh7. PCR worked for only one of the isoforms (isoform b) of human Pcdh7 and it was a truncated version. Pcdh7 cDNA is 3.22 kb (GenBank: AB006756.1) while the truncated version we cloned is 2.5 kb. NC is negative control with primer-free PCR.

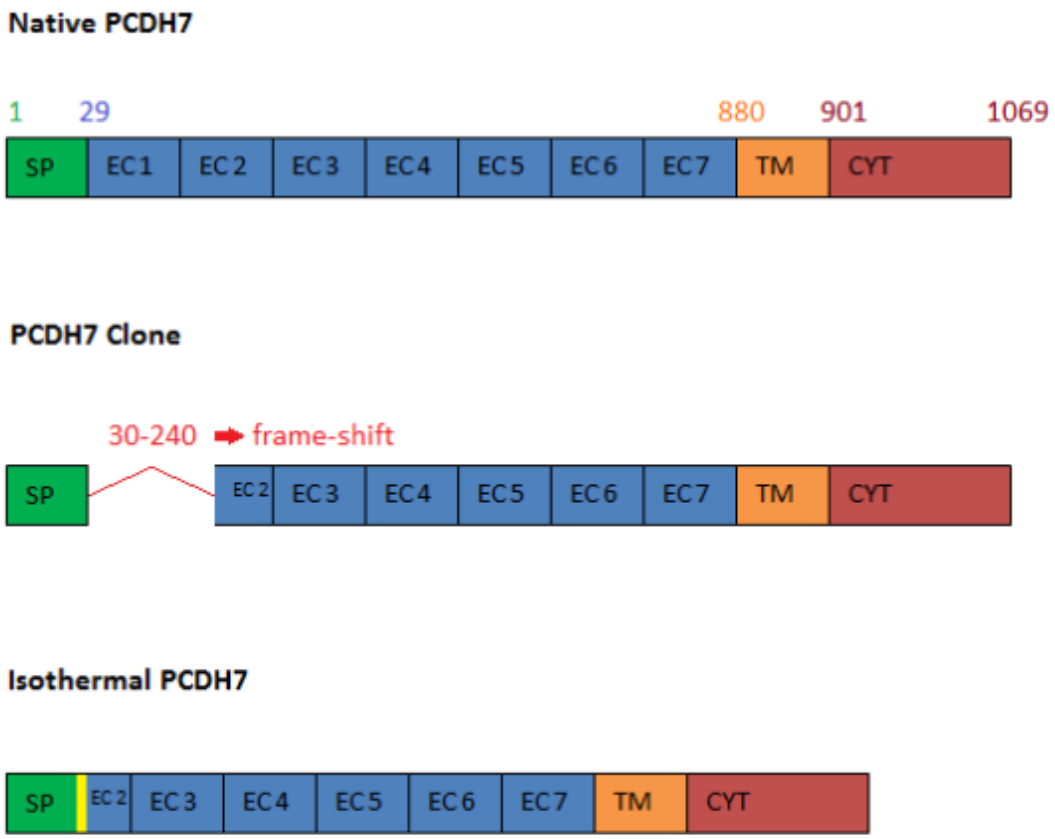

Figure 4.2: Schematic representations of wild-type Pcdh7, truncated Pcdh7 clone and product of isothermal assembly reaction. Numbers on the native construct marks amino acid positions at the borders of signal sequence (SP; green), extracellular domain (EC; blue), transmembrane domain (TM; orange), and cytoplasmic domain (CYT; red). Truncated Pcdh7 lacks EC1 and partial EC2 conserved cadherin repeats at the extracellular domain. This deletion also causes frame-shift. We employed isothermal assembly method to insert additional nucleotides (yellow) to the deletion region to correct the reading frame.

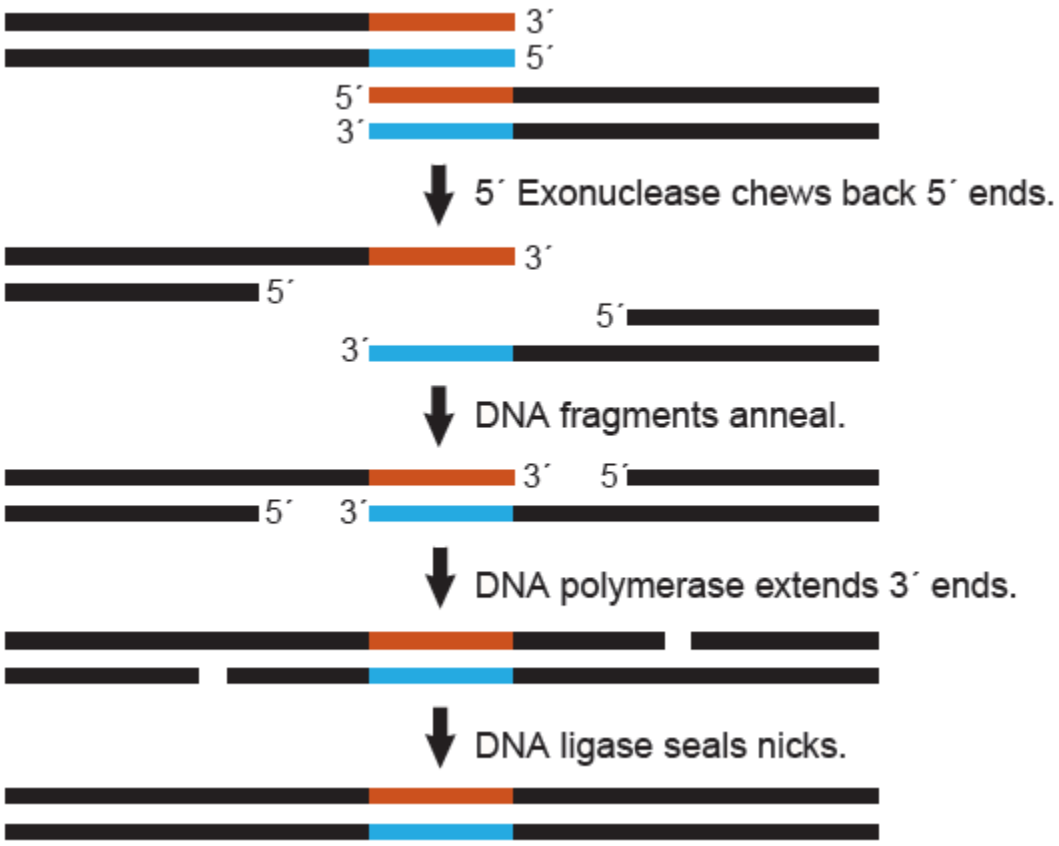

Figure 4.3: Mechanism of isothermal assembly method. Exonuclease chews back 5' ends which produces complementary single DNA strand flanks where DNA fragments anneal. DNA polymerase fills the gaps and DNA ligase seals the nicks. We designed the flank region so that it adds nucleotides between the two fragments in order to fix frame-shift problem. Flanks at the other ends of these two fragments complements to selected digest sites of vector (pEGFP-N1) so that the cloning is done in a single reaction. (Adapted from Gibson *et al.* 2009 [64])

4.1.2 Cloning of Pcdh7 *ΔCYT*

Transmembrane proteins can be regulated by their cytoplasmic interaction partners which may bind to cytoplasmic domain or execute enzymatic modification (such as phosphorylation) on cytoplasmic domain. It is known that Pcdh7 interacts with TAF1 (template-activating factor 1) and PP1α (protein phosphatase type 1 isoform α). There may be other interaction partners which are unknown yet. There is no information in the literature about the role of cytoplasmic domain of Pcdh7 to our knowledge. We aimed to produce a cytoplasmic domain deleted mutant of Pcdh7 (Pcdh7 *ΔCYT*) to investigate physiological role of this domain in the regulation of the protein.

We performed a PCR to amplify the gene from the start codon to the 2745th nucleotide and cloned the insert to pEGFP-N1 and pEGFP-C1 (stop codon included for pEGFP-C1) vectors between selected digestion sites to produce GFP tagged Pcdh7 *ΔCYT* mutant. Sequencing confirmed the success of the cloning. The translated protein has a short juxtamembrane residue and lacks the cytoplasmic domain (Figure 4.4).

4.1.3 Cloning of mutants to assess role of the putative modification site

Enzymatic modification of proteins influences structure and function of target protein. Phosphorylation/dephosphorylation of serine, threonine and tyrosine residues of a protein is a widespread protein modification mechanism. We used an online bioinformatics tool (GPS 2.1 Kinase-specific Phosphorylation Site Prediction [65]) to predict phosphorylation targets on the cytoplasmic domain of Pcdh7 and compared the results with amino acid modifications of Pcdh7 information presented on UniProt (O60245) (See Table 4.1). We focused on predicted targets of CDKs (cyclin dependent kinases) because CDKs regulate cell cycle related events and their activities are also regulated by the cell cycle. We postulated that cell cycle dependent localization of Pcdh7 may be regulated by

phosphorylation of its cytoplasmic domain by a CDK. We found that the region between amino acids 970-1015 is of importance as there are four serine residues which are probable targets of several CDKs. We termed this region as putative modification site (MS) and aimed to produce two mutant version of Pcdh7: The first one lacks MS and terminates at amino acid 970 (MS^-) while the other one has MS and terminates just after MS at amino acid 1015 (MS^+) (Figure 4.4). Using PCR, we cloned mutant genes (MS^- and MS^+) with isothermal Pcdh7 construct (see above) as the reaction template and ligated the inserts to GFP vectors (pEGFP-N1 and pEGFP-C1). Cloning was confirmed by sequencing of the constructs.

Table 4.1: Summary of predicted phosphorylation sites on the cytoplasmic domain of Pcdh7

Position	Amino acid	UniProt	GPS 2.1	Upstream enzymes
948	Tyrosine	✓		
974	Serine	✓	✓	CDK4
982	Serine	✓		
989	Serine	✓	✓	CDC2, CDK2, CDK4
1000	Serine	✓	✓	CDK4, CDK6, CDK5, CDK2, CDC2
1011	Serine	✓	✓	CDK4, CDK5, CDC2, CDK2, CDK6

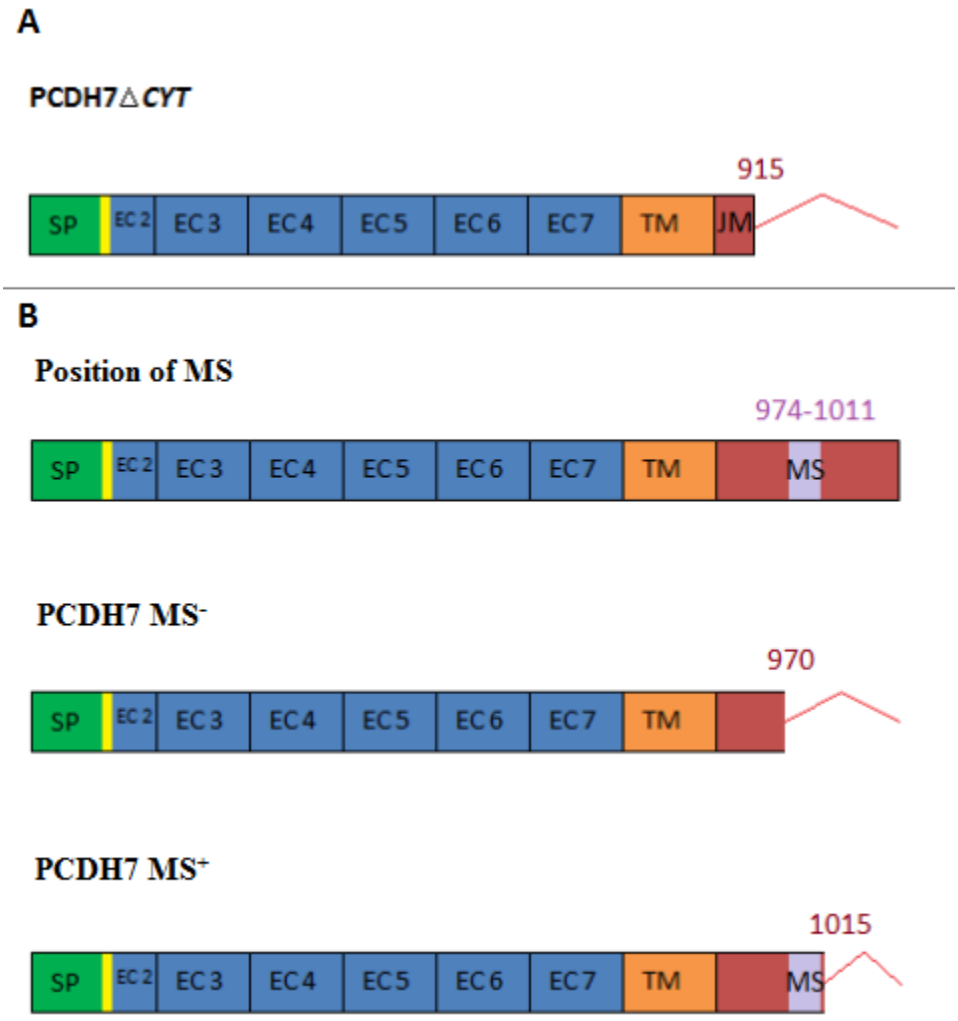

Figure 4.4: Schematic representation of Pcdh7 mutant constructs. The cytoplasmic domain deleted mutant (Pcdh7 *ΔCYT*) is shown in A. Location of the putative modification site (MS) at the cytoplasmic domain of Pcdh7 and the two mutant constructs of MS are presented in B.

