Cellular Role of a Protocadherin Family Member in Mammalian Cells

by

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To My Beloved Husband, Can

ABSTRACT

Cell division is a fundamental process by which all living things propagate. Throughout the cell cycle different cellular complexes undergo dramatic re-organization in a coordinated manner. During the cell division cell surface morphology also undergoes drastic changes. Intracellular mechanisms that control cell division cycle have been widely studied, whereas not much is known about the changes in cell surface during the cell cycle. As cells divide, adherent cells undergo cell retraction and become rounded due to down regulation of adhesive systems and reorganization of the actin cytoskeleton. Cell to cell adhesion is especially mediated by cadherin superfamily of proteins. Protocadherins are the largest subfamily of cadherin superfamily and they are primarily expressed in nervous system. They are found at the cell-to-cell contact site and via homophilic binding activity they mediate cell sorting in a Ca^{2+} dependent manner. The aim of this study is to investigate the cellular role of a protocadherin family member during mitosis. Towards this goal, we performed transfection experiments and by using microscopy techniques we analyzed the subcellular localization of the protocadherin family member during the cell cycle. We also performed RNAi mediated depletion experiments and showed that it is important for the organization of the mammalian mitotic cells.

ÖZET

Hücre bölünmesi, tüm canlıların çoğalması ve büyümesi için gerekli temel bir süreçtir. Hücre döngüsü sırasında farklı hücresel yapılar koordineli bir şekilde yeniden düzenlenir. Hücre yüzey morfolojisi de hücre bölünmesi sırasında köklü değişikliklere uğrar. Hücre bölünmesini kontrol eden hücre içi mekanizmaları detaylı bir biçimde incelenmiş olmasına rağmen hücre bölünmesi sırasında hücre yüzeyinde meydana gelen değişimler ile ilgili bilgimiz sınırlıdır. Hücreler bölünürken yapışık hücreler, yapıştırıcı sistemlerin baskılanması ve aktin hücre iskeletinin yeniden düzenlenmesiyle yuvarlaklaşırlar. Hücre-hücre adezyonu özellikle kadherin protein üst ailesi tarafından meydana getirilir. Protocadherinler kadherin ailesinin en büyük alt ailesidir ve esas olarak sinir sisteminde ifade edilirler. Protocadherinler hücre hücre temas yerlerinde bulunurlar ve de kalsiyum iyonuna bağımlı bir biçimde gerçekleşen homofilik adezyon sayesinde hücre öbekleşmesini sağlarlar. Bu çalışmada, mitoz sırasında bir protocadherin aile üyesinin hücresel rolünü araştırmayı amaçladık. Bu amaçla, transfeksiyon deneyleri yaptık ve de mikroskopi teknikleri kullanarak hücre döngüsü sırasında çalıştığımız protocadherin aile üyesinin hücre içi lokalizasyonunu analiz ettik. Bunun yanında, RNAi aracılı gen susturması deneyleri yaparak, çalıştığımız proteinin memeli mitotik hücrelerinin organizasyonu için önemli olduğunu gösterdik.

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NOMENCLATURE

AFM	Atomic force microscopy
BH-Pcdh	Brain heart protocadherin
BSA	Bovine Serum Albumin
Cdk	Cyclin dependent kinase
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Ectodomain
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
esiRNAs	Endoribonuclease-Prepared Short Interfering RNAs
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
G0	Quiscent state
G1	Gap one
G2	Gap two
GFP	Green Fluorescent protein
HeLa	Human cervical cancer cell line
HRP	Horseradish peroxidase

kDa	Kilodalton
LatB	Latrunculin B
mRNA	messenger ribonucleic acid
MS	Modification site
Opti-MEM	Reduced Serum Media
PAPC	Paraxial protocadherin
PBS	Phosphate buffered saline
PCDH	Protocadherin
PCDH7-CytDel	Cytoplasmic domain deleted Protocadherin 7
PCDH7-FL	Full length Protocadherin 7
PCDH7-MSless	Modification site lacking Protocadherin 7
PCDH7-WithMS	Modification site containing Protocadherin 7
PS	Penicillin-Streptomycin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SP	Signal sequence
SRP	Signal recognition particle
STC	S-Trityl-L-cysteine
TBS	Tris buffered saline
TM	Transmembrane domain
U2OS	Human Bone Osteosarcoma Epithelial Cells
WT	Wild type

Chapter 1

INTRODUCTION

Cell division cycle is the most fundamental subject in the development of multicellular organisms. It is composed of four sequential phases in which growth of cell, DNA replication and cell division takes place and it is strictly controlled by some cell-cycle control mechanisms. With the help of these control mechanisms, the cell grows to a certain point, replicates its DNA and divides to give identical two daughter cells [1].

Intracellular mechanisms that control cell division cycle are well known, however not much is known about the changes in cell surface during cell cycle. At the beginning of mitosis, adherent cells undergo cell retraction and become rounded. After two daughter cells are separated in cytokinesis phase, cells become fully spread and attached again [2]. Cadherins are one of the targets of regulation during the cell cycle because they are adhesion molecules. They are Ca^{2+} dependent homophilic cell-cell adhesion molecules and their adhesion properties are dependent on conserved Trp2 domain in the extracellular part and conserved catenin binding motifs in the intracellular part [3]. Protocadherins which are the largest subfamily of cadherins are not well studied. There is much less information about the adhesion properties of protocadherins. Protocadherins are different from classical cadherins. They do not have Trp2 or catenin binding domains. In a recent study, it was found out that Xenopus paraxial protocadherin down regulates the adhesion activity of classical cadherin, C-cadherin, during the cell cycle and this causes cell de-adhesion.

Therefore, it functions as an indirect cell de-adhesion molecule [4]. Cell de-adhesion causes the cell rounding and the cells in mitosis look similar to cancer cells. De-adhesion property of protocadherins might play a role in cancer cell detachment and metastasis.

In this study, we aim to investigate the molecular mechanism of Protocadherin 7 (Pcdh7) in mammalian cells which is shown firstly as a mitosis selective cell surface protein. For this reason, cells were arrested at interphase and mitosis via double thymidine and STC method and the localization of Pcdh7 protein was examined by confocal microscopy. We thought that the cytoplasmic part is important in Pcdh7 localization, we made cytoplasmic part deleted versions of the protein and checked the localization of the constructs. We depleted the Pcdh7 protein with specific esiRNAs and examined how the cell surface properties of cells changed when they did not have Pcdh7. All results indicated that Pcdh7 localizes on the cell surface at mitosis and within the ER at interphase. Interestingly, its small domain at the end of the cytoplasmic part is the domain where other intracellular partner protein binds or posttranslational modification occurs. This modification or the protein interaction renders the protein within ER at interphase. In addition, when cells do not have Pcdh7, the mitotic rounding pressure has decreased.

In Chapter 2, the corresponding work in the literature is demonstrated. This chapter includes the most recent information about cell division cycle, cell adhesion during mitosis, cadherin and protocadherin structure and function in cell adhesion and most importantly the recent information about our protein of interest Protocadherin 7.

Chapter 3 includes materials and methods and illustrates the details of the experiments. Cell culture, transfection, immunofluorescence, confocal microcopy imaging and quantification, SDS-Page and Western Blotting, cell synchronization to obtain mitosis and interphase arrested cells are explained in this chapter. In the remaining part of this chapter, sucrose gradient centrifugation and biotinylation to obtain membranes of the cells is presented in detail. Finally, the chapter ends with the brief explanation of the

endoribonuclease-prepared short interfering RNAs (esiRNAs) mediated depletion of the PCDH7.

Chapter 4 includes the detailed results of this work. This thesis ends with chapter 5 which includes discussion and the future directions of the study.

Chapter 2

LITERATURE REVIEW

2.1 Cell Cycle

Cell cycle or cell division cycle is a fundamental biological process by which all living cells propagate. It is a series of events which leads to cell division and replication. Main purpose of cell cycle is to pass genetic information neatly to the next generation. When unicellular organisms such as bacteria or yeasts undergo cell division, complete new organism is produced. Whereas for more complex organisms, cells have to undergo complex sequences of cell divisions in order to produce a functioning organism. Producing of new cells is important for the organism in many ways such as providing new cells that will be used to create a tissue or an organ or replacing cells that are dead [5].

Cell division cycle can be slightly different between organisms but the main concept is highly similar in all living organisms. Cell cycle is composed of four sequential phases in which growth of cell, DNA replication and cell division takes place and it is strictly controlled by cell-cycle control mechanisms [1].

2.1.1 Phases of the Cell Cycle and Their Checkpoints

The division cycle of the eukaryotes is composed of four discrete phases: G_1, S (synthesis), G_2 and M (mitosis). G_1, S and G_2 phases are called interphase and it occupies 23 hours of a 24-hour cycle of typical human cell proliferating in a culture. Mitosis phase takes only one hour of the complete 24-hour cycle. Cells are metabolically active and continue to grow during the G1 phase which is a gap between DNA synthesis and cell division. G1 is followed by S phase in which cells replicate their DNA (that is why this phase is called synthesis). G2 phase is the gap between S and M phase where cells continue to grow and synthesize certain essential proteins for mitosis. M phase is where the replicated DNA is segregated into two nuclei and the cell splits to produce two daughter cells [6]. Mitosis phase further dividied into pro-, prometa-, meta-, ana- and telophases preceding cytokinesis.

Some cells in adult organisms such as nerve cells cease to divide and some cells such as skin fibroblasts only divide when there is an external signal that tells them to divide. These cells exit G1 phase to enter quiescent stage of the cycle called G0. At this phase, cells are metabolically active but they do not grow and proliferate [7].

There are several cell cycle checkpoints that ensure the cells undergo cell division cycles only under the appropriate circumstances and the division is executed with high fidelity (Figure 2.1). When there is an incomplete event of the cycle, these control mechanisms halt the progression of the cell cycle. Restriction point is an important gate within the G1 phase, when the conditions are favorable for division through which cells pass and commit to cell division [8]. G1/S and G2/M border checkpoints have a role in detecting the damaged DNA and halt the cycle until the damaged DNA is repaired. At the G2/M checkpoint, precise replication of the DNA is also ensured. At metaphase to anaphase checkpoint, regulators detect the proper lining of chromosomes at metaphase

plate and prevent entry into anaphase if all of the chromosomes are not aligned correctly [9]. If these control mechanisms fail to govern the proper cell division cycle, excessive cell divisions may result in cancer [10, 11].

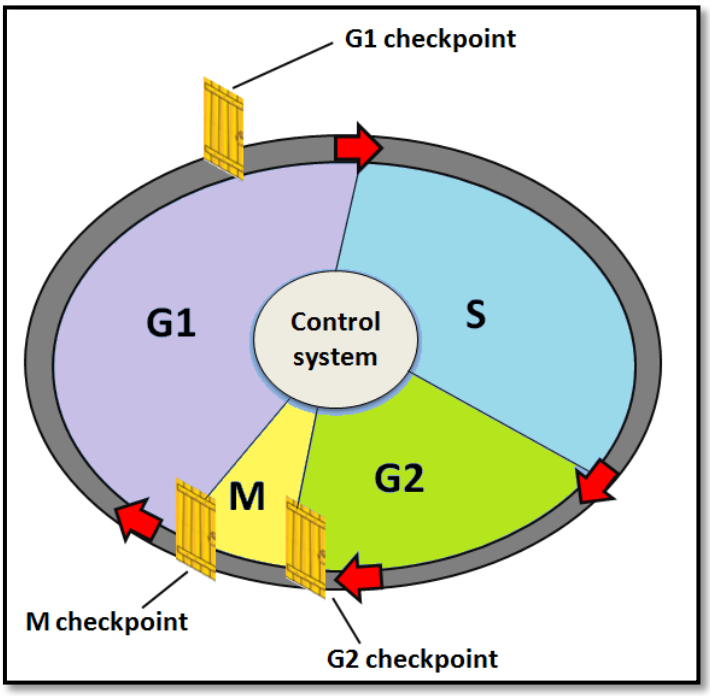

Figure 2.1 Cell cycle checkpoints. There are cell cycle checkpoints that are responsible for the precise completion of the cell cycle. Adapted from [12].

2.1.2 Mitosis

Mitosis stage of the cell cycle takes place within a short time period compared to interphase but during this period cells undergo dramatic changes both physically and biochemically. Replicated chromosomes are separated and segregated into two identical cells. The cytokinesis event divides the cells with two nuclei into two identical cells that have exact same genomic content. Traditionally mitosis is divided into six stages, each defined on the chromosome behavior seen in the microscope as in Figure 2.2 [13].

Prophase is the stage where the replicated chromosomes condense and the mitotic spindle assembles between the two centrosomes that are replicated from one centromere. At prometaphase stage nuclear envelope breaks open and chromosomes are attached to the spindle microtubules via their kinetochores. Chromosomes are aligned at the middle of the spindle poles at the metaphase stage. After proper alignment of the chromosomes, anaphase stage begins where sister chromatids are pulled towards the separate spindle poles. During telophase, daughter chromosomes reach the poles of the spindle and start to decondense. Nuclear envelope starts to enclose around the decondensed genetic material. Final stage of mitosis is cytokinesis where contractile ring contracts and divides the cytoplasm of two cells each with a nucleus [5].