4.1.4 Fluorescence Microscopy of Pcdh7 Constructs

U2OS (human osteosarcoma cell line) cells which stably express mCherry-tubulin were transfected with GFP tagged Pcdh7 constructs (Pcdh7, Pcdh7 *ΔCYT*, Pcdh7 MS^-, and Pcdh7 MS^+) by lipofectamine transient transfection method. The cells were synchronized in mitosis by s-trityl-l-cysteine blocking and in interphase by s-trityl-l-cysteine blocking followed by release. Fluorescent images were taken by confocal microscopy.

Microscopic analysis of Pcdh7 supports the biochemical data that shows Pcdh7 has a cell-cycle dependent localization (Figure 4.5 and 4.6). Pcdh7 has cytoplasmic localization during interphase and it relocates to cell membrane during mitosis. This dynamic localization behavior alters when the protein lacks its cytoplasmic domain. Pcdh7 *ΔCYT* enriches on plasma membrane in mitosis like the full length protein. However, it continues to stay on the membrane when the cell enters interphase. Therefore, Pcdh7 *ΔCYT* has a static localization and it does not shuttle from cytoplasm to cell membrane when the cell divides. This finding affirms the notion that the protein is regulated through its cytoplasmic domain. On the other hand, both MS^- and MS^+ constructs show a similar localization pattern as Pcdh7 *ΔCYT*. Thus, our effort to pinpoint residues of cytoplasmic domain which are necessary for the regulation of dynamic localization turned out to be inadequate. Further mutational and biochemical analysis are necessary to illuminate details of how the cytoplasmic domain functions in direction of Pcdh7.

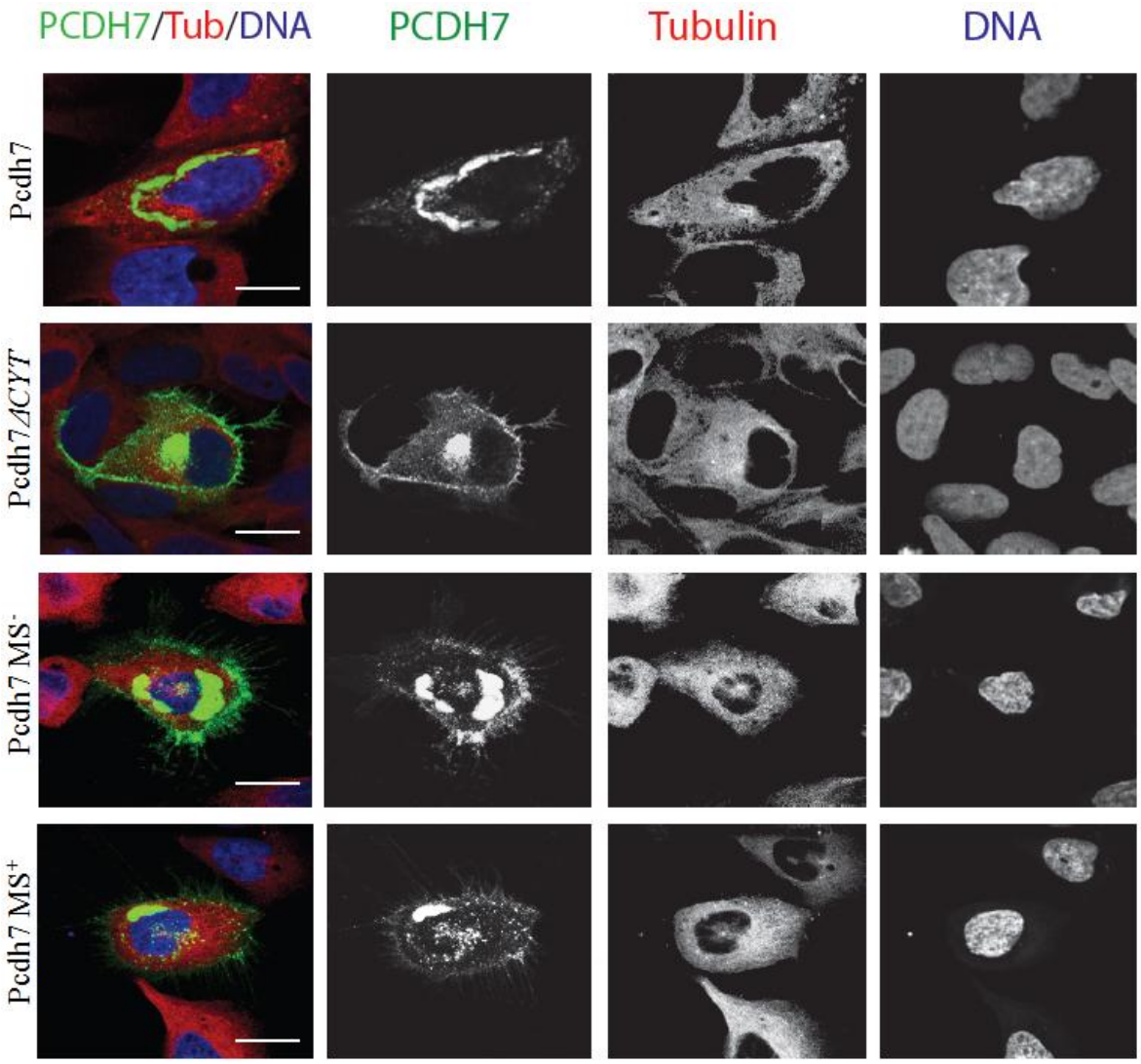

Figure 4.5: Localization of Pcdh7, Pcdh7 *ΔCYT*, Pcdh7 MS$^-$, and Pcdh7 MS$^+$ in U2OS cells during interphase. The native and mutant versions of Pcdh7 are fused to GFP (green). Tubulin is fused to mCherry (red). DNA is stained with DAPI (blue). (Courtesy of Selda Bülbül [66])

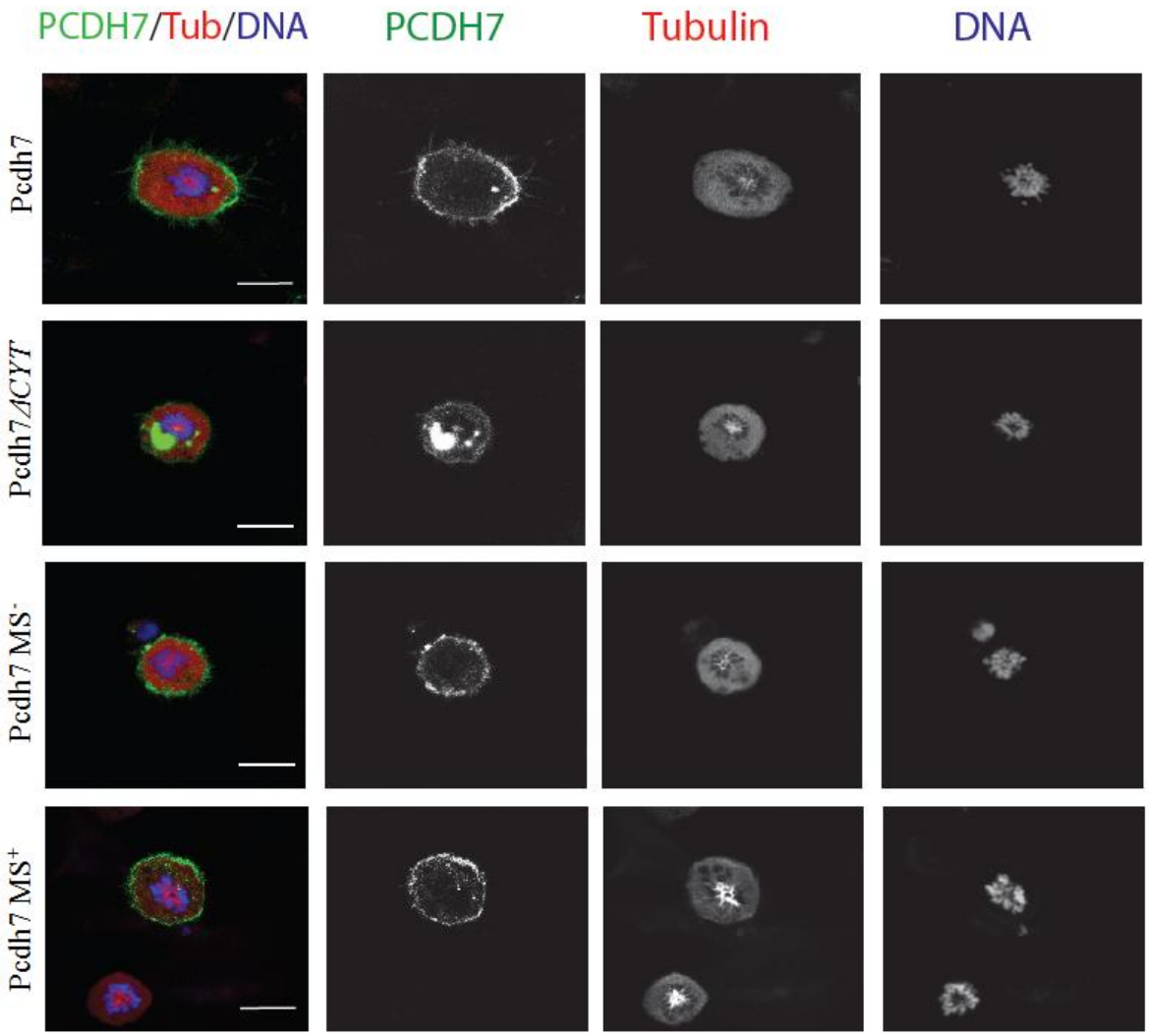

Figure 4.6: Localization of Pcdh7, Pcdh7 *ΔCYT*, Pcdh7 MS$^-$, and Pcdh7 MS$^+$ in U2OS cells during mitosis. The native and mutant versions of Pcdh7 are fused to GFP (green). Tubulin is fused to mCherry (red). DNA is stained with DAPI (blue). (Courtesy of Selda Bülbül [66])

4.2 Identification of Intracellular Interaction Partners of Protocadherin 7

4.2.1 Protein Expression and Purification, Pull-Down Assay, and Mass Spectrometry

Transmembrane proteins interact with several intracellular proteins via their cytoplasmic domain. These interactions may regulate activity of transmembrane protein or they may induce intracellular signaling pathways. In both scenarios, it is important to identify interaction partners of transmembrane proteins because it yields information about function of the protein of interest.

We cloned the cytoplasmic domain of Pcdh7 (901-1072 amino acids) to pGEX-6P-1 vector and transformed *E. coli* BL21 bacteria strain with this plasmid. In liquid culture, IPTG (Isopropyl β-D-1-thiogalactopyranoside) was used to induce Ptac promoter in order to express GST (Glutathione S-transferase; 26 kDa) fused protein. Expressed protein accumulates in cytoplasm of bacteria. Hence, bacteria were lysed with help of lysis buffer and brief sonication. GST fused protein was purified from total protein preparation by affinity chromatography using glutathione beads. After several wash steps, target protein was eluted from beads by elution buffer which has reduced glutathione. Purified protein was dialyzed to PBS with 10% glycerol. Protein concentration was measured with colorimetric BCA (bicinchoninic acid) assay. Success of protein expression and purification experiments was confirmed by SDS-PAGE in comparison to controls. We also expressed and purified GST-tagged GFP to use it as negative control in pull-down assay.