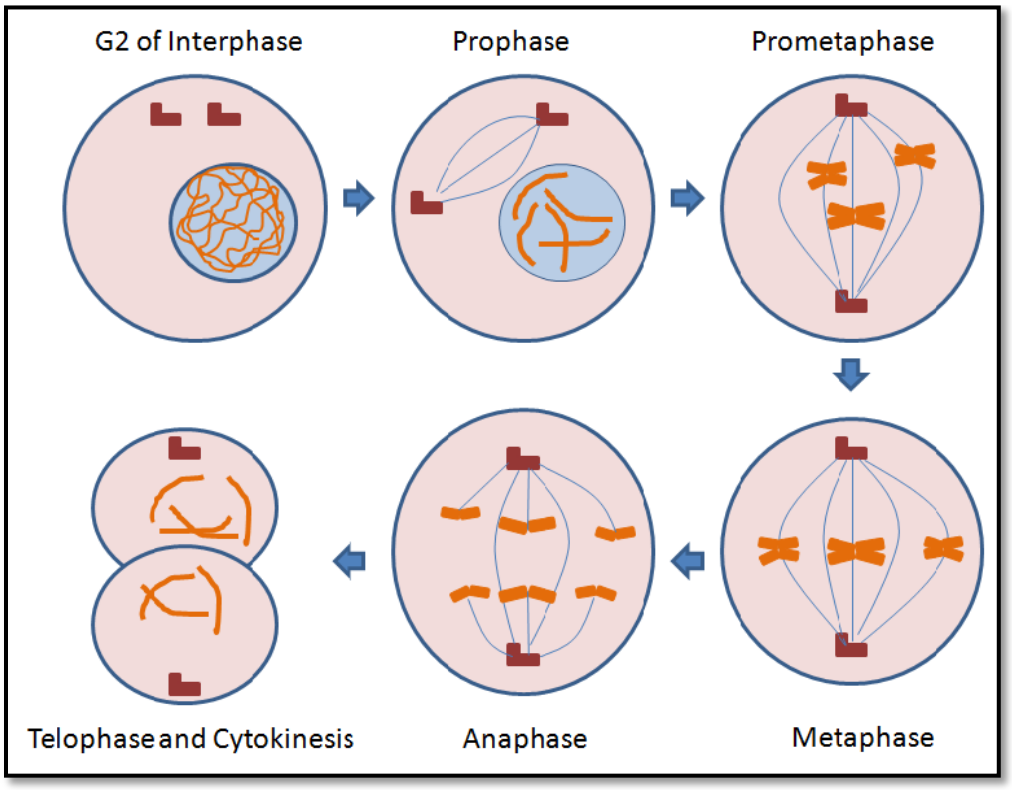

Figure 2.2 Stages of the mitosis. Adapted from [12]

2.2 Cell Adhesion during Mitosis

Changes within the cells' cytoplasm during mitosis have been widely studied over the past decades and it is known that Cdk1 is the master kinase responsible for these drastic changes [13]. On the contrary, changes at the cell's surface during cell division are still a mystery. At the beginning of mitosis fully adhered cells in culture undergo cell retraction and become rounded due to downregulation of adhesive systems and actin stress fibers [14, 15]. This phenomenon is called mitotic cell rounding which is crucial for the cell division by means of geometry. Daughter cells which undergo cell retraction and has rounded morphology, become fully spread according to the mother cells adhesion pattern after

cytokinesis [16]. The dynamics of mitotic cell rounding is not well determined, yet it is thought to be driven by actomyosin cortex contraction and osmotic pressure [17]. Vesicular trafficking also contributes to mitotic cell rounding by means of changing the biochemical properties of the plasma membrane. The balance between exo- and endocytosis during mitosis is altered compared to interphase [18]. Therefore the amounts and types of proteins on the cell surface especially responsible for the cell adhesion (integrins, cadherins etc.) are tightly regulated during mitosis. When cells undergo mitotic divisions, cell attachment to the ECM or cell attachment to other cells change rapidly. There are two large superfamily of proteins that are responsible for these type of attachments: integrins and cadherins. Integrin superfamily of proteins mediate cell to matrix attachment [19, 20], while proteins of the cadherin superfamily mediate cell to cell attachment [21]. During mitotic cell rounding, both integrin and cadherin mediated attachments are distrupted to a certain extent and after cell division new attachments are formed (Figure 2.3).

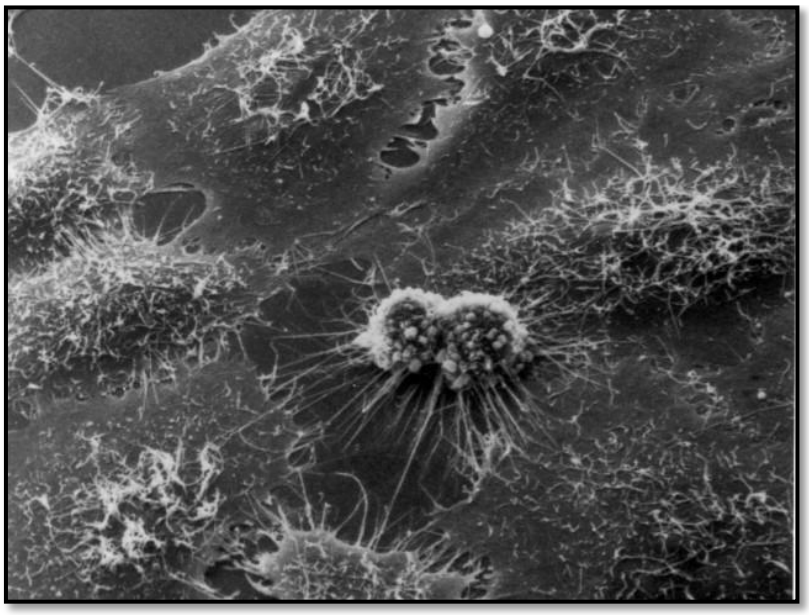

Figure 2.3 Dividing HeLa cells under electron microscopy. Retrieved from Public Domain/Wikimedia Commons

2.2.1 Cadherins

Cadherins are membrane associated glycoproteins and this family of proteins shares common structural features: a large extracellular domain, a transmembrane domain and a relatively small cytoplasmic domain. They are primary mediators of adhesion between cells. The expression of the cadherin subtype is highly regulated during the development of multicellular organisms as the switch in subtype expression leads to tissue segregation and morphogenesis (Figure 2.4) [22]. Cells that express same subtype of cadherin stick together and segregate from other types of cells. Highly selective recognition mediated by cadherins is the major factor in the organization of animal tissues [23].

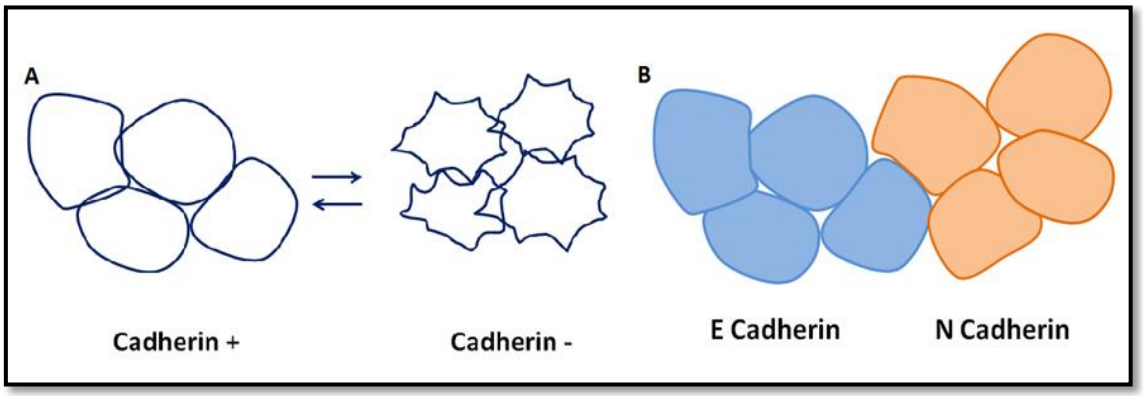

Figure 2.4 Cadherin mediated cell sorting. A) Cadherins mediate firm and ordered cell to cell adhesions. B) In a mixture of cells that express different types of cadherins, ones that express identical type of cadherins prefer to associate with each other and exclude cells that express other types of cadherins. Adapted from [23].

Cadherin mediated adhesion is calcium dependent homophilic cell adhesion. That is to say, it requires calcium ions to fully exert its adhesive property and it binds to the same subtype of cadherin molecule on the opposing cell [24]. Cadherins contain 110 aminoacid long five cadherin ectodomains (EC) of which EC1-4 have high homology but EC5 is less conserved. Calcium binds to the calcium binding regions within the ectodomains and cadherin adopts a stable curved structure needed for adhesion. Tryptophan residue side chain at the membrane distal end of cadherin molecule is inserted into a hydrophobic pocket of cadherin on the opposing cell. That interaction is crucial to form homophilic adhesions [22]. Truncated cadherin that lacks cytoplasmic domain mediate weak adhesion with extracellular domains alone. Whereas the presence of cytoplasmic tail that contains catenin binding domain causes strong adhesion. Catenins link cadherins to the actin filaments so that extracellular homophilic adhesion is transmitted to the cytoskeleton which in turn creates a strong cell to cell adhesion. Finally, it is shown that lateral dimerization is

necessary for the homophilic cell adhesion as monomers mediate only slight adhesion activity (Figure 2.5) [25-28].

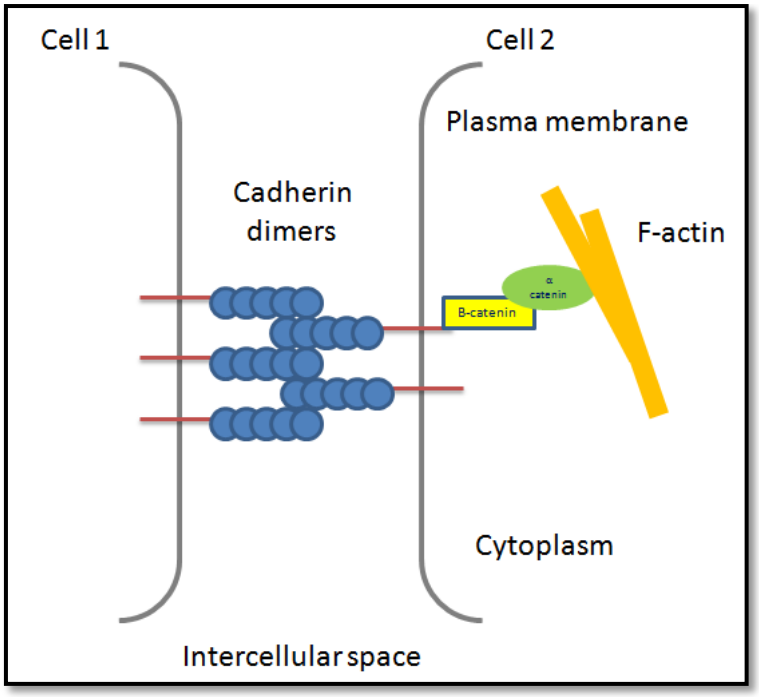

Figure 2.5 Schematic representation of cadherin mediated cell to cell adhesion. Cadherins are laterally dimerized to mediate stable cell adhesion. Cytoplasmic tail binds to catenins which links cadherins to actin cytoskeleton. Adapted from [29].

Cadherin superfamily consists of six subfamilies which differ in protein domain composition, genomic structure and their phylogenetic analysis. These families are classical cadherins, atypical cadherins, desmocollins, desmogleins, flamingo cadherins and protocadherins [3]. Classical cadherins are present in the adherens junctions and desmosomal cadherins are found in desmosomes. Protocadherins are expressed primarily in the nervous system but their biological role is elusive [30].

2.2.2 Protocadherins

Protocadherin (PCDH) family is a large family with more than 80 members and they are primarily expressed in the nervous system where their expression is developmentally regulated. Based on their genomic structures they are divided into two large subgroups: clustered and non-clustered protocadherins [31, 32]. The clustered protocadherin genes are encoded as a large cluster in the genome and they encode neuronal cell surface receptors whose exact function is unknown. Clustered protocadherins are further divided into Pcdhα and Pcdhγ. Homophilic activity of these Pcdhs are so weak that it cannot be detected by bead aggregation assays. Nevertheless, Pcdhγ is thought to play role in synaptic assembly or development [31]. The genes of the non-clustered protocadherins are scattered throughout the genome and they have been divided into three subgroups: PCDHδ1 (PCDH1, PCDH7, PCDH9 and PCDH11), PCDHδ2 (PCDH8, PCDH10, PCDH17, PCDH18 and PCDH19) and solitary PCDHs (PCDH12, PCDH15, PCDH20 and PCDH21) [33]. Similar with the clustered protocadherins, non-clustered protocadherins also take part in the formation and maintenance of neuronal circuits. They have homophilic and/or heterophilic cell to cell adhesion properties. Each non-clustered protocadherin has many isoforms that enable them to have different intracellular signaling connections (Figure 2.6).