For the pull-down assay, total protein preparation of lysed HeLa cells were incubated with 60 µg GST-PCDH7cyt or GST-GFP at 4 °C overnight. Next day, affinity chromatography with glutathione beads was used to collect GST tagged protein with any other interacting proteins bound. Wash steps were performed to discard any non-specific protein in the collect. Captured proteins were heated up to 90 °C in Laemmli sample buffer with DTT. Samples were run in precast 12% SDS-PAGE and stained with PageBlue Protein Staining

Solution. After the gel was fractionated, in-gel tryptic digestion was performed to produce peptides. Peptide purification (including desalting) and concentration was done with peptide cleanup C18 StageTips. Peptide analysis was performed by LC-MS/MS on Orbitrap based mass spectrometer. Peptides were identified by Proteome Discover software.

We repeated the pull-down assay and mass spectrometry analysis three times. 9 proteins were identified in at least two of the replicates and Table 4.2 presents coverage values for each replicate, and overall coverage for each protein. Table 4.3 summarizes functions of these proteins.

Some of the identified proteins are unrelated to Pcdh7 as their functions are distinct from function of Pcdh7. For example, Histone H2B type 2-E works as a constituent of nucleosome complex in nucleus. However, localization of Pcdh7 is limited to cytoplasm and cell membrane. Hence, it is not possible for Pcdh7 to interact with any histone proteins *in vivo*.

On the other hand, three of the identified proteins (Signal recognition particle 14 kDa protein, 78 kDa glucose-regulated protein, Peptidyl-prolyl cis-trans isomerase B) have the probability to interact with Pcdh7 since they are molecular chaperones. However, none of them reveals any specific information about function and/or regulation of Pcdh7.

Protein	Molecular mass (kDa)	ΣCoverage of Exp. 1	ΣCoverage of Exp. 2	ΣCoverage of Exp. 3	Total ΣCoverage
Signal recognition particle 14 kDa protein	14.6	21.32	NA	21.32	**42.64**
78 kDa glucose-regulated protein	72.3	21.71	9.17	NA	**30.88**
Elongation factor 2	95.3	17.48	7.46	5.48	**30.42**
Peptidyl-prolyl cis-trans isomerase B	23.7	19.91	NA	10.19	**30.1**
Histone H2B type 2-E	13.9	7.14	NA	17.46	**24.6**
C-1-tetrahydrofolate synthase, cytoplasmic	107.4	10.90	NA	8.38	**19.28**
Pyruvate kinase	40.2	2.19	16.81	NA	**19**
elongation factor Tu, mitochondrial precursor	49.8	2.20	NA	16.04	**18.24**
Nuclease sensitive element binding protein-1	34.7	11.18	NA	5.90	**17.08**

Table 4.2: Possible intracellular binding partners of Pcdh7. For each experiment, identified proteins of negative control (GST-GFP) were subtracted from results of sample (GST-PCDH7cyt). The experiment was repeated three times. Only the results that are confirmed by at least two of the three experiments were covered in this table.

Protein Name	Protein Function
Signal recognition particle 14 kDa protein	targets secretory proteins to the rough endoplasmic reticulum membrane
78 kDa glucose-regulated protein	facilitates the assembly of multimeric protein complexes inside the ER.
Elongation factor 2	catalyzes the GTP-dependent ribosomal translocation step during translation elongation
Peptidyl-prolyl cis-trans isomerase B	accelerates the folding of proteins
Histone H2B type 2-E	core component of nucleosome
C-1-tetrahydrofolate synthase, cytoplasmic	one-carbon metabolism
Pyruvate kinase	glycolysis
elongation factor Tu, mitochondrial precursor	promotes binding of aminoacyl-tRNA to the A-site of ribosomes during translation
Nuclease sensitive element binding protein-1	mediates pre-mRNA alternative splicing regulation

Table 4.3: Functions of the identified interacting partners. The source is UniProt. Only three of the identified proteins (underlined in the table) may be interacting with Pcdh7 if we consider the functions of these proteins and Pcdh7.

4.2.2 Identification with BioID Method

We employed an alternative strategy for identification of the interaction partners of Pcdh7. BioID (Biotin Identification) system is the basis of this strategy. Protein of interest is fused to a promiscuous *Escherichia coli* biotin protein ligase enzyme (BirA*). This enzyme has a

point mutation (R118G) which weakens its substrate selectivity. As a result, the mutated enzyme targets any protein in proximity. When fused to a target protein, the enzyme tags every protein in proximity with biotin. If tagged proteins are captured with affinity purification and analyzed by mass spectrometry, interaction partners of the target protein can be identified [67] (Figure 4.7).

First, we cloned Pcdh7 and Pcdh7 *ΔCYT* (cytoplasmic domain deleted mutant) to pcDNA3.1 mycBioID vector (mycBirA-R118G-MCS). Then, the fusion constructs (mycBirA*-Insert) and control (mycBirA*) were cloned to pENTR1A vector with blunt-end cloning. After that, fusion constructs and control construct were transferred to pLenti CMV Hygro DEST vector by Gateway Cloning technology. pLenti clones were used in lentiviral transduction of HeLa cells to produce stable cell lines of each sample and the control. Biotin was added into cell cultures so that the fusion protein of each sample selectively biotinylated proximal proteins of it. After incubation with biotin, the cells were lysed and biotinylated proteins were collected by streptavidin-conjugated beads. Finally, collected proteins were prepared for and analyzed by LC-MS/MS mass spectrometry (liquid chromatography-tandem mass spectrometry).

As a result, we could identify 297 proteins for full length Pcdh7, 58 proteins for cytoplasmic domain deleted Pcdh7, 320 proteins for mycBirA*, and 16 proteins for biotin-free control. We subtracted the results of Pcdh7 *ΔCYT* and mycBirA* (negative control) from the results of Pcdh7 to pin down interaction partners specific to cytoplasmic domain of Pcdh7. We set 10% coverage set off to increase validity of this result. The 17 cytoplasmic interaction candidates which satisfy this search are presented in Table 4.4 with their molecular mass and coverage ratings. Information about their cellular locations and functions retrieved from UniProt database is shown in Table 4.5.

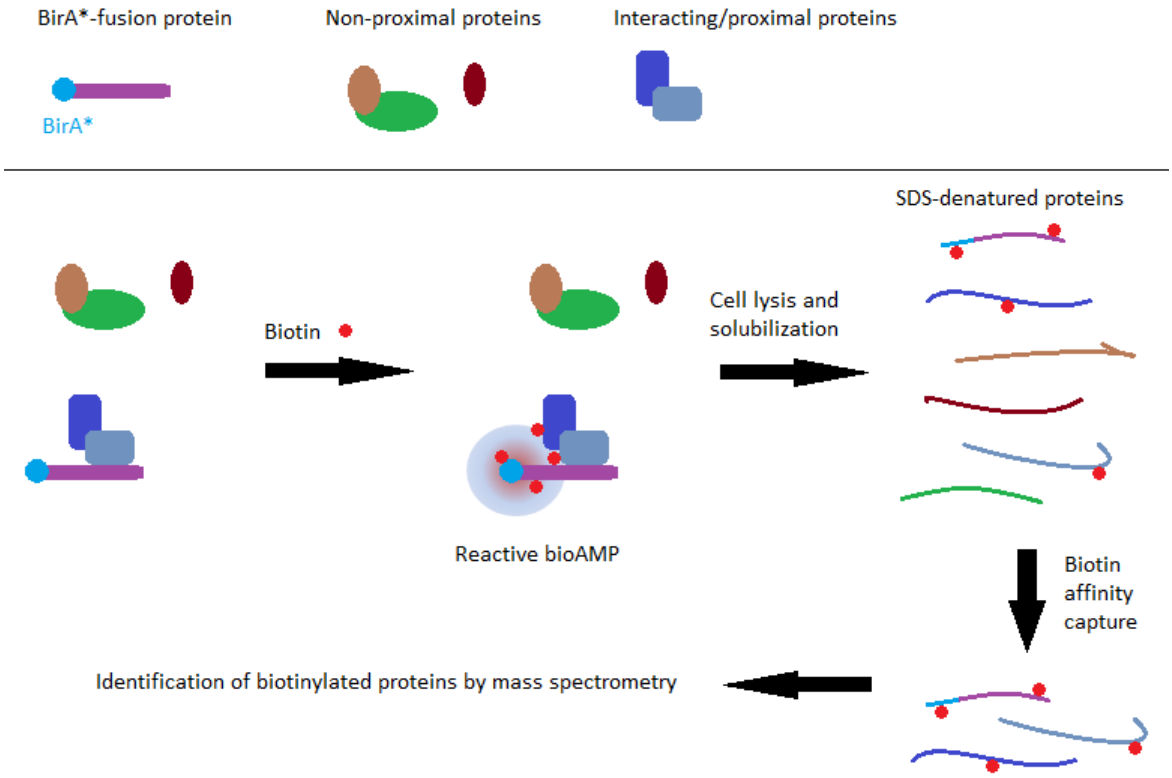

Figure 4.7: Application of BioID method. BirA*-fused protein biotinylates proximal proteins in the cell. Labeled proteins are purified from cell lysate by biotin affinity chromatography and then identified by mass spectrometry. (Adapted from Kyle J. Roux *et al.* 2012 [67])

Some of the resulted proteins are false positive because they have functions unrelated to Pcdh7 to our current knowledge. Their cellular localization can be a partial restriction to assess their validity. For example, a candidate with nuclear localization may still have interaction with Pcdh7 in cytoplasm as some of nuclear proteins (such as some transcription factors) shuttle from cytoplasm to nucleus after activation.

Among the most notable candidates, transcription factor HES-5 is under control of Notch signaling and it affects cell adhesion. Interestingly, it is abundant in fatal brain and heart tumors and Pcdh7 has the highest expression levels in these organs (see Title 2.5). Capping protein (Actin filament) muscle Z-line and Ezrin may also be significant as these proteins regulate cell shape and Pcdh7 may have a role in rounding of cells in mitosis. 14-3-3 protein zeta/delta and Isoform Short of 14-3-3 protein beta/alpha are also interesting because these adaptor proteins have role in regulation of a wide variety of signaling pathways including CDK16, BAX, RAF1, ABL1, and AKT1.

We also wanted to compare results of Pcdh7 and Pcdh7 *ΔCYT* based on localization of the identified proteins. Results of biotin-free control were subtracted from each of the sample group results and remaining proteins were analyzed by Gofact (Gene Ontology) online program to identify percentages of proteins in each cellular component. Percentages of proteins located to plasma membrane and cytoplasm are compared for Pcdh7 and Pcdh7 *ΔCYT* in the graphic presented as Figure 4.8. As expected, labeled cytoplasmic protein number is comparably less for Pcdh7 *ΔCYT* than it is for the full length protein while it is almost the same for membrane proteins. Note that cytoplasmic proteins can be labeled by mycBirA-Pcdh7 *ΔCYT* as this fusion protein has the same cellular route as the full length and is also presented in cytoplasm (though with less amount compared to the full length protein).

Protein Name	Molecular Mass (kDa)	ΣCoverage
Similar to Protein LDOC1	11.0	25.51
high mobility group nucleosome-binding domain-containing protein 3 isoform HMGN3d	13.9	19.23
CIP29 protein	16.8	18.67
Transcription factor HES-5	18.2	17.47
Capping protein (Actin filament) muscle Z-line, beta	13.6	16.53
cDNA FLJ59939, highly similar to Protein disulfide-isomerase	24.5	16.14
cDNA FLJ53048, highly similar to Phosphoglycerate mutase 1	16.1	15.28
14-3-3 protein zeta/delta	27.7	15.10
cDNA FLJ53078, highly similar to Splicing factor, arginine/serine-rich 1	16.2	13.99
Isoform 1 of Calcyclin-binding protein	26.2	13.60
Growth arrest and DNA damage-inducible proteins-interacting protein 1	25.4	12.61
Isoform 2 of U2 snRNP-associated SURP motif-containing protein	118.2	12.45
Isoform 2 of DAZ-associated protein 1	40.5	11.90
Isoform Short of 14-3-3 protein beta/alpha	27.8	11.89
Isoform SRP55-1 of Serine/arginine-rich splicing factor 6	39.6	11.63
Ezrin	69.4	11.60
cDNA FLJ59206, highly similar to Eukaryotic translation initiation factor 4B	66.1	10.56

Table 4.4: Possible intracellular interaction partners of Pcdh7 detected by BioID method. Proteins which were exclusively detected for only Pcdh7 full length were subjected to 10% coverage limit to produce this table. The proteins are listed from top to bottom with decreasing coverage ratings.