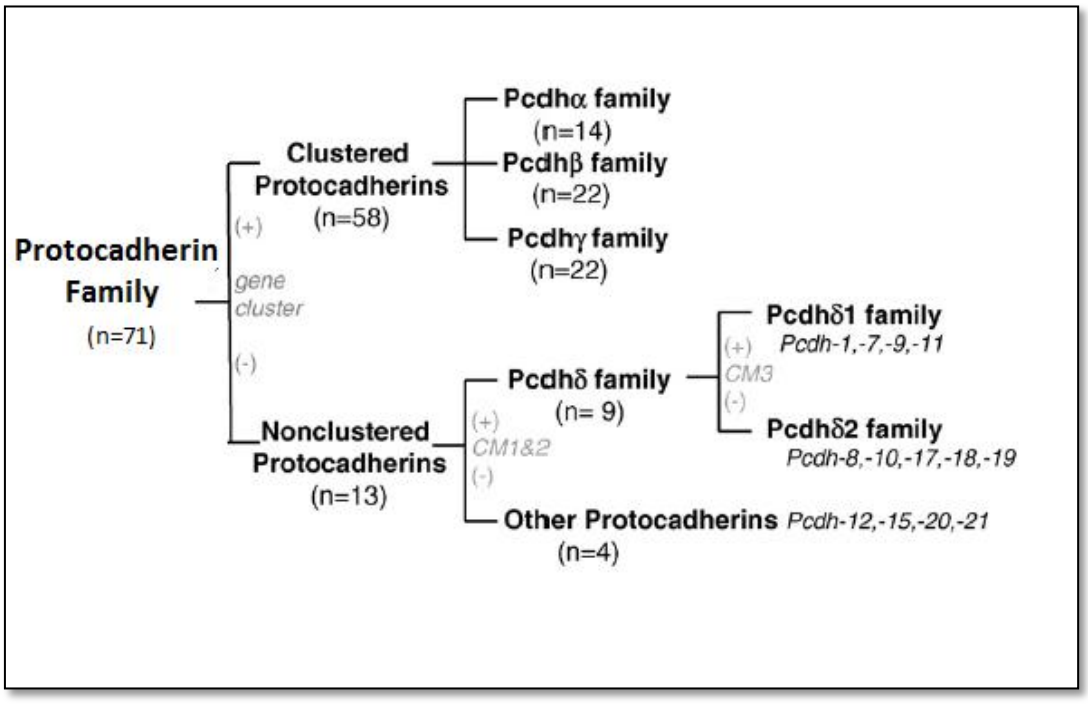

Figure 2.6 Protocadherin family members. Based on their genomic structures protocadherin family is divided into two groups: clustered and nonclustered. Clustered protocadherin family is composed of α, β and γ protocadherin families. Nonclustred protocadherins consist of Pcdhδ and solitary protocadherins. Adapted from [34].

Unlike cadherins, protocadherins have more than five cadherin domains in the extracellular domain and they do not have the characteristic properties of the cadherin ectodomains [35]. Their cytoplasmic domains that lack catenin binding site are highly variable and is able to interact with various proteins. Protocadherins have been found in a variety of organisms whereas cadherins are found only in vertebrates. Based on this knowledge, it is thought that current classical cadherins evolved from one of the protocadherin type [36, 37].

There are variety of reports about the functions of protocadherins. According to transfection experiments, protocadherins are found at cell to cell contact sites and via homophilic binding activity they mediate cell sorting in a Ca^{2+} dependent manner. However protocadherins are much weaker compared to cadherins in the context of cell adhesion. One important reason of the weak adhesion is that protocadherins do not interact with cytoskeletal proteins. Obata and his colleagues reported that when they construct chimeric protein composed of Pcdh2's extracellular domain and the E-cadherin's cytoplasmic domain, it shows stronger adhesive activity compared to wild type Pcdh2 protein [38]. This clearly indicates that, protocadherins because they do not bind to the cytoskeleton, they do not mediate strong adhesion. They do not interact with catenins yet their cytoplasmic domains interact with various cytoplasmic proteins. For instance, γ-Pcdh cytoplasmic domain interacts with FAK (Focal adhesion kinase) and Pyk2 (Proline rich tyrosine kinase 2) and inhibits their kinase activity [39]. It is also reported that there exist a protocadherin which contains sequence that interacts with SH3 domain [37].

Generally, the role of protocadherins in cell adhesion is unclear. Adhesion mediated by protocadherins is much weaker than cadherins [40-43]. In addition, protocadherins do not only mediate cell adhesion, sometimes they antagonize cell adhesion. Xenopus paraxial protocadherin was shown to cause cell sorting via inhibiting C-cadherin mediated adhesion [4]. Therefore, each protocadherin has unique interaction partners and they mediate cell adhesion or play indirect roles by modulating classical cadherin adhesion activity.

2.2.3 Protocadherin 7 (Brain Heart protocadherin, BH-Pcdh, Pcdh7)

The mechanisms of classical cadherin adhesion have been widely studied, whereas not much is known about the protocadherins. Protocadherin 7 mechanism of action has not been studied extensively. Protocadherin 7 is a member of Pcdhδ1 family of nonclustered protocadherins. It is predominantly expressed in brain and heart (which is why they are called brain-heart protocadherins) and lower levels in various other tissues. It is a single pass type I membrane protein. Pcdh7 has an extracellular domain consisting of seven repeats of cadherin motif (EC 1-7), a transmembrane domain and a cytoplamic domain. EC2 motif of Pcdh7 is unique because it has a 55 amino acid insertion in the middle. Pcdh7 has three isoforms denoted as –a, -b and –c. Each isoform has single open reading frame which encodes 1069, 1072 and 1200 aminoacids, respectively. These three isoforms differ in the length of their cytoplasmic tails and Pcdh7-c isoform has the longest cytoplasmic tail and it has a 47 amino acid deletion in the EC2-3 region. The overall structure of the Pcdh7 shows high similarity with Pcdh1 [44]. mRNA of the Pcdh7-c is shown to be expressed highly in cerebral cortex neurons. Moreover, Pcdh7-c intracellular domain interacts with protein phosphatase 1 alpha and inhibits the action of phosphatase. The intracellular interaction partners of other isoforms remain elusive. It has been demonstrated that a and b isoform mediate homophilic calcium dependent adhesion but isoform c does not [40].

Figure 2.7 Protocadherin 7 isoforms. Unlike Pcdh7a and Pcdh7b isoforms, Pcdh7c isform cytoplasmic tail is longer and it has a 47aa deletion between the EC2 and EC3. Adapted from [40].

Pcdh7 is highly related with cancer. It has been shown that its mRNA expression is different in normal and cancerous cell lines. Only 9kb mRNA was detected in normal tissues whereas shorter, 4.5kb mRNA version of Pcdh7 was detected in human lung carcinoma cell line A549, HeLa S3, human gastric cell lines MKN28 and KATO-III. Furthermore, 4p15 region where the Pcdh7 gene localizes is a region of loss of heterozygosity in some head and neck squamous carcinomas [44]. In addition, genome wide analysis of survival in early stage non-small-cell lung cancer revealed that two of the top ten single nucleotide polymorphisms (SNPs) with highest hazard ratios are located in PCDH7 gene [45].

Cancer cells ignore cell cycle checkpoints and once these checkpoints are overridden, they begin to proliferate wildly and mitosis takes place in an uncontrollable way. This

means most of the cells in the cancerous tissue undergo mitosis repeatedly. Therefore, finding a cell surface biomarker that is found on the cell surface specifically at mitosis is important to evaluate tissue responses to anti mitotic drugs. Based on the results of Mass spectroscopy analysis of membranes in mitosis and interphase, it is found that the amount of protocadherin 7 protein on the cell surface increases during mitosis (Nurhan Özlü, PhD's unpublished data). In this Master's thesis project, our motivation was to investigate the molecular mechanism of mitosis selective biomarker candidate protocadherin 7, Pcdh7.

Chapter 3

MATERIALS AND METHODS

3.1 Cell culture and Transfection

U2OS mCherry Tubulin cells were grown in Geneticin (400ug/ul) containing McCoy's 5A medium (10% FBS, 1%PS), respectively, under 5% CO2 at 37°C. Cells were seeded and cultured on glass coverslips in a 12-well plate. Constructs were transiently transfected with using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. 1ug DNA (see Appendix A for the vectors) and 5ul Lipofectamine were transfected and cells were cultured for up to 48h. DNA and Lipofectamine were diluted in 100µl of Opti-MEM (Invitrogen) and incubated for 5min. These were then combined for 20 minutes; 200µl added on top of serum free medium containing wells and incubated at 37ºC 5% CO2 for 24h. The solution of DNA/Lipofectamine complexes and serum free medium were then aspirated and replaced with 1ml complete medium. After 48h, cells were fixed and/or stained and analyzed under the confocal microscope.

3.1.2 Immunofluorescence and Fixed Imaging

For immunofluorescence experiments U2OS cells stably expressing mCherry tubulin were transfected with different vector constructs. After 48 hour of transfection, coverslips

were washed with PBS and fixed with 3% Paraformadehyde (EM-grade, EMS) for 15 minutes. After several times washing with TBST (0.01% Triton X-100/ TBS), cells' nuclei were stained with 1ug/ml DAPI (in Abdil) for 10 minutes. Coverslips were washed with TBST several times. Finally, the stained cells were mounted in mounting medium (Sigma-Aldrich, M1289) and sealed with nail polish.

3.1.3 Confocal microscopy and Quantification

PCDH7::GFP, PCDH7-CytDel::GFP, PCDH7-MSless::GFP, PCDH7-WithMS::GFP expressing U2OS mCherry Tubulin cells were imaged with a Nikon Eclipse 90i laser scanning confocal microscope controlled by EZ-C1 Imaging software. Images were acquired using 60X or 100X oil immersion objective lenses. For quantification of the Protocadherin 7 protein on the membrane, two areas were circled for each cell: the circle that has the whole cell (larger circle) and the circle that has the whole cytoplasm but lacks the membrane (smaller circle). The integrated densities of the circles were determined by using ImageJ [Wayne Rasband, NIH] software. For each cell, the integrated density difference between the larger and the smaller circle was calculated. Then this difference was divided to the smaller circle to normalize the data. The data of the individual experiments was combined and the graphs were drawn.

3.2 Whole Cell Lysate Preparation

Cells were cultured on 10cm plates. When cells reach 90% confluency, plates were washed with PBS and for detachment from the plates cells were incubated with trypsin/EDTA (Sigma Aldrich). After 5 min, trypsin/EDTA was deactivated with cell culture media (depend on the cell type) and the cell-media mixture was transferred to the

15ml falcon tubes. After 5 minutes centrifugation at 1200rpm at 4°C, cells were pelleted. Pellets were washed with PBS and centrifuged again to obtain cell pellet again. Subsequently, these cells were lysed using lysis buffer (0.1 % Triton X-100/ PBS). Before being used in Western blot analysis, lysates were precleared by centrifugation and denaturated with sample buffer (100mM DTT/Laemmli) by incubation at 80°C for 5 min and protein concentrations were determined using a BCA protein assay (Thermo Sicentific) as described in the manufacturer's manual.

3.2.1 SDS-PAGE and Western Blotting

Electrophoretic separation and analysis of the proteins for confirmation of expression and purification steps were performed by SDS-PAGE following by western blotting. Equal amounts (75 ug) of protein samples were electrophoresed on a 12% SDS-PAGE. Gels subjected to 160 V for 1.5 h for the separation of proteins. Then, gel was transferred to nitrocellulose membrane (Whatman Protran BA85) with transfer cell at 100V for 1h. After 30 minutes blocking with 4% milk (in 0.01 % Tween20/ TBS), the membrane was incubated with primary antibodies overnight at 4°C. Then the membrane was incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) 1h at room temperature. Proteins were visualized with the ECL (Pierce ECL western blotting substrate 32106) system according to the manufacturer’s instructions.

3.3 Latrunculin B treatment

U2OS mCherry Tubulin cells were transfected with PCDH7-CytDel::GFP fusion gene and before the time of fixation cells were treated with DMSO or 1uM Latrunculin B (Santa Cruz) drug (in culture media) for 1h at 37°C. After an hour, cell were washed several times

with PBS (+0.1 mM $CaCl_2$, 1mM $MgCl_2$) and fixed immediately as in section 3.1.2. Images were acquired using a Nikon Eclipse 90i laser scanning confocal microscopy using 60X oil immersion objective lenses and the quantification for the protein localization was done by using the ImageJ [Wayne Rasband, NIH] software.

3.4 Cell Synchronization with Double Thymidine Block and STC

HeLa cells were arrested at interphase and with the double thymidine block protocol. To arrest cells at mitosis, cells were treated with kinesin 5 inhibitor, S-trityl-L- cysteine (STC) for 12 hours after a double thymidine block and release. Shortly, cells were seeded on plates and at 80% confluency washed with PBS and incubated for 20 hours with DMEM high glucose complete media (10%FBS, 1% Pen-Strep, 1% Glutamine) + 2mM Thymidine (Calbiochem). Thymidine was removed by washing 3 times with PBS and fresh DMEM high glucose complete media was added to release cells. After 8 hours of release, cells were incubated for another 19 hours with DMEM high glucose complete media (10%FBS, 1% Pen-Strep, 1% Glutamine) + 2mM Thymidine for second thymidine block. At the 19^{th} hour cells will be at the interphase stage and pooled for subsequent analysis. To arrest cells at mitosis, at the 19^{th} hour, cells were washed 3 times with PBS to remove thymidine and incubated with 10 μM S-trityl-L-cysteine (STC) (Alfa Aesar) in DMEM high glucose complete media for 12 hours. At the end of the 12^{th} hour, 95% of cells were at the mitosis phase and pooled for subsequent analysis.

3.5 Sucrose Gradient Membrane Fractionation and Biotinylation

Mitosis and interphase stage arrested HeLa cells' membranes were isolated with the sucrose gradient and biotinylation protocol (see Appendix C for experimental workflow).