Protein Name	Cellular Location	Function	Notes
Similar to Protein LDOC1	Nucleus	Negative regulation of cell proliferation	1. Leucine zipper protein down-regulated in cancer cells
high mobility group nucleosome-binding domain-containing protein 3 isoform HMGN3d	Nucleus	Chromatin modification	1. Interacts with the ligand binding domain of the thyroid receptor (TR) (in vitro).
CIP29 protein	Nucleus	?	1. SAP domain-containing ribonucleoprotein
Transcription factor HES-5	Nucleus	Transcriptional regulation	1. Expressed in fetal heart and brain tumors. 2. Notch signaling pathway 3. Cell adhesion
Capping protein (Actin filament) muscle Z-line, beta	Cytoplasm	Actin cytoskeleton organization	-
cDNA FLJ59939, highly similar to Protein disulfide-isomerase	?	Cell redox homeostasis	-
cDNA FLJ53048, highly similar to Phosphoglycerate mutase 1	Cytoplasm	Glycolysis	-
14-3-3 protein zeta/delta	Cytoplasm	Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways	1. Interacts with CDK16, BAX, RAF1, ABL1, AKT1 and some other proteins
cDNA FLJ53078, highly similar to Splicing factor, arginine/serine-rich 1	?	Nucleic acid binding	-
Isoform 1 of Calcyclin-binding protein	Cytoplasm and nucleus	calcium-dependent ubiquitination and subsequent proteasomal degradation of target proteins	1. Cardiac muscle cell differentiation

Growth arrest and DNA damage-inducible proteins-interacting protein 1	Mitochondrion and nucleus	Acts as a negative regulator of G1 to S cell cycle phase progression by inhibiting cyclin-dependent kinases	-
Isoform 2 of U2 snRNP-associated SURP motif-containing protein	Nucleus	Alternative splicing	-
Isoform 2 of DAZ-associated protein 1	Cytoplasm and nucleus	RNA-binding protein, which may be required during spermatogenesis	-
Isoform Short of 14-3-3 protein beta/alpha	Cytoplasm	Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways	1. Reactome: MAPK cascade, Ras signaling, EGFR signaling, cellular membrane organization
Isoform SRP55-1 of Serine/arginine-rich splicing factor 6	Nucleus	Alternative splicing	-
Ezrin	Cell membrane and cytoplasm	Probably involved in connections of major cytoskeletal structures to the plasma membrane	1. Regulation of cell shape
cDNA FLJ59206, highly similar to Eukaryotic translation initiation factor 4B	?	Protein biosynthesis	-

Table 4.5: Cellular locations and functions of the possible intracellular binding partners of Pcdh7. Information about cellular localization and functions of the proteins presented in Table 4.4 is listed here. Source is UniProt database.

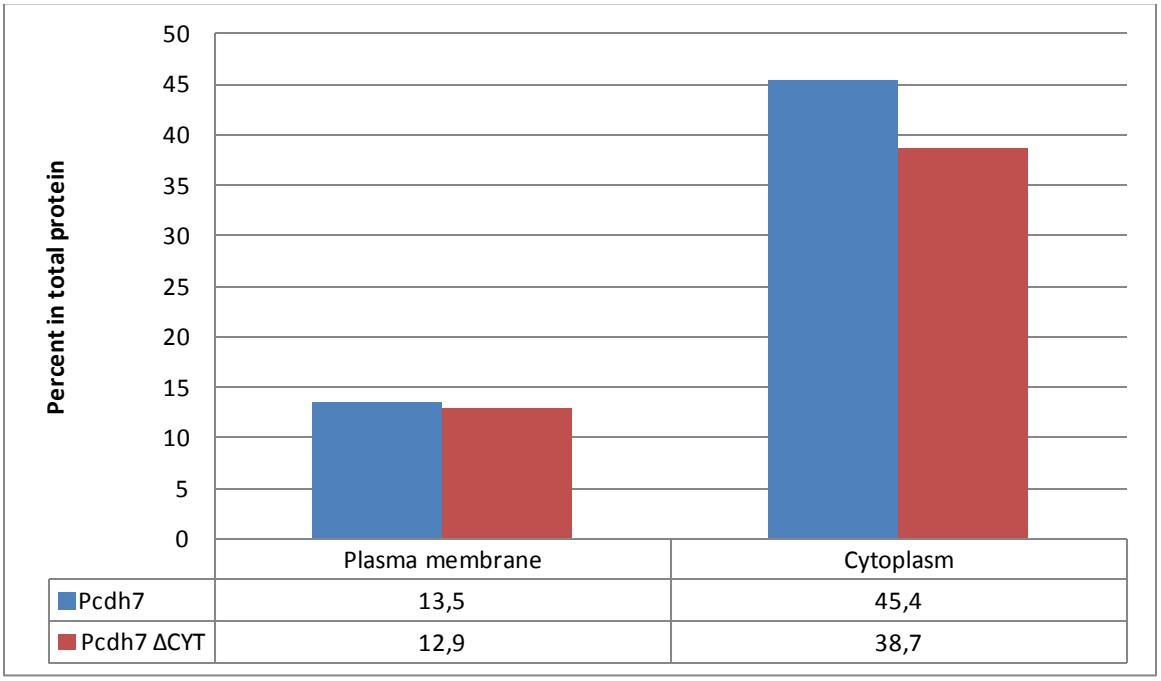

Figure 4.8: Comparison of identified proteins based on their cellular location. Percentages of membrane proteins are almost the same whereas Pcdh7 has more cytoplasmic interaction partners than Pcdh7 *ΔCYT*.

4.3 Functional Analysis of Pcdh7

4.3.1 Bead Aggregation Assay for Adhesion Activity

Although classical cadherins establish cell-cell adhesion, protocadherins either do not have adhesion function or they mediate weak adhesion. Since Pcdh7 localizes to cell membrane in mitosis when the cell has weakest adhesion to its surrounding, we suspected that Pcdh7 may have a de-adhesion function in fact. To test role of Pcdh7 in adhesion, we employed a simple *in vitro* assay which is called bead aggregation assay.

Extracellular domain (EC) of Pcdh7 (241-862 amino acids) was cloned as a fusion of GST tag and the protein was expressed and purified as it is explained for cytoplasmic domain above (Figure 4.9). HeLa cell culture media was collected and filtered to be used as a medium of the experiment. 30 µl glutathione bead and 120 µg of purified protein (GST-PCDH7ec as the sample or GST-GFP as the negative control) were incubated in 500 µl filtered HeLa media at 4 °C for 2 hours. Then, beads were washed with PBS (+BSA) solution and they were added onto depression glass slides in the same solution. 2mM $CaCl_2$ (cadherin proteins need Ca^{2+} ions for adhesive effect) or EDTA (EDTA chelates Ca^{2+} ions) was added to the bead suspensions. The beads were incubated at 37 °C for 1 hour and then their images were taken by JuLi Smart Cell Imager.

Bead aggregates formed after incubation with differing degrees for each condition (Figure 4.10 and 4.11). As expected, bead aggregation level was higher when Ca^{2+} ions were present in the medium than when they were absent. Existence of a protein (PCDH7ec or GFP) bound to the beads increased aggregation compared to only beads condition. However, PCDH7ec had slightly decreased aggregation level compared to negative control GFP (see Ca post condition). Indeed, Pcdh7ec should have caused more aggregates than the negative control if it has adhesive function. Therefore, this result may indicate de-adhesive

function of the protein. Another scenario is that Pcdh7 works as a heteromultimeric complex and absence of its partner(s) interfered the results.

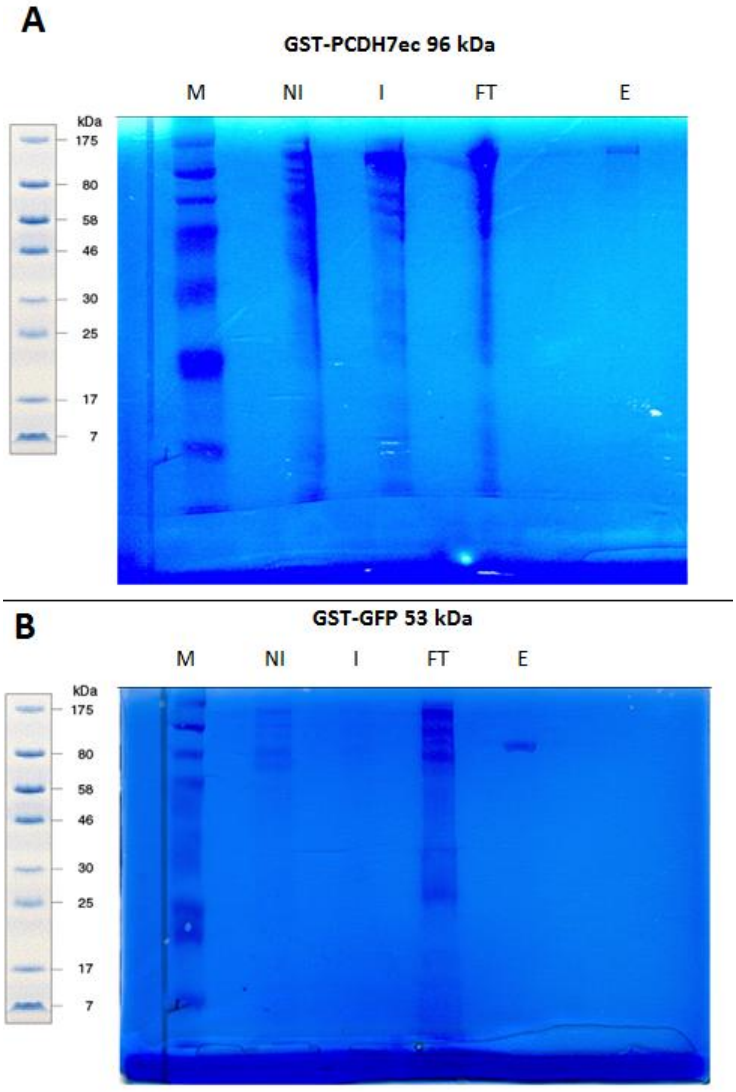

Figure 4.9: SDS-PAGE analysis of protein expression and purification. [GST-PCDH7ec (A) and GST-GFP (B)] M: Marker; NI: Non-induced (before IPTG induction); I: Induced (after IPTG induction); FT: Flow-through (unbound proteins); E: Elute (purified protein)

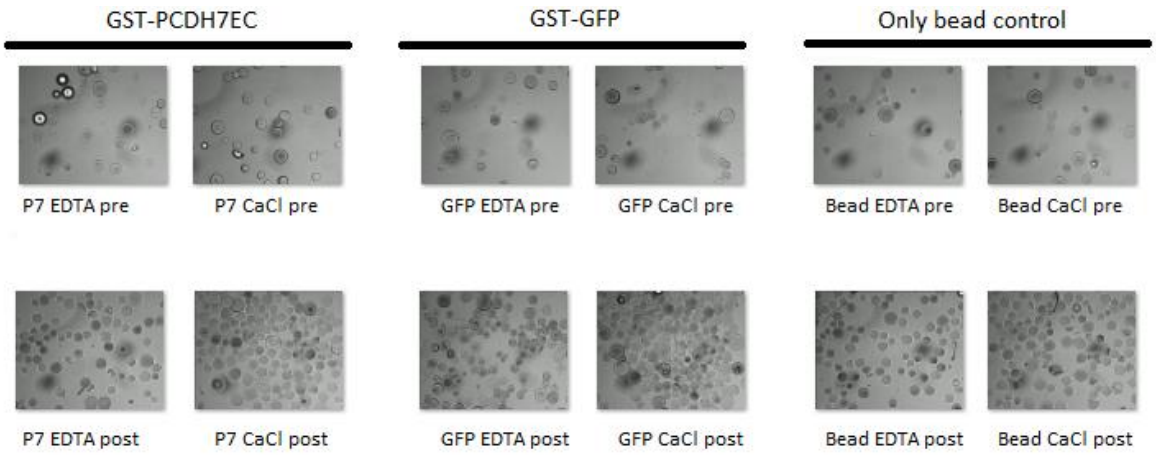

Figure 4.10: Bead aggregation assay. Each panel shows the images for different proteins (left: PCDH7ec; central: negative control, GFP; right: only bead control where there is no protein added). The top line shows the images taken before the incubation (pre) and the images at the bottom line show after incubation (post). The left row of each panel represents the condition for absence of Ca^{2+} ions (chelated by EDTA) whereas the right row is for the presence of Ca^{2+} ions.

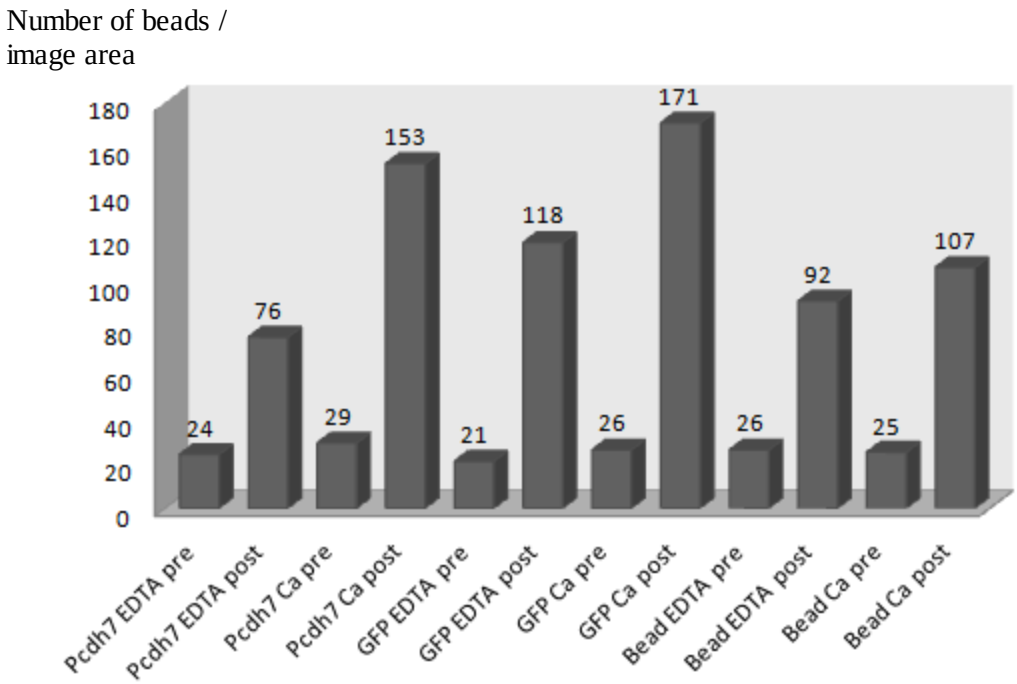

Figure 4.11: Graphical comparison of the bead aggregation assay results. Presence of Ca^{2+} ions increases bead aggregation. Existence of a protein also increases aggregation. However, PCDH7ec caused less aggregates than the negative control GFP. Read the text and the Discussion chapter for explanation of this result. Numbers of beads in each image area were counted by ImageJ software (Plugins > Analyze > Cell Counter) (ImageJ 1.46r by Wayne Rasband, National Institutes of Health, USA).

Chapter 5

DISCUSSION

The cell undergoes dramatic changes through the cell cycle phases. Although cell cycle dependent intracellular changes have been studied comprehensively, we know little about the changes on the cell surface. Cell membrane has several important roles which include cell adhesion, cell migration, cell communication, and material transport into and out of the cell. Cell surface biochemistry and spatiotemporal organization of its components are also dynamic through the cell cycle. Most notably, a cell's adhesion to the extracellular matrix and neighbor cells weakens in mitosis and cell shape becomes rounded. A synchronous reorganization of cell surface proteins and the cytoskeleton enables this dramatic morphological alteration. Protocadherins, the biggest subfamily of cadherins, are thought to be involved in cell adhesion and cell recognition and they are not exempt from this dynamism of the plasma membrane. However, protocadherins had been in the shadow of cadherins until recently.

In this study, we focused on a member of protocadherin subfamily which is Pcdh7. The current knowledge about Pcdh7 is very limited. However, it is proposed that this protein is important for cell adhesion and cell identity (especially for neurons) and it is thought to have a link with tumor formation. But, neither its exact function nor its mechanism of action is known.

We cloned human Pcdh7 and used fluorescent imaging to track its localization. We were able to show that Pcdh7 has intracellular localization in interphase and it moves form

endoplasmic reticulum to plasma membrane when the cell starts dividing (Figure 5.1). Together with our unpublished mass spectrometry based proteomics data, we revealed cell cycle dependent localization of Pcdh7 for the first time in the literature.

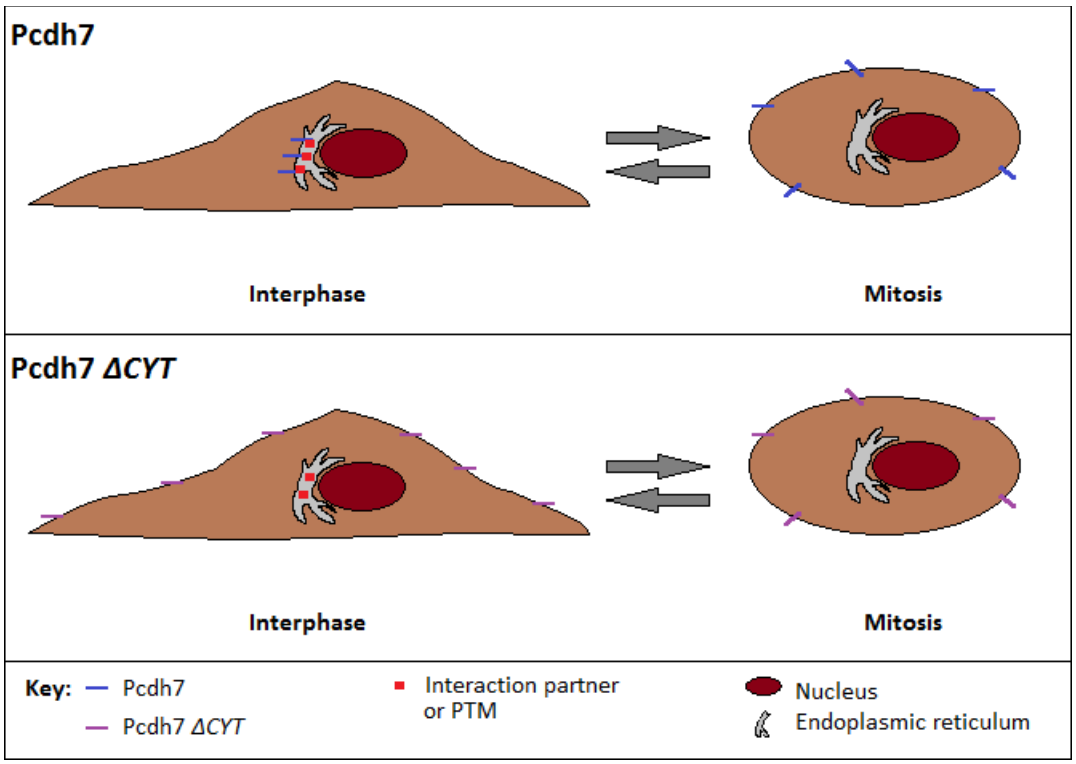

Figure 5.1: Cell cycle dependent localization of Pcdh7. Pcdh7 stays in ER in interphase and locates to cell surface during mitosis. Reversal binding of an interaction partner to the cytoplasmic domain or modification of residues in the cytoplasmic domain directs this spatiotemporal regulation.

We also studied role of the cytoplasmic domain of Pcdh7 on regulation of its localization. We cloned a mutant (Pcdh7 *ΔCYT*) which lacks the entire cytoplasmic domain and analyzed it in mitotic and interphase cells which were transfected to express this construct. Fluorescent microscopy analysis showed that Pcdh7 *ΔCYT* has a static localization and it is always located on the cell membrane, regardless of the cell cycle phases. This result supports the notion that cytoplasmic domain of Pcdh7 is necessary for proper regulation of the protein's localization.

Then, we wanted to pin down the important residues on the cytoplasmic domain and created two mutant versions of the protein to assess a region which was identified by bioinformatic approach to have at least four amino acids which are targets of CDKs. However, both of these mutants (MS^- and MS^+) behaved the same way as of Pcdh7 *ΔCYT*. We propose that post-translational modifications of Pcdh7 can be mapped by mass spectrometry. If Pcdh7 proteins of mitotic and interphase cells are compared by this method, any modification on the cytoplasmic domain which regulates the protein in a cell cycle dependent manner will be identified.

Proteins can also be regulated by their binding partners. Interaction partners are also important to reveal function of a protein. To identify interaction partners of Pcdh7, we employed two approaches. For pull-down assay, we expressed and purified the cytoplasmic domain fused to GST and used it to collect interacting proteins from cell lysates. The other method includes usage of a promiscuous biotin ligase fusion protein to label proximal proteins in cell. Then, labeled proteins were purified by biotin-streptavidin affinity chromatography. For both of the methods, collected proteins were identified by LC-MS/MS. We identified several interaction partner candidates by these two methods. Importantly, some of these proteins are chaperone proteins to aid their partners in ER. Note that, we showed that Pcdh7 localizes to ER in interphase. Strikingly, other candidates include transcription factors and adaptor proteins which are related to cell adhesion,

regulation of cell shape, important signaling pathways and cancer. However, we approach these results prudently and suggest more repeats before a conclusion. Nevermore, the results of these two approaches overlap to some degree, which affirms validity of our experiments.

Finally, we aimed to demonstrate possible adhesion effect of Pcdh7. We expressed and purified GST tagged extracellular domain of Pcdh7 and used it in bead aggregation assay. Aggregate formation was similar among the sample (Pcdh7) and the negative controls (GFP and only bead control). This is evidence that Pcdh7 does not have homophilic adhesion activity. Either Pcdh7 forms heteromultimeric complex with other protocadherins or cadherins for adhesive function or it does not have any role in adhesion. Actually, Pcdh7 may have de-adhesive effect since it localizes to cell membrane when a cell has weakest adhesion. This possibility is also consistent with correlation between Pcdh7 and increased metastatic activity (see Title 2.5).

The work presented in this thesis builds a base for future work on Pcdh7. In a wider perspective, it includes novel findings about a member of protocadherin family, which is the biggest subgroup of cadherins but not well studied yet. Importantly, this study has potential to be used in clinic medicine as Pcdh7 can be used as mitotic marker in cancer diagnosis, prognosis and even treatment as a drug target.

BIBLIOGRAPHY

1. Tan, S.Y. and J. Brown, *Rudolph Virchow (1821-1902): "pope of pathology".* Singapore Med J, 2006. **47**(7): p. 567-8.
2. Nurhan Özlü, S.A., Gürkan Mollaoglu, Bernhard Y. Renard, Edwin Tang, Ina Poser, Wiebke Timm, Timothy Mitchison, Judith Steen, *Quantitative comparison of the Human Cancer Cell-Surface Proteome between Interphase and Mitosis*. 2013.
3. Alberts, B., J.H. Wilson, and T. Hunt, *Molecular biology of the cell*. 5th ed. 2008, New York: Garland Science. xxxiii, 1601, 90 p.
4. Williams, G.H. and K. Stoeber, *The cell cycle and cancer.* J Pathol, 2012. **226**(2): p. 352-64.
5. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
6. Mehta, D. and A.B. Malik, *Signaling mechanisms regulating endothelial permeability.* Physiol Rev, 2006. **86**(1): p. 279-367.
7. Lauffenburger, D.A. and A.F. Horwitz, *Cell migration: a physically integrated molecular process.* Cell, 1996. **84**(3): p. 359-69.
8. Cramer, L.P., *Forming the cell rear first: breaking cell symmetry to trigger directed cell migration.* Nat Cell Biol, 2010. **12**(7): p. 628-32.
9. Howell, A.S. and D.J. Lew, *Morphogenesis and the cell cycle.* Genetics, 2012. **190**(1): p. 51-77.
10. Cai, L. and B.P. Tu, *Driving the cell cycle through metabolism.* Annu Rev Cell Dev Biol, 2012. **28**: p. 59-87.
11. Kronja, I. and T.L. Orr-Weaver, *Translational regulation of the cell cycle: when, where, how and why?* Philos Trans R Soc Lond B Biol Sci, 2011. **366**(1584): p. 3638-52.
12. Fisher, D., et al., *Phosphorylation network dynamics in the control of cell cycle transitions.* J Cell Sci, 2012. **125**(Pt 20): p. 4703-11.
13. Olsen, J.V., et al., *Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis.* Sci Signal, 2010. **3**(104): p. ra3.
14. Stein, S.M. and J.M. Berestecky, *Inhibition of growth by masking of arginine moieties in protein at the cell surface.* Cancer Res, 1974. **34**(11): p. 3112-6.
15. McCusker, D. and D.R. Kellogg, *Plasma membrane growth during the cell cycle: unsolved mysteries and recent progress.* Curr Opin Cell Biol, 2012. **24**(6): p. 845-51.
16. Boucrot, E. and T. Kirchhausen, *Endosomal recycling controls plasma membrane area during mitosis.* Proc Natl Acad Sci U S A, 2007. **104**(19): p. 7939-44.
17. Dao, V.T., et al., *Dynamic changes in Rap1 activity are required for cell retraction and spreading during mitosis.* J Cell Sci, 2009. **122**(Pt 16): p. 2996-3004.