All procedures were carried out at 4°C or in crushed ice. Firstly, cells were washed several times with ice cold PBS (+0.1 mM $CaCl_2$, 1mM $MgCl_2$) and then intact cells were incubated with 0.5mg/ml NHS-ss-biotin in PBS (+0.1 mM $CaCl_2$, 1mM $MgCl_2$) for 30 minutes at 4°C. Cells are then rinsed with PBS (+0.1 mM $CaCl_2$, 1mM $MgCl_2$) with 100mM glycine pH 8.0 to make sure all of the unreacted biotin was quenched. Cells were washed several times with Lysis Buffer 1 (10mM Hepes, 1.5mM $MgCl_2$, 10mM NaCl, 1mM EDTA). Cells in Lysis Buffer 1 were scraped from the plates with the cell scraper, collected into 15ml falcon tubes and pelleted by centrifugation (2000rpm, 5min at 4°C). Supernatant was removed and pellets were resuspended with Lysis Buffer 1 plus protease inhibitor (Pierce). Suspension was left to sit on ice for 15 minutes for cell swelling. After that, suspension was placed in a 1ml Dounce homogenizer fitted with a tight pestle and was subjected to 20-30 downstrokes of the pestle (air pocket avoided on return stroke). The degree of cell rupture was checked by phase contrast microscopy. When the cell rupture was not sufficient, the rupturing was continued by syringing the suspension several times. DNA was pelleted twice by centrifugation at 2000rpm 1 minute at 4°C. Supernatant was used for the further steps. Fresh ice cold sucrose solutions were prepared (1.1M, 1.3M, 1.7M and 1.9M). 2ml of each sucrose solutions were decanted slowly into the ultracentrifuge tubes (1.1M on the top and 1.9M on the bottom) by avoiding mixing. About 800ul cell supernatant was added on top of sucrose layers and ultracentrifuged at 25000rpm for 16h at 4°C. Supernatant was removed and pellet was washed carefully with dH_2O. After that, pellet was dissolved in Lysis Buffer 2 (10mM TrisCl pH 7.5, 0.5%SDS, 2% NP-40, 150mM NaCl, 1mM EDTA, 1mM EGTA, 10mM Iodoacetamide). 100ul of 500ul suspension was taken and same amount of Laemmli sample buffer (with 100mM DTT) was added and boiled at 80°C for 5 min. 75ul of packed streptavidin beads were added to remaining 400ul supernatant and incubated at 4°C for 12 hours while rotating. After 12 hours 100ul aliquot of unbound supernatant was taken and again boiled in Laemmli sample

buffer (100mM DTT). Protein bound beads were washed three times with Lysis Buffer 2 and proteins were eluted from beads by adding 50ul of SDS sample buffer (100mM DTT/Laemmli) and incubating at 50°C for 30 minutes. Samples were stored at -20°C for further experiments.

3.6 esiRNA mediated depletion of PCDH7 gene

Endoribonuclease-prepared short interfering RNAs (esiRNAs) for PCDH7 was purchased from Sigma Aldrich and used to knockdown the gene. At day 0, HeLa S3 PC cells were seeded to 12 well plates. At day 1, when cells reached to 50% confluency they are transfected with the following protocol. Mixture A with 4ul Oligofectamine (Invitrogen) and 12ul OPTI-MEM (Invitrogen) was prepared. Mixture B with 8ul of esiRNA and 54ul of OPTI-MEM was prepared and after 5 minutes in room temperature, mixture A and mixture B was mixed. Final mixture was incubated at room temperature for 20 minutes. HeLa S3 cells were washed with pure DMEM and 510ul pure DMEM was added onto the wells. After 20 minutes of incubation, transfection complexes were pipet mixed and added to each well. After 4 hours, 290ul of 30% FBS containing DMEM was added on top of wells to give the final 10% concentration of FBS. The solution of RNA/Oligofectamine complexes medium were then aspirated and replaced with 1ml complete medium. After 48h cells' media were aspirated and cells were washed several times with ice cold PBS. HeLa cells were scraped with cell scraper in ice cold PBS. Then, the cell suspension was transferred into 15 ml falcon tube and centrifuged at 1200rpm for 5 minutes at 4°C. Supernatant was aspirated and pellet was dissolved in lysis buffer (PBS+ 0.1 Triton X) at 4°C. After centrifugation at 75000rpm at 4°C, all the cells debris and DNA were pelleted. Supernatant was taken and same amount of SDS sample buffer (100mM DTT/Laemmli) was added and heated at 80°C for 5 minutes. Samples were stored at -20°C.

Samples were checked with Western Blotting for gene knockdown. (Protocol was adapted from Yusuke Toyoda, PhD from Max Planck Institues.)

The antibodies used for western blotting are listed in Table 3.1.

Table 3.1 Western Blotting antibodies and dilutions

Antibody	Host	company	Catalogue no	Dilution
Anti-phosphoH3 pSer10	Rabbit	Thermo Scientific	PA5-12526	1:200
Anti-beta Actin (AC-15)	Mouse	Thermo Scientific	MA1-91399	1:7500
Anti-EGFR (1005) sc-03	Mouse	Santa Cruz	F1412	1:100
Anti-PCDH7 antibody [2G6]	Mouse	Abcam	ab139274	1:500
Anti-rabbit IgG, HRP-linked	Goat	Cell Signaling	#7074	1:2000
Anti-mouse IgG, HRP-linked	Horse	Cell Signaling	#7076	1:2000

The antibodies used for immunostaining are listed in Table 3.2.

Table 3.2 Immunostaining antibodies and dilutions

Antibody	Host	company	Catalogue no	Dilution
Anti-α tubulin (DM1a)	Mouse	Cell Signaling	#3873	1:2000
Anti KDEL [10C3]	Mouse	Abcam	Ab12223	1:200
Syntaxin 6 (C34B2)	Rabbit	Cell Signaling	#2869	1:100
Anti-rabbit 594 conjugated	Donkey	Invitrogen	725861	1:1000
Anti-mouse IgG 594 conjugated	Donkey	Invitrogen	714258	1:1000

Chapter 4

RESULTS

4.1 Cell Cycle Dependent Localization of Pcdh7

We firstly aimed to demonstrate Pcdh7's cell cycle dependent localization with a biochemical approach since Nurhan Özlü, PhD. showed that Pcdh7 protein is a mitotically enriched cell surface protein (unpublished data, see Appendix A). Briefly, we arrested HeLa S3 cells at mitosis and interphase stages of the cell cycle by double thymidine (arrest at G1/S border) and STC (M phase arrest) method then labeled with NHS-SS-LC-Biotin which selectively binds primary amine groups of membrane proteins. We used NHS-SS-LC-Biotin because NHS-ester of this reagent reacts with primary amines of proteins or peptides. The reagent does not permeate the cell membrane of the intact cell, consequently only primary amines on the cell surface will be biotinylated. After biotinylation, for the purpose of enrichment of membrane proteins, we did sucrose gradient membrane fractionation. By that we aimed to obtain membrane proteins and with streptavidin bead incubation we tried to isolate only biotinylated membrane proteins. Proteins were eluted from the streptavidin beads for Western blotting. In order to show cell cycle dependent localization, western blotting analysis was performed (Figure 4.1). To check the cell cycle arrest success, we immunobloted the mitosis and interphase arrested samples with Phosho-Histone-H3. Phosphorylation of histone H3 at serine 10 is tightly correlated with

chromosome condensation during both mitosis and meiosis [46-48]. Therefore, we checked the mitotic arrest by using phosporylation of H3 at serine 10 recognizing antibody. To evaluate membrane fraction and labeling efficiency, we immunobloted the membrane fraction and whole cell lysate samples with cell surface protein EGFR and the likely cytoplasmic contaminant actin. EGFR protein ratio to the total protein was increased in membrane fraction samples. Very abundant protein actin was also recovered to some extent in the membrane fraction yet compared to whole cell lysate its amount is highly reduced. Based on these two indicators we confirmed that membrane fraction and labeling was successful to obtain cell surface proteins. Pcdh7 protein levels are same in mitosis and interphase arrested cells whereas it is more abundant in the membrane fraction of cells that were arrested at mitotis stage. This result demonstrates that Pcdh7 is localized on the cell surface in a cell cycle dependant manner.

After biochemical representation of the localization of Pcdh7, we wanted to display its cell cycle dependent localization by imaging of transiently expressed GFP tagged PCDH7 gene. We treated cells with STC 36 hours after the transfection and at 48^{th} hour we took images of mitosis and interphase cells with a confocal microscope. We have used unsynchronized U2OS cells that are stably expressing mcherry tubulin. At interphase, Pcdh7 localizes mostly within the cell (Figure 4.2). On the other hand, Pcdh7 decorates the surface of mitotic cells (Figure 4.2). This result again demonstrates that Pcdh7 localizes to the cell surface at mitosis.

To further demonstrate the increased membrane localization of Pcdh7 in a cell cycle dependent manner, we transfected cells with GFP tagged PCDH7 gene (see Appendix B) and arrested them at mitosis and interphase by using double thymidine block and STC treatment. Then, we obtained images of transfected cells with confocal microscope and quantified the fluorescence intensities of membranes of mitosis and interphase cells with Image J software [Wayne Rasband, NIH]. Graph was produced based on the fluorescence

intensities of cell membranes and it clearly shows that Pcdh7 localization on the cell surface in mitotic cells is higher than the localization of protein on the cell surfaces' of interphase cells (Figure 4.5).

4.2 Cellular localization of Pcdh7

Interphase cells that overexpress GFP tagged PCDH7 gene displays bulk GFP localization near the nucleus. After we demonstrated the mitosis selective cell surface localization of Pcdh7, we wondered what the intracellular organelle is in which Pcdh7 localizes at interphase. There were two possibilities; Golgi apparatus or endoplasmic reticulum (ER). Pcdh7 is a membrane protein and membrane proteins are synthesized on the membrane of the ER and they are sent to Golgi apparatus for posttranslational modification and packaged into vesicles. Then vesicles are fused to the cell membrane and the membrane protein is localized on the cell surface. Therefore we speculated that before its journey to the cell surface it should reside within the Golgi where it might be modified and packaged for final destination. To answer this question, we first transfected HeLa S3 cells with PCDH7::GFP fusion gene and stained with Golgi marker Anti-Syntaxin 6 antibody. Syntaxin 6 protein is a member of SNARE proteins and localized to the trans-Golgi and within endosomes [49-51]. Pcdh7 and Golgi marker Syntaxin 6 does not seem to colocolize, therefore we concluded that Pcdh7 does not localize within the Golgi at interphase (Figure 4.6). The second organelle that is responsible for the transportation of membrane proteins is endoplasmic reticulum. Therefore, we suspected that if Pcdh7 does not localize within the Golgi, it should localize within ER. To reveal its cellular localization, again we transfected HeLa S3 cells with PCDH7::GFP fusion gene and stained with Anti-KDEL antibody which is an ER marker antibody. KDEL is a specific common retention sequence for the proteins that are retained within the ER [52]. It is clearly evident that Pcdh7 colocalizes with the ER marker antibody. Consequently, we convinced that Pcdh7 localizes within the endoplasmic reticulum (Figure 4.7).

4.3 Importance of cytoplasmic domain on the localization of Pcdh7

As membrane proteins are being transcribed their signal sequence part is transcribed first. Signal recognition particle (SRP) binds to the signal sequence of nascent protein which is still being attached to ribosome and brings to its membrane associated receptor (SR) on the endoplasmic reticulum where the remaining translation occurs in a membrane integrated manner. Signal sequence is cleaved after translocation to the ER membrane [53]. When the protein folds correctly, protein modification takes place and protein translocates to Golgi or is retained in the ER by some kind of specific retention mechanism. Pcdh7 is a mitosis selective cell surface protein and it localizes mostly within the ER at interphase. These results make us think that some specific modifications might lead to the protein's residence shift from ER to the membrane at the entry of mitosis. And this modifications should be on the cytoplasmic part where faces to the lumen of the ER. To clarify this, we first deleted the whole cytoplasmic domain of the protein with the hope of seeing any localization change. Luckily, we observed that cytoplasmic part deleted Pcdh7 localizes on the cell surface but still most of the protein resides within the ER which we thought as an overexpression side effect (Figure 4.3B). This result shows that cytoplasmic domain undergoes some modification and this modification or specific interactions with ER proteins cause retainment of protein within the ER at interphase. When cells enter mitosis, some specific modification occurs on the cytoplasmic domain and it directs Pcdh7 to the cell surface. In order to find the specific part on the cytoplasmic domain, we bioinformatically examined the cytoplasmic domain and found certain part (so called "modification site") that could be the targets of modification. Therefore, our next aim was to examine the role of modification site.

4.4 Role of modification site (MS) on the localization of Pcdh7

There are three serine residues in the literature located at 989th, 100th and 1011th aminoacids that are phosphorylated on the cytoplasmic part of the Pcdh7. We thought that these phosphorylations might be the possible modification sites that cause the retention of protein within the cell at interphase. In addition, we found out bioinfomatically using GPS 2.1 (Group-based Prediction System) tool that the location between 974th and 1011th amino acids are targets of CDKs. We thought this site should be important as cyclin dependent kinases regulate cell cycle. We called between the residues 974th and 1011th as modification site (MS) and we made a construct that has modification site but lacks the rest of the N terminal part (PCDH7-WithMS). Also we made a construct that has cytoplasmic domain until the MS site and called it PCDH7-MSless (for futher information about cloning and constructs see the thesis of Gürkan Mollaoğlu [54] and also see Appendix B). By doing that we wanted to find the part of the cytoplasmic domain that is responsible for the retention of protein in ER at interphase. We transfected U2OS cells stably expressing mcherry tubulin with each of PCDH7-MSless:GFP and PCDH7-WithMS::GFP fusion constructs and observed under the confocal microscope. When we examine PCDH7-MSless::GFP expressing cells, we observed that this protein also localizes on the cell surface (Figure 4.3C). Therefore, we concluded that the retention causing part is not the cytoplasmic part located between transmembrane domain and the so called modification site. This result gives us hope that when we transfect PDH7-WithMS::GFP, the protein is going to be localized within the cell. We did not expected to observe cell surface localization when we transfected PCDH7-WithMS::GFP fusion protein. Whereas there is no difference in localization between the protein that lacks whole cytoplasmic domain (PCDH7-CytDel) and the protein that has modification site (PCDH7-WithMS), Pcdh7 with modification site localizes within the ER and on the cell surface (Figure 4.3D).