18. Rosenblatt, J., *Mitosis: moesin and the importance of being round.* Curr Biol, 2008. **18**(7): p. R292-3.
19. Stewart, M.P., et al., *Hydrostatic pressure and the actomyosin cortex drive mitotic cell rounding.* Nature, 2011. **469**(7329): p. 226-30.
20. Jinguji, Y. and H. Ishikawa, *Electron microscopic observations on the maintenance of the tight junction during cell division in the epithelium of the mouse small intestine.* Cell Struct Funct, 1992. **17**(1): p. 27-37.
21. Okayama, H., *Cell cycle control by anchorage signaling.* Cell Signal, 2012. **24**(8): p. 1599-609.
22. Matthews, H.K. and B. Baum, *The metastatic cancer cell cortex: an adaptation to enhance robust cell division in novel environments?* Bioessays, 2012. **34**(12): p. 1017-20.
23. Chappuis-Flament, S., et al., *Multiple cadherin extracellular repeats mediate homophilic binding and adhesion.* J Cell Biol, 2001. **154**(1): p. 231-43.
24. Morishita, H. and T. Yagi, *Protocadherin family: diversity, structure, and function.* Curr Opin Cell Biol, 2007. **19**(5): p. 584-92.
25. Sano, K., et al., *Protocadherins: a large family of cadherin-related molecules in central nervous system.* EMBO J, 1993. **12**(6): p. 2249-56.
26. Morishita, H., et al., *Structure of the cadherin-related neuronal receptor/protocadherin-alpha first extracellular cadherin domain reveals diversity across cadherin families.* J Biol Chem, 2006. **281**(44): p. 33650-63.
27. Mutoh, T., et al., *Cadherin-related neuronal receptor 1 (CNR1) has cell adhesion activity with beta1 integrin mediated through the RGD site of CNR1.* Exp Cell Res, 2004. **294**(2): p. 494-508.
28. Triana-Baltzer, G.B. and M. Blank, *Cytoplasmic domain of protocadherin-alpha enhances homophilic interactions and recognizes cytoskeletal elements.* J Neurobiol, 2006. **66**(4): p. 393-407.
29. Hambsch, B., et al., *{gamma}-Protocadherins, presenilin-mediated release of C-terminal fragment promotes locus expression.* J Biol Chem, 2005. **280**(16): p. 15888-97.
30. Murata, Y., et al., *Interaction with protocadherin-gamma regulates the cell surface expression of protocadherin-alpha.* J Biol Chem, 2004. **279**(47): p. 49508-16.
31. Sago, H., et al., *Cloning, expression, and chromosomal localization of a novel cadherin-related protein, protocadherin-3.* Genomics, 1995. **29**(3): p. 631-40.
32. Obata, S., et al., *Protocadherin Pcdh2 shows properties similar to, but distinct from, those of classical cadherins.* J Cell Sci, 1995. **108 (Pt 12)**: p. 3765-73.
33. Frank, M., et al., *Differential expression of individual gamma-protocadherins during mouse brain development.* Mol Cell Neurosci, 2005. **29**(4): p. 603-16.
34. Reiss, K., et al., *Regulated ADAM10-dependent ectodomain shedding of gamma-protocadherin C3 modulates cell-cell adhesion.* J Biol Chem, 2006. **281**(31): p. 21735-44.
35. Yamagata, K., et al., *Arcadlin is a neural activity-regulated cadherin involved in long term potentiation.* J Biol Chem, 1999. **274**(27): p. 19473-1979.

36. Hirano, S., Q. Yan, and S.T. Suzuki, *Expression of a novel protocadherin, OL-protocadherin, in a subset of functional systems of the developing mouse brain.* J Neurosci, 1999. **19**(3): p. 995-1005.
37. Emond, M.R., et al., *A complex of Protocadherin-19 and N-cadherin mediates a novel mechanism of cell adhesion.* J Cell Biol, 2011. **195**(7): p. 1115-21.
38. Chen, X. and B.M. Gumbiner, *Crosstalk between different adhesion molecules.* Curr Opin Cell Biol, 2006. **18**(5): p. 572-8.
39. Chen, X., et al., *A protocadherin-cadherin-FLRT3 complex controls cell adhesion and morphogenesis.* PLoS One, 2009. **4**(12): p. e8411.
40. Haas, I.G., et al., *Presenilin-dependent processing and nuclear function of gamma-protocadherins.* J Biol Chem, 2005. **280**(10): p. 9313-9.
41. Bonn, S., P.H. Seeburg, and M.K. Schwarz, *Combinatorial expression of alpha- and gamma-protocadherins alters their presenilin-dependent processing.* Mol Cell Biol, 2007. **27**(11): p. 4121-32.
42. Kaneko, R., et al., *Allelic gene regulation of Pcdh-alpha and Pcdh-gamma clusters involving both monoallelic and biallelic expression in single Purkinje cells.* J Biol Chem, 2006. **281**(41): p. 30551-60.
43. Redies, C., K. Vanhalst, and F. Roy, *delta-Protocadherins: unique structures and functions.* Cell Mol Life Sci, 2005. **62**(23): p. 2840-52.
44. Kirov, G., et al., *Variation in the protocadherin gamma A gene cluster.* Genomics, 2003. **82**(4): p. 433-40.
45. Noonan, J.P., et al., *Extensive linkage disequilibrium, a common 16.7-kilobase deletion, and evidence of balancing selection in the human protocadherin alpha cluster.* Am J Hum Genet, 2003. **72**(3): p. 621-35.
46. Miki, R., et al., *Identification and characterization of coding single-nucleotide polymorphisms within human protocadherin-alpha and -beta gene clusters.* Gene, 2005. **349**: p. 1-14.
47. Esumi, S., et al., *Monoallelic yet combinatorial expression of variable exons of the protocadherin-alpha gene cluster in single neurons.* Nat Genet, 2005. **37**(2): p. 171-6.
48. Kim, S.Y., et al., *The expression of non-clustered protocadherins in adult rat hippocampal formation and the connecting brain regions.* Neuroscience, 2010. **170**(1): p. 189-99.
49. Suo, L., et al., *Protocadherin clusters and cell adhesion kinase regulate dendrite complexity through Rho GTPase.* J Mol Cell Biol, 2012. **4**(6): p. 362-76.
50. Schreiner, D. and J.A. Weiner, *Combinatorial homophilic interaction between gamma-protocadherin multimers greatly expands the molecular diversity of cell adhesion.* Proc Natl Acad Sci U S A, 2010. **107**(33): p. 14893-8.
51. Ying, J., et al., *Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation.* Oncogene, 2006. **25**(7): p. 1070-80.

52. Wang, C., et al., *Downregulation of PCDH9 predicts prognosis for patients with glioma.* J Clin Neurosci, 2012. **19**(4): p. 541-5.
53. Yoshida, K., et al., *Cloning, expression analysis, and chromosomal localization of BH-protocadherin (PCDH7), a novel member of the cadherin superfamily.* Genomics, 1998. **49**(3): p. 458-61.
54. Yoshida, K., et al., *BH-protocadherin-c, a member of the cadherin superfamily, interacts with protein phosphatase 1 alpha through its intracellular domain.* FEBS Lett, 1999. **460**(1): p. 93-8.
55. Kim, S.Y., et al., *Non-clustered protocadherin.* Cell Adh Migr, 2011. **5**(2): p. 97-105.
56. Yoshida, K., *Fibroblast cell shape and adhesion in vitro is altered by overexpression of the 7a and 7b isoforms of protocadherin 7, but not the 7c isoform.* Cell Mol Biol Lett, 2003. **8**(3): p. 735-41.
57. Berx, G. and F. van Roy, *Involvement of members of the cadherin superfamily in cancer.* Cold Spring Harb Perspect Biol, 2009. **1**(6): p. a003129.
58. Miyake, K., et al., *The protocadherins, PCDHB1 and PCDH7, are regulated by MeCP2 in neuronal cells and brain tissues: implication for pathogenesis of Rett syndrome.* BMC Neurosci, 2011. **12**: p. 81.
59. Huang, Y.T., et al., *Genome-wide analysis of survival in early-stage non-small-cell lung cancer.* J Clin Oncol, 2009. **27**(16): p. 2660-7.
60. Zhao, H. *Cell mechanics-cytoskeleton-membrane protein transduction loop mediates brain metastasis of breast cancer cells [abstract].* in *the 102nd Annual Meeting of the American Association for Cancer Research*. 2011. Orlando, FL: Cancer Research.
61. Bradley, R.S., A. Espeseth, and C. Kintner, *NF-protocadherin, a novel member of the cadherin superfamily, is required for Xenopus ectodermal differentiation.* Curr Biol, 1998. **8**(6): p. 325-34.
62. Rashid, D., et al., *A requirement for NF-protocadherin and TAF1/Set in cell adhesion and neural tube formation.* Dev Biol, 2006. **291**(1): p. 170-81.
63. Piper, M., et al., *NF-protocadherin and TAF1 regulate retinal axon initiation and elongation in vivo.* J Neurosci, 2008. **28**(1): p. 100-5.
64. Gibson, D.G., et al., *Enzymatic assembly of DNA molecules up to several hundred kilobases.* Nat Methods, 2009. **6**(5): p. 343-5.
65. Xue, Y., et al., *GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy.* Mol Cell Proteomics, 2008. **7**(9): p. 1598-608.
66. Bülbül, S., *Cellular role of a protocadherin family member in mammalian cells*. 2013, Koç University.
67. Roux, K.J., et al., *A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells.* J Cell Biol, 2012. **196**(6): p. 801-10.

VITA

Gürkan Mollaoğlu was born in Istanbul, Turkey, on August 3, 1988. He graduated from Haydarpaşa High School, Istanbul, in 2007 and then received his B.Sc. degree in Molecular Biology and Genetics from Istanbul University, Istanbul, in 2011. From September 2011 to July 2013, he worked as a teaching and research assistant at Koç University, Istanbul. He studied his M.Sc. research on "Molecular Analysis of Protocadherins in Mammalian Cells."