In order to show the increased membrane localization of the cytoplasmic part deleted version of Pcdh7 and wild type Pcdh7, we quantified the fluorescence intensities of the membranes of cells that express each construct with Image J software [Wayne Rasband, NIH]. Quantification result depicts that cytoplasmic part deleted versions of Pcdh7 localizes on the membrane much more than the wild type protein (Figure 4.8).

We have showed Pcdh7 is a mitosis selective cell surface protein with both Western blotting and immunofluorescence technique. As a consequence, we wondered if the cytoplasmic part deleted versions of Pcdh7 also localizes on the cell surface at mitosis. In order to clarify that, we transfected U2OS cells (stably expressing mCherry tubulin) with our constructs and we arrested cells at mitosis with STC. By using confocal microscopy, we obtained images of cells that were transfected with our constructs. Cytoplasmic deleted versions of the protein also localize on the surface of the cells which were arrested at mitosis (Figure 4.4B, C and D).

The cell surface fluorescence intensities of the cytoplasmic part deleted versions were also quantified with Image J software [Wayne Rasband, National Institutes of Health, USA]. We compared the fluorescence intensities of each construct on the membrane of interphase and mitosis arrested cells. The graph which depicts the results of the quantification of membranes was plotted and for each constructs the fluorescence intensities of the membranes of mitotically arrested cells were higher than the fluorescence intensities of the membranes of interphase cells which mean PCDH7-CytDel, PCDH7-MSless and PCDH7-WithMS localization on the cell surface increase at mitosis (Figure 4.9).

4.5 Localization of Pcdh7 on the cell surface is actin dependent

Cadherins interact with actin cytoskeleton to mediate strong and stable cell to cell adhesion. Although protocadherins do not have catenin binding domains on their cytoplasmic parts that can link them to the actins, there may be weak interactions. Therefore, we expected that Pcdh7 also interacts with actin and this interaction is responsible for the dynamic localization of the protein.

In order to verify this assumption, we had tried to distrupt actin cytoskeleton with Latrunculin B agent. Latrunculin B is an actin monomer sequestering agent that blocks fast actin polymerization [55]. Firstly, we transfected U2OS cells, stably expressing mCherry tubulin, with PCDH7-CytDel construct and 1 hour before fixation of cells, we treated with the 1µM Latrunculin B (LatB) agent. We diluted LatB in DMSO, so as a control we treated cells with DMSO (same amount with the LatB solution). We treated cells with DMSO in order to show that the data we obtain did not result from DMSO.

When we examined cells under the confocal microscope, we could identify that PCDH7-CytDel localizes on the surface of the cells. In addition, after 1 hour incubation with DMSO, PCDH7-CytDel still localizes on the cell surface. On the other hand, when we distrupt actin cytoskeleton with Latrunculin B agent, we could see that the morphology of the cells were changed and also PCDH7-CytDel does not localize on the cell surface (Figure 4.10). Protein localizes within the ER. This results indicates that localization shift from ER to the cell surface is actin dependent.

The cell surface fluorescence intensities of the non treated, Latrunculin B or DMSO treated cells were quantified with Image J software [Wayne Rasband, National Institute of Health, USA.] (Figure 4.11).

4.6 Role of Protocadherin 7 on the cell surface

The question which arises after we found that Pcdh7 localizes on the cell surface at mitosis is what role it has on the cell surface during mitosis. Protocadherins which are investigated so far have cell to cell adhesion property, yet according to our results and Nurhan Özlü, PhD's Mass spectroscopy result, PCDH7 localization increases on the cell surface at mitosis. Therefore, we suspected that it has a de-adhesion property as cells enter mitosis they round up. In order to reveal its role we first wanted to examine what is happening in the case of loss of function. To provide information in this direction we tried to knock down the PCDH7 gene with PCDH7 specific esiRNAs (Sigma Aldrich). These short interfering RNAs only target our gene of interest and silence it gene specifically. We transfected HeLa S3 cells with the aid of Oligofectamine (Invitrogen) transfection reagent, and we could silence the PCDH7 successfully (Figure 4.12).

Loss of adhesion to the substratum cause cell rounding which is not depend on cell cycle because trypsinized interphase and mitosis cells that lost their retraction fibers are almost spherical. Cell rounding is the balance between osmotic pressure and the actomyosin cortex contraction [17]. With the light of this knowledge, we wanted to display Pcdh7 protein's role on the cell surface at mitosis. We could successfully deplete the protein. He depleted the Pcdh7 with esiRNA and examined the rounding pressure of the cells at mitosis that lack Pcdh7. He used Atomic force microscopy (AFM) to analyze the shape of the cells and transmitted light microscopy to analyze the cell width. The data obtained from constant height cantilever was when cells lacked Pcdh7; their rounding pressure at mitosis decreased compared to control cells (Figure 4.13). The rounding force is generated by an intact actomyosin cortex and contraction of actomyosin cortex causes rounding pressure. This means, Pcdh7 localization on the cell surface has an effect on the actomyosin contraction so that the rounding force of the cell was decreased.

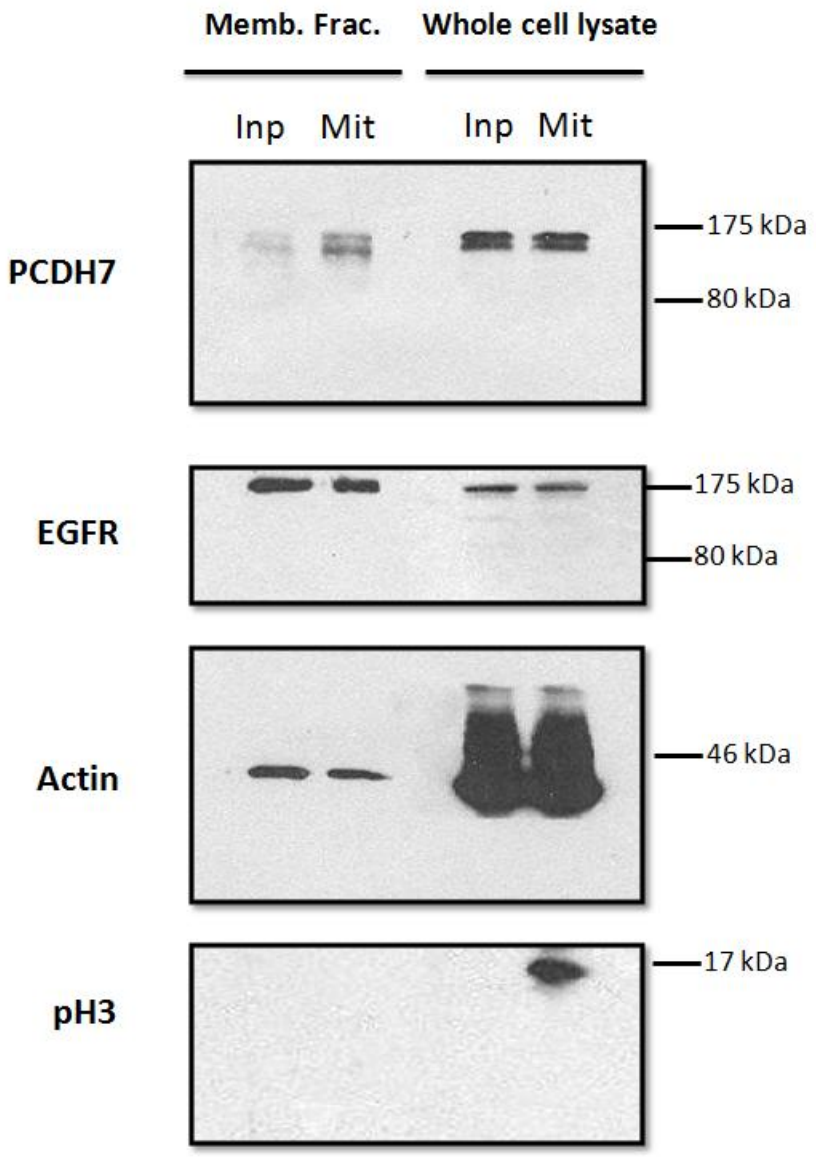

Figure 4.1 Western blot confirmation of the mitosis selective cell surface localization of Pcdh7. Membrane fraction and whole cell lysate samples were subjected to SDS-PAGE and blotted with the Anti-PCDH7, Anti-EGFR, Anti-β-Actin and Anti-pHistoneH3 antibodies, as indicated on the left. Molecular masses of the protein marker were indicated on the right.

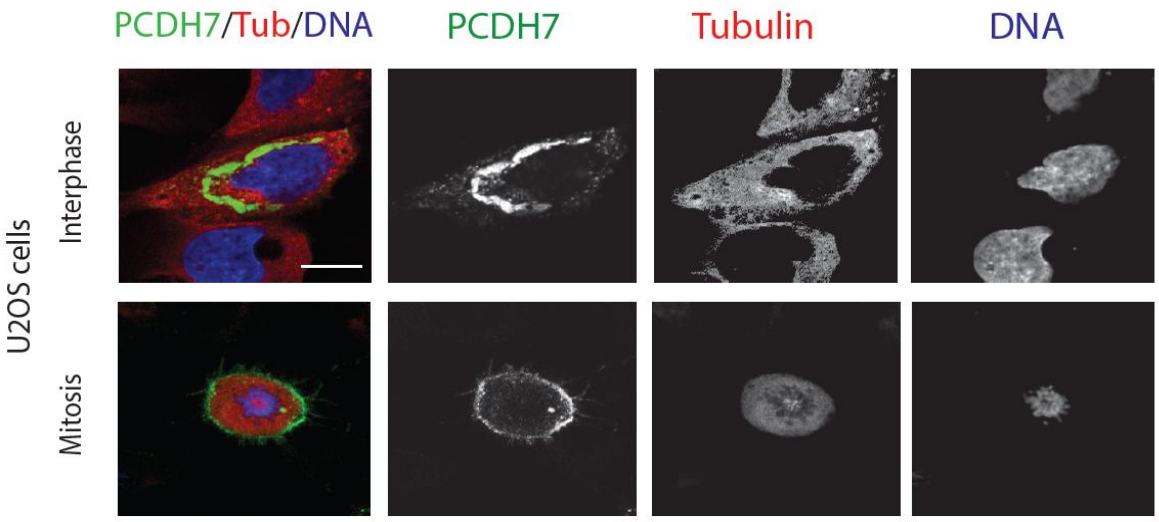

Figure 4.2 Mitosis selective cell surface localization of Pcdh7. PCDH7-EGFP transfected unsynchronized U2OS cells, stably expressing mcherry tubulin, at interphase (top) and mitosis (bottom). PCDH7, green; tubulin, red; DNA, blue. Scale bar, 20 μm.

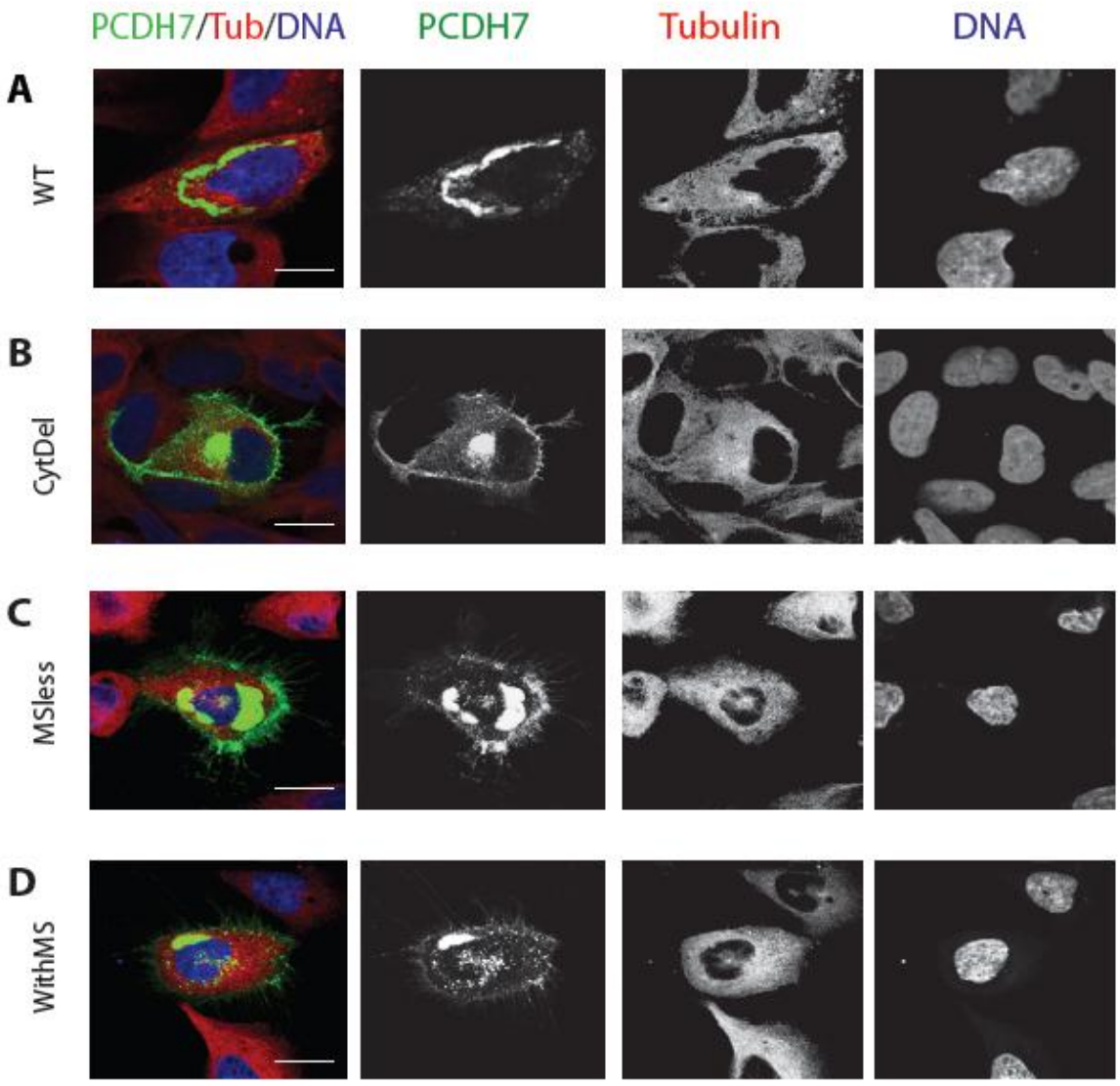

Figure 4.3 Confocal images representing the localization of Pcdh7 at interphase. PCDH7-EGFP-N1 (A), CytDel-PCDH7-EGFP-N1 (B), MSless-PCDH7-EGFP-N1 (C) and WithMS-PCDH7-EGFP-N1 (D) transfected unsynchronized, interphase U2OS cells, stably expressing mcherry tubulin. PCDH7, green; tubulin, red; DNA, blue. Scale bars, 20 µm.

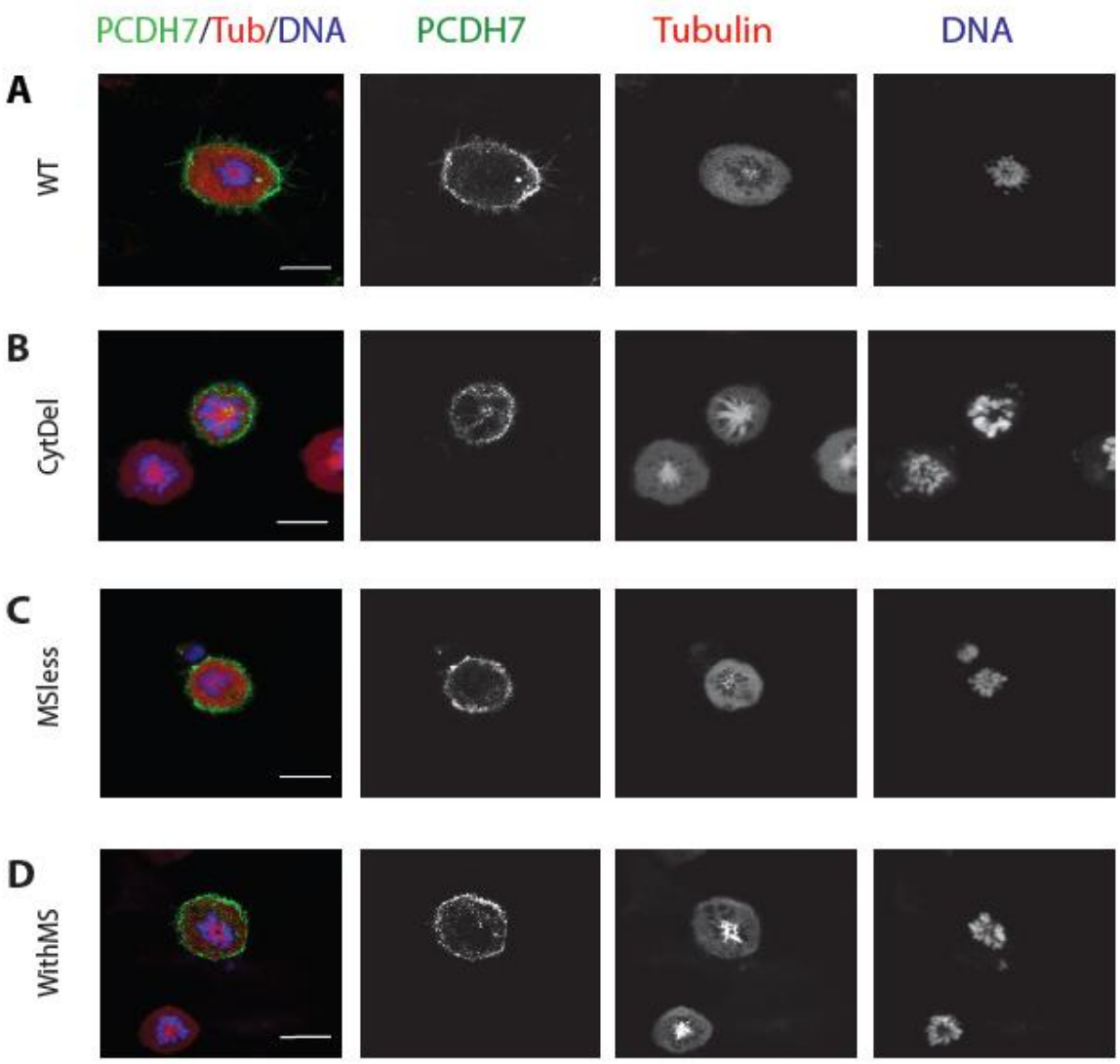

Figure 4.4 Confocal images representing the localization of Pcdh7 at mitosis. PCDH7-EGFP-N1 (A), CytDel-PCDH7-EGFP-N1 (B), MSless-PCDH7-EGFP-N1 (C) and WithMS-PCDH7-EGFP-N1 (D) transfected STC treated, mitotic U2OS cells, stably expressing mcherry tubulin. PCDH7, green; tubulin, red; DNA, blue. Scale bars, 20 µm.

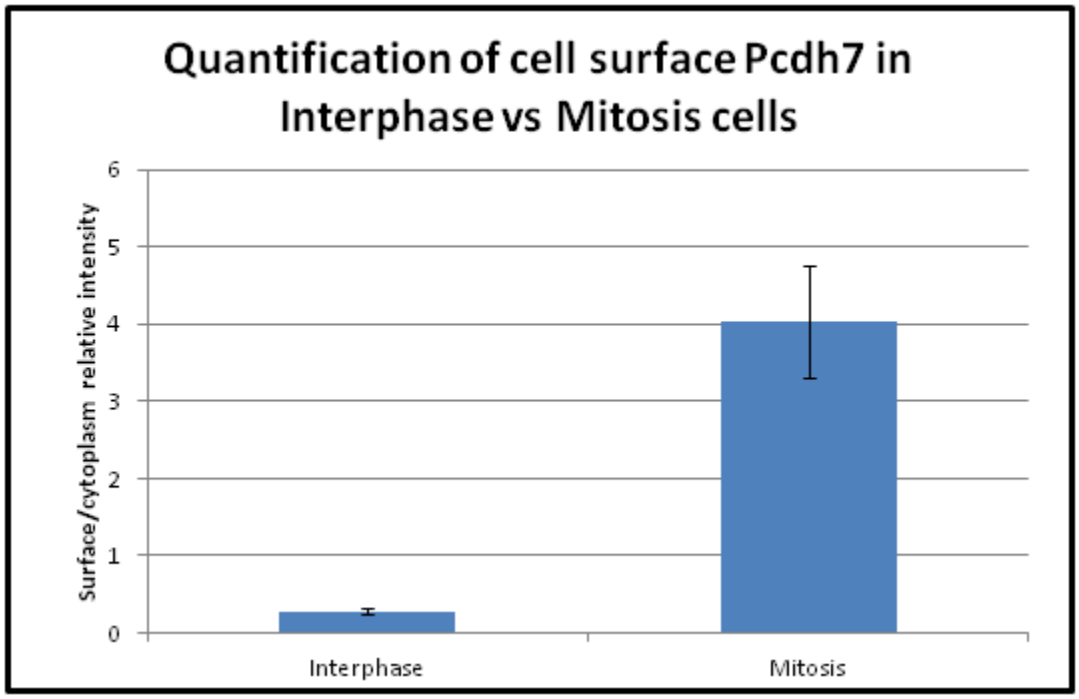

Figure 4.5 Cell surface Pcdh7 fluorescence quantification graph. Pcdh7 protein localization on the cell surface of mitotic cells is higher than the interphase cells.

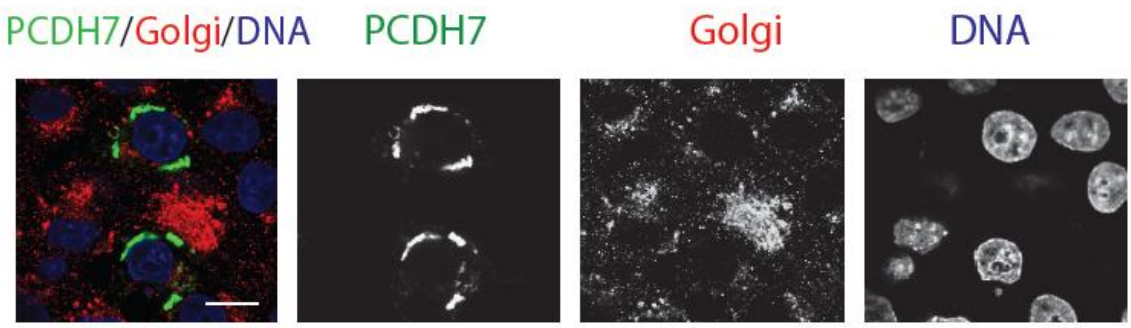

Figure 4.6 Pcdh7 does not localize within the Golgi at interphase. HeLa Kyoto cells were transfected with PCDH7-EGFP fusion gene and stained with Golgi marker antibody. PCDH7, green; Golgi, red; DNA, blue. Scale bars, 20 µm.

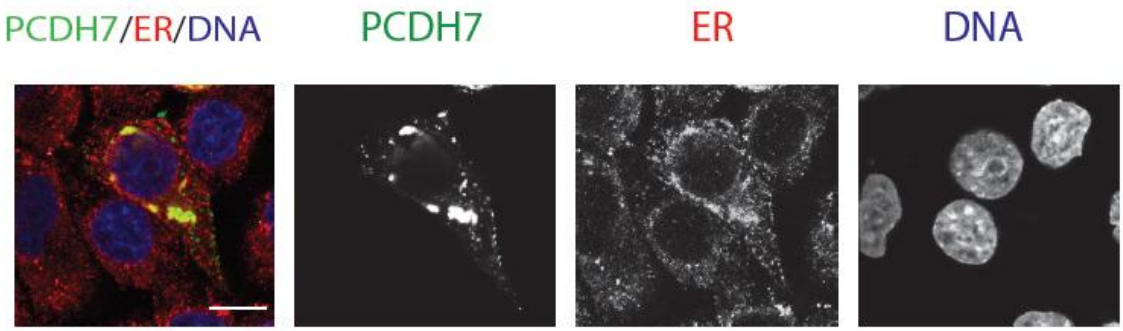

Figure 4.7 Pcdh7 localizes within the ER at interphase. HeLa Kyoto cells were transfected with PCDH7-EGFP fusion gene and stained with ER marker antibody. PCDH7, green; ER, red; DNA, blue. Scale bars, 20 µm.

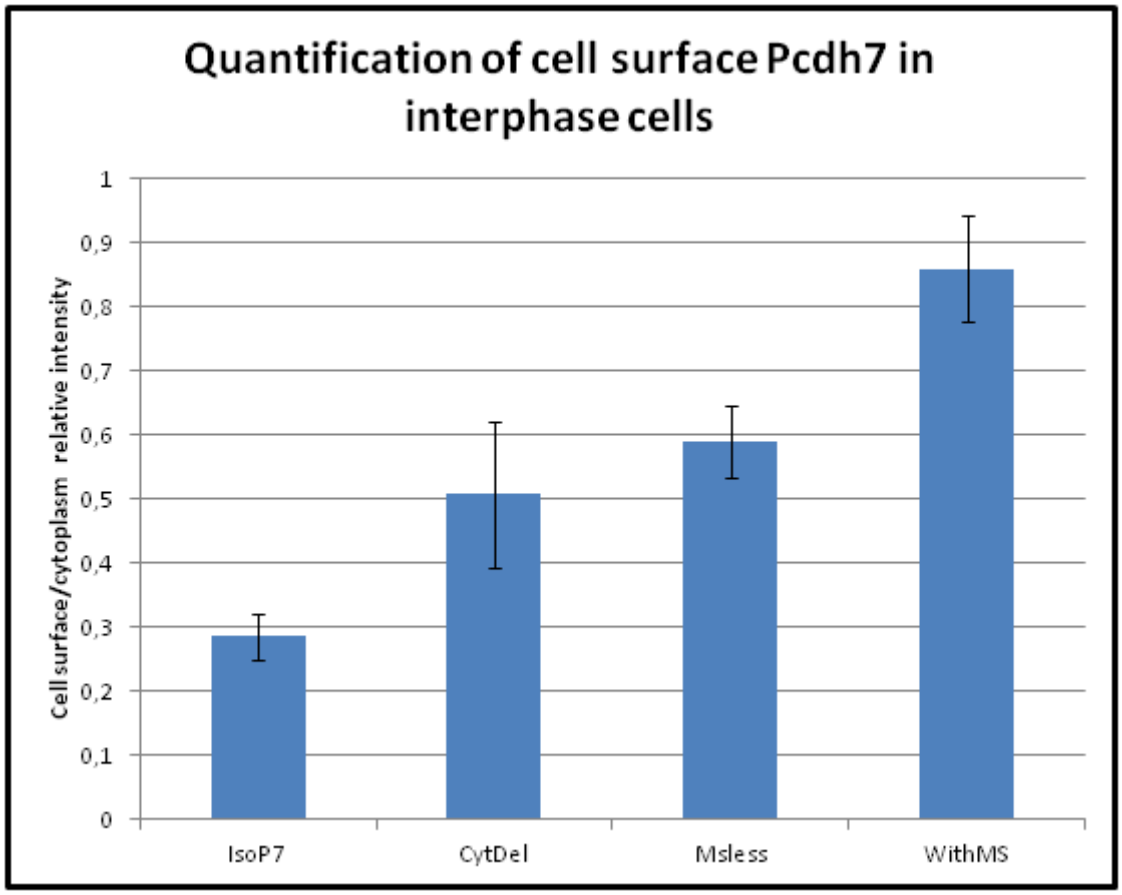

Figure 4.8 Quantification graph of membrane localization of Pcdh7 constructs at interphase. When Pcdh7 lack whole or partial cytoplasmic domain, membrane localization on the protein has increased.