Appendix A: Cloning and Site-Directed Mutagenesis of Human Aurora Kinase B

Human Aurora Kinase B (Homo sapiens aurora-related kinase 2) (GenBank: AF008552.1) was cloned to pEGFP-N1 vector (Clontech #6085-1) and threonine (T/Thr) amino acid at the 35th site was changed to either alanine (A/Ala) or aspartic acid (D/Asp) with Site-directed Mutagenesis. Aurora B was amplified from HeLa cDNA (20 ng/µl) (see above for cDNA production) with PCR (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#48 10 µM 1.0 µl; Reverse primer P#49 10 µM 1.0 µl; dH_2O 11.8 µl; HeLa cDNA 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.2 µl); PCR conditions (Initial denaturation 98 °C – 3 min; Denaturation 98 °C – 15 sec; Annealing 65 °C – 30 sec; Extension 72 °C – 40 sec; Final extension 72 °C – 10 min; 30 cycles). PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. Then, PCR products were purified with Sigma GenElute PCR Clean-Up Kit #NA1020; according to the manufacturer's instructions. PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl; DNA 5.0 µl; EcoRI NEB #R3101S 1.0 µl; XhoI NEB #R0146S 1.0 µl; dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour). Digestions products were also purified with PCR Clean-Up Kit. pEGFP-N1 vector plasmid was also digested with the same RE couple with the same reaction conditions above. Digested vectors were run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vectors were dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 10.0 µl CIP NEB #M0290S 5.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested

inserts and plasmids were used in the ligation reaction with vector: insert ratio of 1:3. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer and ligation calculation was done with "Ligation Calculator" online program. Ligation reaction was performed (10X T4 Buffer 1.0 µl; T4 Ligase NEB #M0202S 1.0 µl; Vector 1.0 µl; Insert 6 µl; dH_2O 1 µl) – negative control reaction had no insert – ; reaction conditions (4 °C overnight). Ligation products were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pEGFP-N1 vector had kanamycin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.5 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#48 10 µM 1.0 µl; Reverse primer P#49 10 µM 1.0 µl; dH_2O 11.3 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.2 µl); PCR conditions (Initial denaturation 95 °C – 3 min; Denaturation 95 °C – 30 sec; Annealing 65 °C – 30 sec; Extension 72 °C – 1 min 30 sec; Final extension 72 °C – 10 min; 30 cycles). PCR products were run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (1 ml of 50 mg/ml kanamycin stock into 1 L LB agar) and 5 ml LB (50 µg/ml kanamycin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with

pEGFP forward and reverse sequencing primers (P#5 and P#6) by Macrogen Company. After confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (50 μg/ml kanamycin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT. Site-directed mutations were introduced to Aurora B in pEGFPN-1 (30 ng/μl) with accordingly designed primer pairs in PCR (10X PfuTurbo Buffer: 2.5 μl; DMSO 100% 1.0 μl; dNTP mix 10 mM 0.7 μl; Forward primer P#50/P#52 10 μM 1.0 μl; Reverse primer P#51/P#53 10 μM 1.0 μl; dH_2O 17.3 μl; DNA 0.5 μl; PfuTurbo DNA Polymerase Stratagene #600252 1.0 μl) – negative control with no primers; primer pairs per each reaction (T35D: P#50 and P#51; T35A: P#52 and P#53); PCR conditions (Initial denaturation 98 °C – 2 min; Denaturation 98 °C – 30 sec; Annealing 70 °C – 30 sec; Extension 72 °C – 6 min; Final extension 72 °C – 10 min; 25 cycles). After the PCR finished and samples were cooled to 4 °C, 1.5 μl of DpnI NEB #R0176S enzyme was added to each PCR tube to cleave and discard the non-mutated template DNA. Tubes were briefly centrifuged in bench-top centrifuge machine and placed back into the PCR machine. They were incubated at 37 °C for 1 hour. PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. The samples and control were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 μl DNA + 100 μl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 μl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 μl of supernatant > Resuspend the pellet > Plate 100 μl onto LB agar plate with selective antibiotic) – pEGFP-N1 vector had kanamycin resistance. Plates were incubated at 37 °C overnight. Single colonies were chosen from sample plates and incubated in 5 ml LB (50 μg/ml kanamycin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria

cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with pEGFP forward and reverse sequencing primers (P#5 and P#6) by Macrogen Company. After confirmation by sequencing, mutant DNAs were produced in large quantity with Midiprep as described above.

Appendix B: Site-Directed Mutagenesis of Nusap1

Mus musculus nucleolar and spindle associated protein 1 (Nusap1) (Gene ID: 108907) was mutated at two amino acid sites and three mutant genes were produced with Site-directed Mutagenesis: S246A, S290A and double mutant (S: serine; A: alanine). NUSAP-pEGFP-N1 construct had been produced by Dr. Nurhan Özlü, Koç University, Department of Molecular Biology and Genetics, Istanbul, Turkey. Single mutations were introduced to native gene in this construct. Double mutation was produced as introduction of S290A to S246A in a second PCR round. Site-directed mutations were introduced to NUSAP in pEGFPN-1 (40 ng/µl) and NUSAP S246A in pEGFP-N1 (40 ng/µl) with accordingly designed primer pairs in PCR (10X PfuTurbo Buffer: 2.5 µl; dNTP mix 10 mM 0.5 µl; Forward primer P#54/P#56 10 µM 1.0 µl; Reverse primer P#55/P#57 10 µM 1.0 µl; dH_2O 18.5 µl; DNA 1.0 µl; PfuTurbo DNA Polymerase Stratagene #600252 0.5 µl) – negative control with no primers; primer pairs per each reaction (S246A: P#54 and P#55; S290A: P#56 and P#57); PCR conditions (Initial denaturation 95 °C – 1 min; Denaturation 95 °C – 30 sec; Annealing 64 °C – 30 sec; Extension 68 °C – 6 min; Final extension 68 °C – 10 min; 16 cycles). After the PCR finished and samples were cooled to 4 °C, 1.5 µl of DpnI NEB #R0176S enzyme was added to each PCR tube to cleave and discard the non-mutated template DNA. Tubes were briefly centrifuged in bench-top centrifuge machine and placed back into the PCR machine. They were incubated at 37 °C for 1 hour. PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. The samples and control were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min >

Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pEGFP-N1 vector had kanamycin resistance. Plates were incubated at 37 °C overnight. Single colonies were chosen from sample plates and incubated in 5 ml LB (50 µg/ml kanamycin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with pEGFP forward and reverse sequencing primers (P#5 and P#6) by Macrogen Company. After confirmation by sequencing, mutant DNAs were produced in large quantity with Midiprep as described above.

Appendix C: Cloning of CLIC1 and CLIC4 to pEGFP Vectors

Human chloride intracellular channel proteins CLIC1 (GenBank: AF109197.1) and CLIC4 (GenBank: AF097330.1) were cloned to pEGFP-N1 (Clontech #6085-1) and pEGFP-C1 (Clontech Catalog #6084-1) vectors. CLIC1 and CLIC4 were amplified from HeLa cDNA (20 ng/µl) (see above for cDNA production) with PCR (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#58/P#60/P#62/P#64 10 µM 1.0 µl; Reverse primer P#59/P#61/P#63/P#65 10 µM 1.0 µl; dH_2O 11.7 µl; HeLa cDNA 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.3 µl) – negative control with no primers; primer pairs per each reaction (CLIC1-pEGFP-N1: P#58 and P#59; CLIC1-pEGFP-C1: P#60 and P#61; CLIC4-pEGFP-N1: P#62 and P#63; CLIC4-pEGFP-C1: P#64 and P#65); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 56 °C – 30 sec; Extension 72 °C – 1 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. Then, PCR products were purified with Sigma GenElute PCR Clean-Up Kit #NA1020; according to the manufacturer's instructions. PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl; DNA 5.0 µl; [HindIII NEB #R3104S 1.0 µl; BamHI NEB #R3136S 1.0 µl] for CLIC1 / [BamHI NEB #R3136S 1.0 µl; XhoI NEB #R0146S 1.0 µl] for CLIC4; dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour). Digestions products were also purified with PCR Clean-Up Kit. pEGFP-N1 and pEGFP-C1 vector plasmids were also digested with the same RE couples with the same reaction conditions above. Digested vectors were run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vectors were

dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 10.0 µl CIP NEB #M0290S 5.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested inserts and plasmids were used in the ligation reaction with vector: insert ratio of 1:3. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer and ligation calculation was done with "Ligation Calculator" online program. Ligation reaction was performed (10X T4 Buffer 1.0 µl; T4 Ligase NEB #M0202S 1.0 µl; Vector 1.0 µl; Insert 2 µl; dH_2O 5 µl) – negative control reaction had no insert – ; reaction conditions (4 °C overnight). Ligation products were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pEGFP vectors had kanamycin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#58/P#60 10 µM 1.0 µl; Reverse primer P#59/P#61 10 µM 1.0 µl; dH_2O 15.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl) – primer pairs per each reaction (CLIC1: P#58 and P#59; CLIC4: P#62 and P#63); PCR conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 56 °C – 30 sec; Extension 72 °C – 1 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (1 ml of 50

mg/ml kanamycin stock into 1 L LB agar) and 5 ml LB (50 µg/ml kanamycin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with pEGFP forward and reverse sequencing primers (P#5 and P#6 for pEGFP-N1 / P#5 and P#15 for pEGFP-C1) by Macrogen Company. After confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (50 µg/ml kanamycin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT.

Appendix D: Site-Directed Mutagenesis of CLIC4

We aimed to introduce two single amino acid changes (C35A and F37D [C: cysteine; A: alanine; F: phenylalanine; D: aspartic acid]) individually or as double mutation to CLIC4 gene. I made site-directed mutagenesis of CLIC4 (C35A) with using CLIC4 in pEGFP-N1 (40 ng/µl) as template in PCR (10X PfuTurbo Buffer: 2.5 µl; dNTP mix 10 mM 0.5 µl; Forward primer P#66 10 µM 1.0 µl; Reverse primer P#67 10 µM 1.0 µl; dH_2O 18.5 µl; DNA 1.0 µl; PfuTurbo DNA Polymerase Stratagene #600252 0.5 µl) – negative control with no primers; PCR conditions (Initial denaturation 95 °C – 1 min; Denaturation 95 °C – 30 sec; Annealing 55 °C – 1 min; Extension 68 °C – 6 min; Final extension 68 °C – 10 min; 16 cycles). After the PCR finished and samples were cooled to 4 °C, 1.0 µl of DpnI NEB #R0176S enzyme was added to each PCR tube to cleave and discard the non-mutated template DNA. Tubes were briefly centrifuged in bench-top centrifuge machine and placed back into the PCR machine. They were incubated at 37 °C for 1 hour. PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. The samples and control were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pEGFP-N1 vector had kanamycin resistance. Plates were incubated at 37 °C overnight. Single colonies were chosen from sample plate and incubated in 5 ml LB (50 µg/ml kanamycin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's

instructions. Purified plasmids were sequenced with pEGFP forward sequencing primer (P#5) by Macrogen Company. After confirmation by sequencing, mutant DNA were produced in large quantity with Midiprep as described above.

Appendix E: Cloning of CLIC1 and CLIC4 to pGEX Vector

Human chloride intracellular channel proteins CLIC1 (GenBank: AF109197.1) and CLIC4 (GenBank: AF097330.1) were cloned to pGEX-6P-1 GE Healthcare #28-9546-48 vector for the aim of peptide expression. Insert DNAs were amplified from CLIC1 in pEGFP-N1 (30 ng/µl) and CLIC4 in pEGFP-N1 (30 ng/µl) with PCR (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#68/P#70 10 µM 1.0 µl; Reverse primer P#69/P#71 10 µM 1.0 µl; dH_2O 11.7 µl; template DNA 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.3 µl) – primer pairs per each reaction (CLIC1: P#68 and P#69; CLIC4: P#70 and P#71); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 56 °C – 30 sec; Extension 72 °C – 1 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. Then, PCR products were purified with Sigma GenElute PCR Clean-Up Kit #NA1020; according to the manufacturer's instructions. PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (10X NEB4 Buffer 2.5 µl; DNA 5.0 µl; BamHI NEB #R3136S 1.0 µl; XhoI NEB #R0146S 1.0 µl; dH_2O 15.5 µl); reaction conditions (37 °C – 1 hour). Digestions products were also purified with PCR Clean-Up Kit. pGEX-6P-1 vector plasmid was also digested with the same RE couple with the same reaction conditions above. Digested vector was run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vector was dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 13.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested inserts and plasmids were used in the

ligation reaction with vector: insert ratio of 1:3. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer and ligation calculation was done with "Ligation Calculator" online program. Ligation reaction was performed (10X T4 Buffer 1.0 µl; T4 Ligase NEB #M0202S 1.0 µl; Vector 1.0 µl; Insert 1 µl; dH_2O 6 µl) – negative control reaction had no insert – ; reaction conditions (4 °C overnight). Ligation products were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – pGEX vector had ampicillin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#68/P#70 10 µM 1.0 µl; Reverse primer P#69/P#71 10 µM 1.0 µl; dH_2O 15.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl) – primer pairs per each reaction (CLIC1: P#68 and P#69; CLIC4: P#70 and P#71); PCR conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 56 °C – 30 sec; Extension 72 °C – 1 min 30 sec; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (10 ml of 10 mg/ml ampicillin stock into 1 L LB agar) and 5 ml LB (100 µg/ml ampicillin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the

manufacturer's instructions. Purified plasmids were sequenced with pGEX5 forward and pGEX3 reverse sequencing primers (P#36 and P#37) by Macrogen Company. After confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (100 μg/ml ampicillin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT.