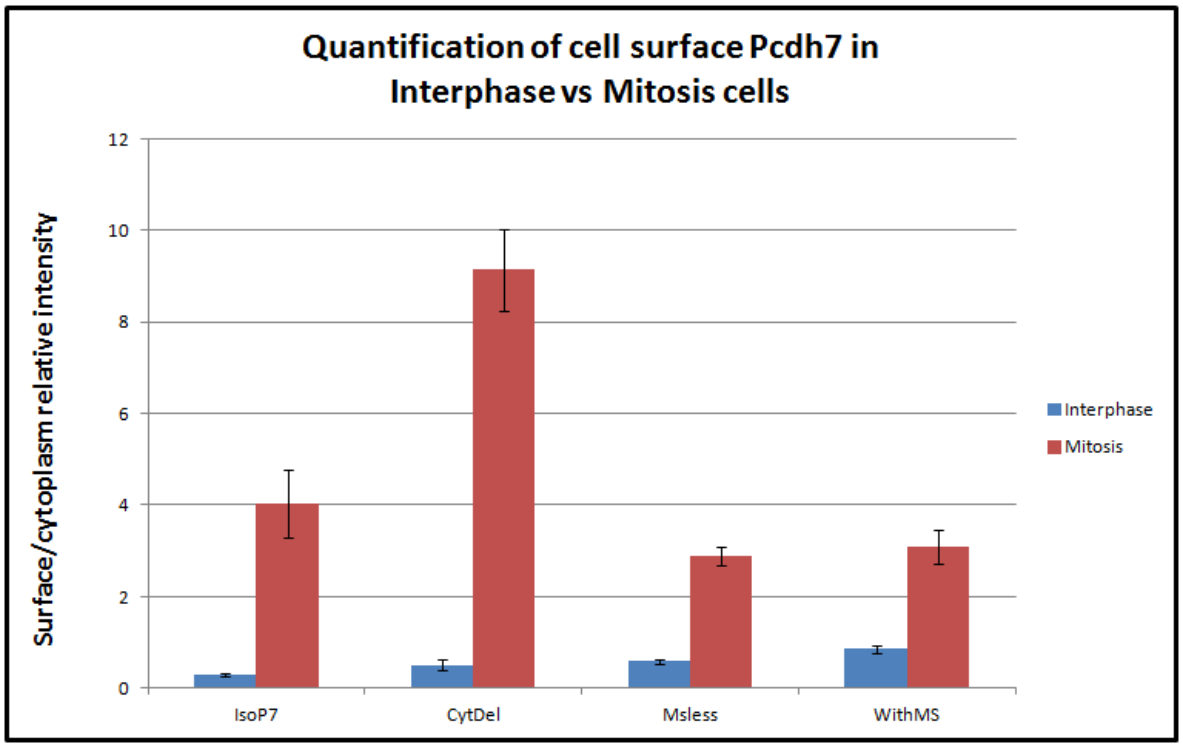

Figure 4.9 Quantification of cell surface Pcdh7 in interphase versus mitosis cells. For each construct, Pcdh7 localization on the cell surface is higher in mitosis cells.

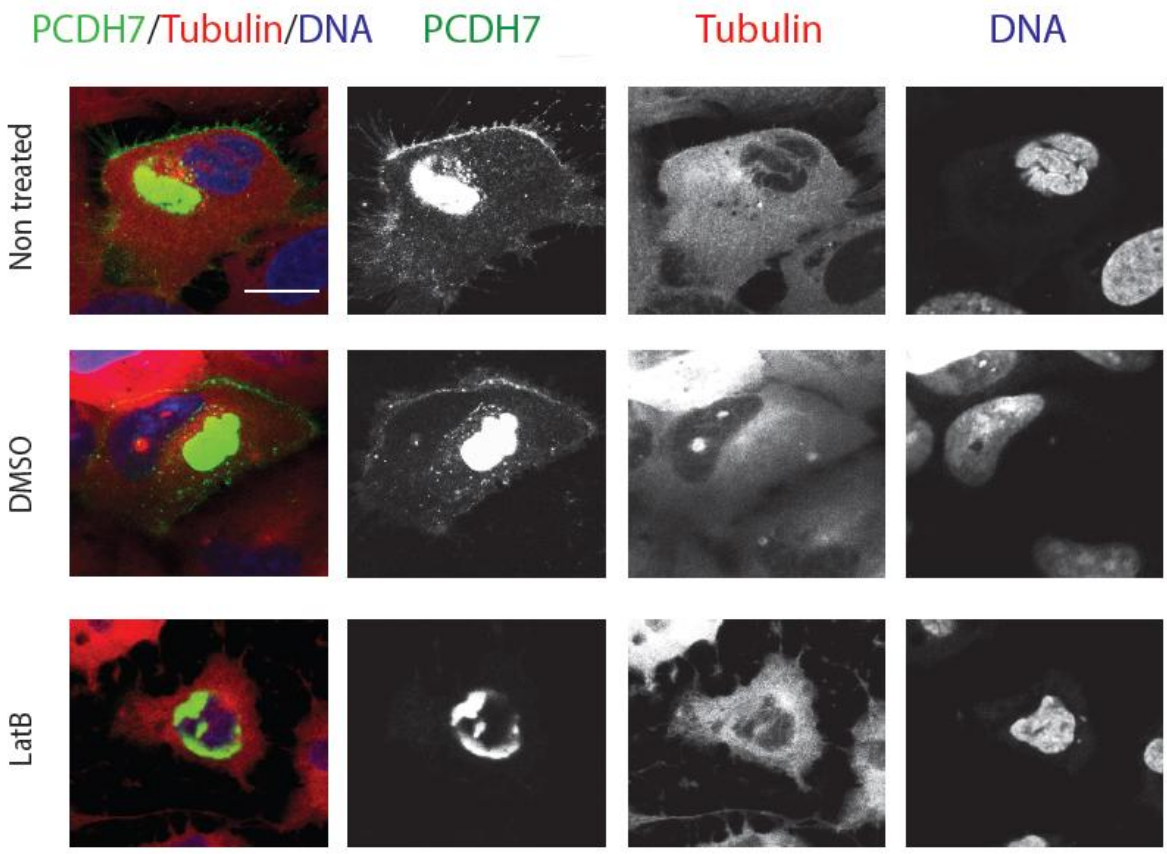

Figure 4.10 Pcdh7 localization on the cell surface is actin dependent. U2OS cells, stably expressing mcherry tubulin were transfected with PCDH7-CytDel::GFP fusion gene and before fixation cells were treated 1h with DMSO or Latrunculin B. PCDH7, green; tubulin, red; DNA, blue. Scale bars, 20 µm.

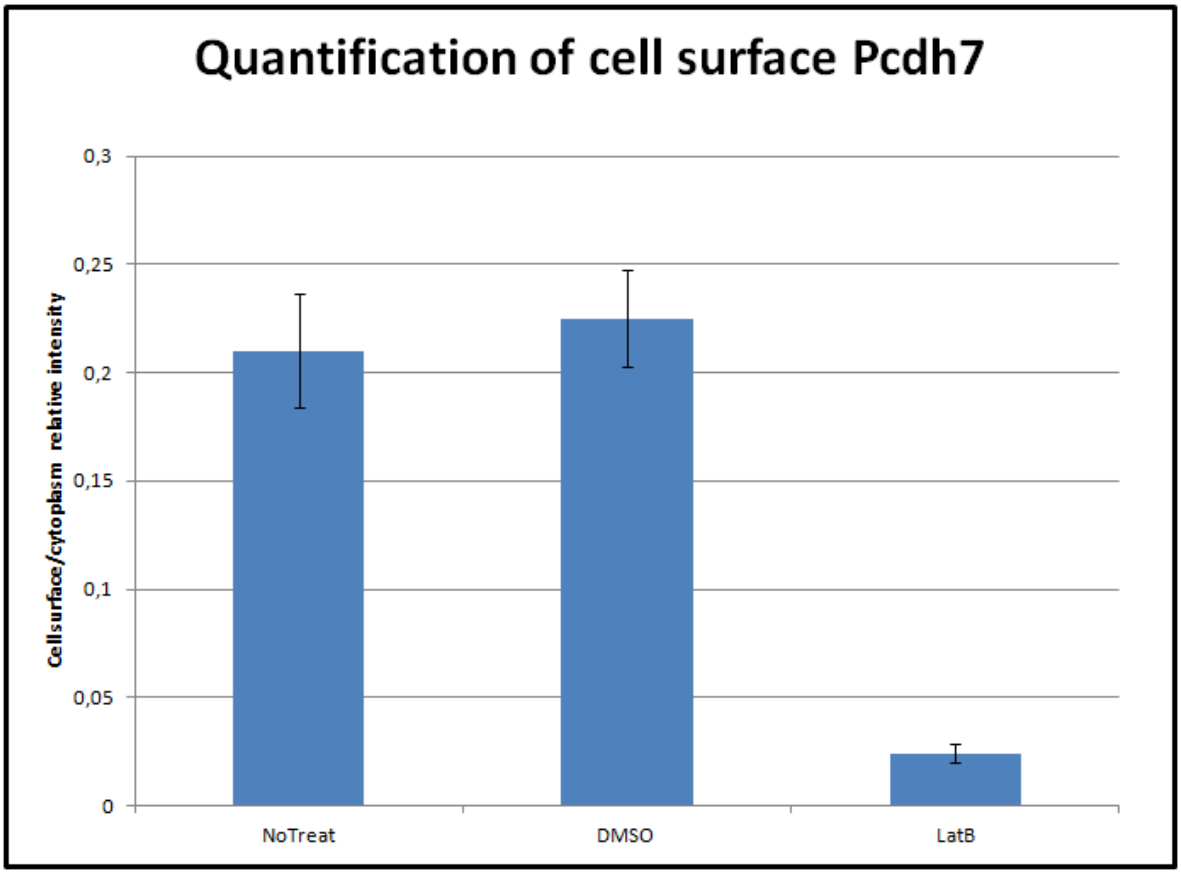

Figure 4.11 Quantification of cell surface Pcdh7 in Latrunculin B treated cells. U2OS cells stably expressing mCherry tubulin were treated with 1µM Latrunculin B for 1 hour. As a control cells were treated with DMSO or not treated with any substance.

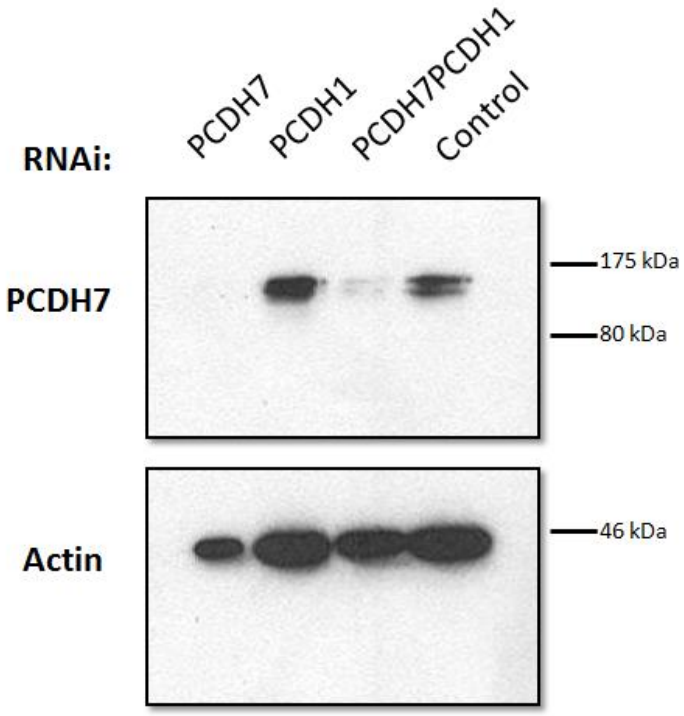

Figure 4.12 esiRNA mediated depletion of PCDH7 gene. Knockdown of the PCDH7 gene was showed with PCDH7 antibody and actin was immunoblotted as a loading control. HeLa S3 cells were treated with indicated esiRNAs. In the lane 1, cells treated with PCDH7 specific esiRNA. In the lane 2, cells treated with PCDH1 specific esiRNA. In the lane 3, cells were treated with both PCDH7 and PCDH1 esiRNAs. In the last lane, HeLa S3 cells were not treated with none of the esiRNAs.

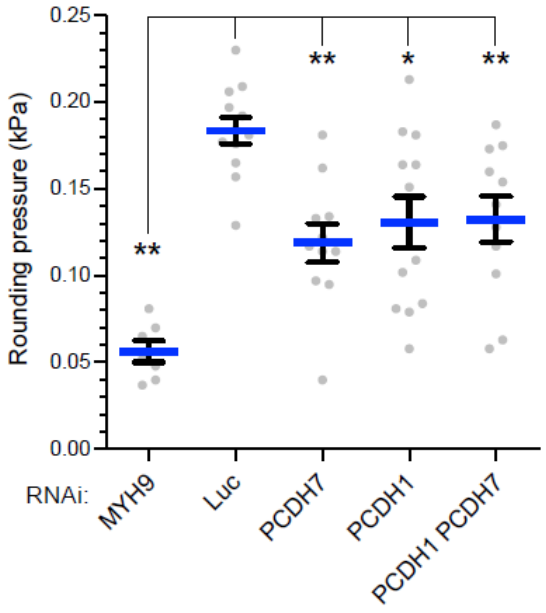

Figure 4.13 Constant height assay with PCDH7 depleted cells. Rounding pressure of the cells decreased when PCDH7 has been depleted. (Courtesy of Yusuke Toyoda, PhD, MPI-Dresden). Depletion of MYH9 was used as a positive control while Luc depletion was used as a negative control. PCDH7 and PCDH1 was depleted by using gene specific esiRNAs. In the last RNAi sample, PCDH7 and PCDH1 was depleted at the same time.