Appendix F: Cloning of CLIC1 and CLIC4 to BioID Vectors

Human CLIC1 and CLIC4 (see above for their cloning to pEGFP vectors) were cloned to BioID-N (Addgene Plasmid 35700: pcDNA3.1 mycBioID) and BioID-C (Plasmid 36047: pcDNA3.1 MCS-BirA(R118G)-HA) vectors. Insert DNAs were amplified from CLIC1 in pEGFP-N1 (30 ng/µl) and CLIC4 in pEGFP-N1 (30 ng/µl) with PCR (10X Phusion HF Buffer: 4.0 µl; DMSO 100% 0.6 µl; dNTP mix 10 mM 0.4 µl; Forward primer P#72/P#74/P#76/P#78 10 µM 1.0 µl; Reverse primer P#73/P#75/P#77/P#79 10 µM 1.0 µl; dH_2O 11.7 µl; template DNA 1.0 µl; Phusion HF DNA Polymerase NEB #F-530S 0.3 µl) – primer pairs per each reaction (CLIC1-BioID-N: P#72 and P#73; CLIC1-BioID-C: P#74 and P#75; CLIC4-BioID-N: P#76 and P#77; CLIC4-BioID-C: P#78 and P#79); PCR conditions (Hot-Start 98 °C; Initial denaturation 98 °C – 30 sec; Denaturation 98 °C – 15 sec; Annealing 56 °C – 30 sec; Extension 72 °C – 1 min; Final extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control the success of PCR reaction. Then, PCR products were purified with Sigma GenElute PCR Clean-Up Kit #NA1020; according to the manufacturer's instructions. PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (10X NEB4 Buffer 2.5 µl; BSA 0.25 µl;DNA 5.0 µl; [XhoI NEB #R0146S 1.0 µl; BamHI NEB #R3136S 1.0 µl] for CLIC1 and CLIC4 for BioID-N REs / [NheI NEB #R3131S 1.0 µl; BamHI NEB #R3136S 1.0 µl] for CLIC1 and CLIC4 for BioID-C REs; dH_2O 15.25 µl); reaction conditions (37 °C – 1 hour). Digestions products were also purified with PCR Clean-Up Kit. BioID vector plasmids were also digested with the same RE couples with the same reaction conditions above. Digested vectors were run in 1% agarose gel electrophoresis and then digested DNA molecules were purified from the gel with Invitrogen Quick Gel Extraction Kit #K2100-12. Digested vectors were

dephosphorylated with alkaline phosphatase enzyme (10X NEB3 Buffer 5.0 µl; DNA 30.0 µl; dH_2O 13.0 µl CIP NEB #M0290S 2.0 µl); reaction conditions (37 °C – 1 hour). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested inserts and plasmids were used in the ligation reaction with vector: insert ratio of 1:3. Concentration measurements of prepared DNA molecules were made with Thermo Scientific Nanodrop Spectrophotometer and ligation calculation was done with "Ligation Calculator" online program. Ligation reaction was performed (10X T4 Buffer 1.0 µl; T4 Ligase NEB #M0202S 1.0 µl; Vector 1.0 µl; Insert n µl; dH_2O n-7 µl) – insert amounts (n) were different for all inserts, ranging from 1.0 µl to 3 µl; negative control reactions had no insert – ; reaction conditions (4 °C overnight). Ligation products were used to transform chemical competent *E. coli* DH5α bacteria according to the standard transformation protocol (5 µl DNA + 100 µl bacteria > 30 min incubation on ice > 1 min heat shock at 42 °C > 2 min incubation on ice > Addition of 900 µl LB and incubation at 37 °C – 1 hour with 700 rpm mixing > Centrifuge at 8000 rpm for 10 min > Discard 800 µl of supernatant > Resuspend the pellet > Plate 100 µl onto LB agar plate with selective antibiotic) – BioID vectors had ampicillin resistance. Plates were incubated at 37 °C overnight. Next day, single colonies from sample plates were picked and tested with Colony PCR method for success of the cloning: Each of the picked single colonies were suspended in 5 µl dH_2O and 3 µl of them were used as DNA template in the PCR (10X Taq Buffer 2.0 µl; DMSO 100% 1.0 µl; dNTP mix 10 mM 0.5 µl; $MgCl_2$ 25 mM 1.5 µl; Forward primer P#72/P#74/P#76/P#78 10 µM 1.0 µl; Reverse primer Reverse primer P#73/P#75/P#77/P#79 10 µM 1.0 µl; 10 µM 1.0 µl; dH_2O 15.7 µl; template DNA 2.0 µl; Taq DNA Polymerase #EP0402 0.3 µl) – primer pairs per each reaction (CLIC1-BioID-N: P#72 and P#73; CLIC1-BioID-C: P#74 and P#75; CLIC4-BioID-N: P#76 and P#77; CLIC4-BioID-C: P#78 and P#79); PCR conditions (Initial denaturation 94 °C – 3 min; Denaturation 94 °C – 30 sec; Annealing 56 °C – 30 sec; Extension 72 °C – 1 min; Final

extension 72 °C – 10 min; 35 cycles). PCR products were run in 1% agarose gel electrophoresis to control which of the selected colonies had cloned insert. The colonies which had passed the test with Colony PCR were incubated on LB-agar plates (10 ml of 10 mg/ml ampicillin stock into 1 L LB agar) and 5 ml LB (100 µg/ml ampicillin) at 37 °C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Fermentas Miniprep Kit #K0503; according to the manufacturer's instructions. Purified plasmids were sequenced with BGH-R reverse primer (P#46) for BioID-N constructs and CMV-F forward primer (P#47) for BioID-C constructs by Macrogen Company. After confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (100 µg/ml ampicillin) and incubated at 37 °C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with Sigma GenElute Plasmid Midiprep Kit #NA0200-1KT.

Appendix G: Primer List

Primer Code (P#)	Primer Sequence (5' to 3')
1	GCGCAAGCTTATGCTGAGGATGCGGACC
2	GCGCGGATCCGCGCCCTCCCTGGGATATTTA
3	GCGCAAGCTTCGCTGAGGATGCGGACC
4	GCGCGGATCCGCTTAGCCCTCCCTGGGATATTTA
5	TTTAGTGAACCGTCAGATC
6	AACAGCTCCTCGCCCTTG
7	CCGGACTCAGATCTCGAGCTCAAGCTTATGCTGAGGATGCGGACCGCGGGA
8	CAGCCGGTACCGGAGGAGCTGCTTGGCGGCCGCCAGGCTGAGC
9	CAAGCAGCTCCTCCGGTACCGGCTGAGCAGCGTGTTCGAGCTG
10	CACCATGGTGGCGACCGGTGGATCCGCGCCCTCCCTGGGATATTTAA
11	CCGGACTCAGATCTCGAGCTCAAGCTTCGATGCTGAGGATGCGGACCGCGGGA
12	CAGCCGGTACCGGAGGAGCTGCTTGGCGGCCGCCAGGCTGAGC
13	CAAGCAGCTCCTCCGGTACCGGCTGAGCAGCGTGTTCGAGCTG
14	CAGTTATCTAGATCCGGTGGATCCGCTTAGCCCTCCCTGGGATATTTAA
15	CCTCTACAAATGTGGTATGG
16	GCGCAAGCTTATGCTGAGGATGCGGACC

17	GCGCGGATCCGCGCCGGCTTCATAGCCATTTTTA
18	GCGCAAGCTTCGCTGAGGATGCGGACCGCGGGA
19	GCGCGGATCCTTAGCCGGCTTCATAGCCATTTTTATTTTTGG
20	GCGCAAGCTTATGCTGAGGATGCGGACC
21	GCGCGGATCCGCCTTCTCATTGACACTATCATACCTC
22	GCGCAAGCTTCGCTGAGGATGCGGACC
23	GCGCGGATCCTTACTTCTCATTGACACTATCATACC
24	GCGCAAGCTTATGCTGAGGATGCGGACC
25	GCGCGGATCCGCTCCTGCAGTTGGTGACTGG
26	GCGCAAGCTTCGCTGAGGATGCGGACC
27	GCGCGGATCCTTATCCTGCAGTTGGTGAC
28	GCGCGGATCCAGCAGCGTGTTCGAGCTG
29	GCGCGAATTCTTAACTGAGTCTCTGTTTGCTAATTTCA
30	GCGCGGATCCAGCAGCGTGTTCGAGCTG
31	GCGCGAATTCTTACATCACTACAATTAAGATGATTAGAATCA
32	GCGCGGATCCATTGTCATTGGCGTAGTTGC
33	GCGCGAATTCTTAGCCCTCCCTGGGATA
34	GCGCGGATCCGCAAGGTACTGCAGGTCCAA
35	GCGCGAATTCTTAGCCCTCCCTGGGATA
36	GGCAAGCCACGTTTGGTG
37	GGAGCTGCATGTGTCAGAGG
38	GCGCGAATTCCTGAGGATGCGGACC
39	GCGCGGATCCTTAGCCCTCCCTGGGATA
40	GCGCGCTAGCATGCTGAGGATGCGGAC
41	GCGCGGATCCGCCCTCCCTGGGATATTTAA

42	GCGCGAATTCCTGAGGATGCGGACCGCGGGA
43	GCGCGGATCCTTAGCCGGCTTCATAGCCATTTTTATTTTTGG
44	GCGCGCTAGCATGCTGAGGATGCGGACC
45	GCGCGGATCCGCCGGCTTCATAGCCATTTTTA
46	TAGAAGGCACAGTCGAGG
47	CGCAAATGGGCGGTAGGCGTG
48	GGCCCTCGAGATGGCCCAGAAGGAGAACTCC
49	GGCCGAATTCGGGCGACAGATTGAAGGGCAG
50	CGGAAAGAGCCTGTCGACCCATCTGCACTTGTC
51	GACAAGTGCAGATGGGTCGACAGGCTCTTTCCG
52	CGGAAAGAGCCTGTCGCCCCATCTGCACTTGTC
53	GACAAGTGCAGATGGGGCGACAGGCTCTTTCCG
54	CCAAGAGGAAGGCTCGCTGTACCCTGTACTCCT
55	AGGAGTACAGGGTACAGCGAGCCTTCCTCTTGG
56	ACACCAGCCAGAAAGGCTCCACACGTGACTGCA
57	TGCAGTCACGTGTGGAGCCTTTCTGGCTGGTGT
58	GCGCAAGCTTATGGCTGAAGAACAACCGCAGG
59	GCGCGGATCCGCTTTGAGGGCCTTTGCCACTTG
60	GCGCAAGCTTCGGCTGAAGAACAACCGCAGG
61	GCGCGGATCCTTATTTGAGGGCCTTTGCCACTTG
62	GCGCCTCGAGATGGCGTTGTCGATGCCGCTGA
63	GCGCGGATCCGCCTTGGTGAGTCTTTTGGCTACATCACTA
64	GCGCCTCGAGCGGCGTTGTCGATGCCGCTGAA
65	GCGCGGATCCTTACTTGGTGAGTCTTTTGGCTACA
66	GAAAGCATAGGAAACGCCCCCTTTTCCCAGAGGCTC

67	GAGCCTCTGGGAAAAGGGGGCGTTTCCTATGCTTTC
68	GCGCGGATCCGCTGAAGAACAACCGCAGG
69	GCGCCTCGAGTTATTTGAGGGCCTTTGCCACTTG
70	GCGCGGATCCGCGTTGTCGATGCCGCTGAA
71	GCGCCTCGAGTTACTTGGTGAGTCTTTTGGCTACA
72	GCGCCTCGAGGCTGAAGAACAACCGCAGG
73	GCGCGGATCCTTATTTGAGGGCCTTTGCCACTTG
74	GCGCGCTAGCATGGCTGAAGAACAACCGCAGG
75	GCGCGGATCCTTTGAGGGCCTTTGCCACTTG
76	GCGCCTCGAGGCGTTGTCGATGCCGCTGAA
77	GCGCGGATCCTTACTTGGTGAGTCTTTTGGCTACA
78	GCGCGCTAGCATGGCGTTGTCGATGCCGCTGA
79	GCGCGGATCCCTTGGTGAGTCTTTTGGCTACATCACTA
80	GCAGGCTTTAAAGGAACCAATTCAGTCGACATGCTGAGGATGCGGACCGCGGGA
81	GAGATCCTCTTCTGAGATGAGTTTTTGTTCCTTGGCGGCCGCCAGGCTGA
82	GAACAAAAACTCATCTCAGAAGAGGATCTCCAGCTCCTCCGGTACCGGCTG
83	GAGTGCGGCCGCGAATTCGGTACCGGATCCTTAGCCCTCCCTGGGATATTTA