Chapter 5

DISCUSSION

Throughout cell division different cellular complexes undergo dramatic re-organization in a coordinated manner. Cell surface morphology also undergoes drastic changes during mitosis. Intracellular mechanisms that control cell division cycle have been widely studied over the past few years, whereas not much is known about the changes in cell surface during cell cycle. While cells are dividing, adherent cells undergo cell retraction and become rounded due to down regulation of adhesive systems and reorganization of the actin cytoskeleton. Cell to cell adhesion is especially mediated by cadherin superfamily of proteins. Protocadherins are the largest subfamily of cadherin superfamily and they are found at the cell-to-cell contact site. It is thought that via homophilic binding activity they mediate cell sorting in a Ca^{2+} dependent manner. Cadherin family proteins are investigated extensively as it is known that they are the primary cell to cell adhesion proteins yet recent data indicate that protocadherins are important in cell adhesion as much as cadherins.

In this Master's project, I aimed to investigate the cellular mechanism of one of the protocadherin family members, Protocadherin 7 (Pcdh7) in mammalian cells. Although it is known that PCDH7 is expressed primarily in brain and heart and also it is highly related to tumor formation, the molecular mechanism of the protein is unknown.

Based on the results of Mass spectroscopy analysis of membranes of mitosis and interphase arrested cells, it is found that the amount of Protocadherin 7 protein on the cell surface increases during mitosis (Nurhan Özlü, PhD's unpublished data). Therefore I first

wanted to display endogenous Protocadherin 7 localization at mitosis and interphase with a biochemical approach. I biotinylated cell surface proteins of mitosis and interphase stage arrested cells and by sucrose gradient centrifugation, we obtained biotinylated membrane proteins. My results are highly correlated with the Mass Spectroscopy data that Pcdh7 localization on the cell surface increases during mitosis which strengthens the idea that it is a deadhesion protein.

After biochemical representation, I wanted to show its cell cycle dependent localization shift with fluorescence imaging. Human PCDH7 protein with GFP tag was cloned. I successfully showed that at interphase, Pcdh7 localizes within endoplasmic reticulum. During mitosis, protein shuttles to the cell surface and localizes on the cell membrane. I first expected that at interphase protein should localize within the Golgi which is the final location of membrane proteins but fluorescence imaging clearly indicated that protein localizes within the ER.

Membrane proteins' function and localization is mostly regulated by the cytoplasmic domain. Thus, the question arises of whether the localization of Protocadherin 7 is regulated by the cytoplasmic domain? To address this question, a mutant PCDH7 which lacks the entire cytoplasmic domain (PCDH7-CytDel) was cloned and transiently transfected interphase and mitosis arrested cells. Fluorescence imaging showed that PCDH7-CytDel localizes on the cell surface cell cycle independently. Therefore, this result discloses that cytoplasmic domain of the Pcdh7 determines the cell cycle dependent localization of the protein.

I showed that cytoplasmic part of the Pcdh7 is important for the exact localization of the protein, yet I wanted to investigate much more to find certain subdomains that are responsible for localization control. By using bioinformatic tools, we found at least three amino acids that are targets of CDKs and we marked this region as our candidate modification site (MS). Two mutant PCDH7s with or without this modification site

(PCDH7-MSless and PCDH7-WithMS) was cloned and I visualized their localization. Interestingly, both of these mutants localized on the cell surface. These results indicate that small part at the very C terminal of the cytoplasmic domain is responsible for the retention of the protein within ER at interphase. Although I did not exactly found the minimum domain that is under cell cycle dependent regulation, I narrowed it down for further examination.

Protocadherins are the subfamily of cadherin superfamily yet unlike cadherins they do not have catenin binding domains that link them to the actin cytoskeleton. But I thought there may be weak interaction through some other unknown domains and this interaction may regulate the localization of the protein. To show whether the localization is actin dependent or not I used actin monomer sequestering agent, Latrunculin B to distrupt actin cytosekelton and examined the localization of the protein. When I treat cells with LatB and distrupted actin cytoskeleton, protein localizes within ER but not on the surface of the cell. This means Pcdh7 interacts with actin and deformations in the actin cytoskeleton cause retention of the protein inside the cell.

Finally, we performed RNAi mediated depletion of PCDH7 to see the possible role of the protein in the context of mitotic cell rounding. In order for cells to be round up during mitosis, actomyosin contraction is necessary for generating a rounded shape against adhesion. Actomyosin inhibitors reduce the rounding pressure of the cells therefore it is certain that the main reason of mitotic cell rounding is actomyosin contraction. The results of the constant height assays indicate that Protocadherin 7 depletion cause a drop of force exerted by a rounded mitotic cells. This means actomyosin cortex contraction decreased to a certain point. Protocadherin 7 existence on the cell surface remarkably affects the proper contraction of the actomyosin cortex. This result correlates with Protocadherin 7 interaction with actin that Protocadherin 7 localization on the cell surface is actin dependent and in return Protocadherin 7 mediates the proper contraction of the actomyosin cortex.These

results might give a clue of what role the protein has on the cell surface during mitosis yet it needs further investigations to understand its exact role (Figure 5.1).

For many years protocadherins role in cell adhesion has been undervalued and cadherins are thought to be the stars of cell adhesion. Yet recent studies have proved that together with cadherins they are key players in the cell adhesion. In this work, I have concentrated on the molecular mechanisms of protocadherin family protein, Protocadherin 7 and I demonstrated that it is a mitosis selective cell surface protein. This striking discovery leads us to think Pcdh7 as a cell surface biomarker for mitotic arrest that can be used to be targeted to treat cancerous tissue. Taking all into consideration, this study represented in this thesis may serve as guidance to obtain more knowledge about protocadherins, especially Protocadherin 7, by means of understanding their mechanism of action.

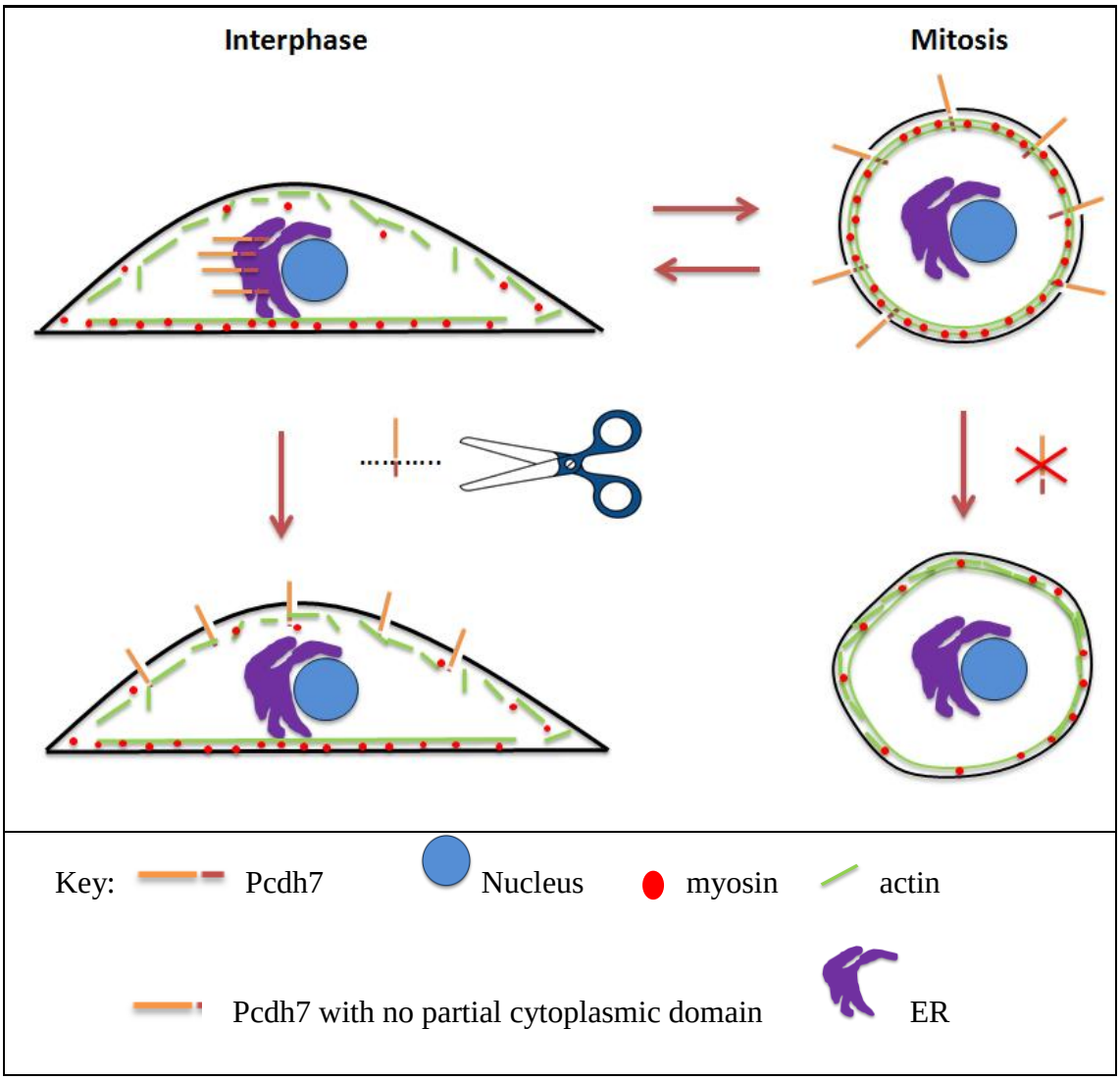

Figure 5.1 Mitosis selective cell surface localization of Pcdh7. At interphase, Pcdh7 localizes within the ER. During mitosis, because of post translational modification or the interaction partner binding to the cytoplasmic domain, it localizes on the cell surface. Pcdh7 is required for proper actomyosin contraction, without Pcdh7 cell cortex softens.

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VITA

Selda Bülbül was born in Bursa, Turkey, on May 9, 1989. She graduated from İzmir Atatürk Anatolian High School, İzmir, in 2006 and then received her B.Sc. degree in Molecular Biology and Genetics from Boğaziçi University, Istanbul, in 2011. From September 2011 to July 2013, she worked as a teaching and research assistant at Koç University, Istanbul. She studied her M.Sc. research on "Cellular Role of a Protocadherin Family Member in Mammalian Cells."

Appendix A: Mass Spectroscopy Results

About 750 cell surface proteins were identified with Mass spectroscopy. The table shows examples of mitotis or interphase enriched proteins.

M: Mitosis enriched

N: No change

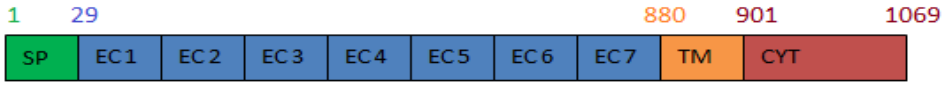
I: Interphase enriched

Gene Name	Protein	M/I_Exp1	M/I_Exp2	M-I
PCDH7	Protocadherin7	5.8	6.6	M
PCDH1	Protocadherin1	4.7	3.3	M
ITGB1	Integrin beta1	1	0.9	N
EGFR	EGF Receptor	0.8	0.9	N
TFRC	Transferrin Recep	0.3	0.3	I
GPR56	G-protein coup.	0.4	0.4	I

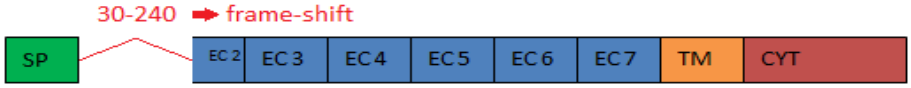
Appendix B: Diagrams of the Protein Constructs

Diagrams illustrating the protein constructs that was done by Gürkan Mollaoğlu.

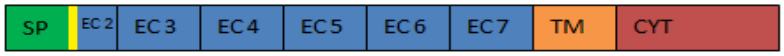
Native PCDH7



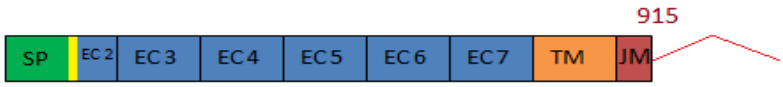
PCDH7 Clone



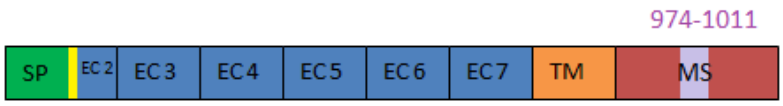
Isothermal PCDH7



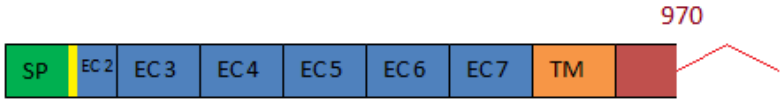
PCDH7△*CYT*



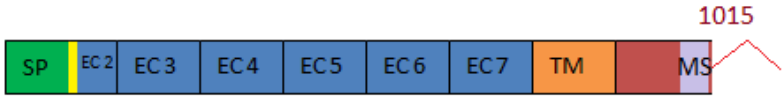
Position of MS



PCDH7 MSless

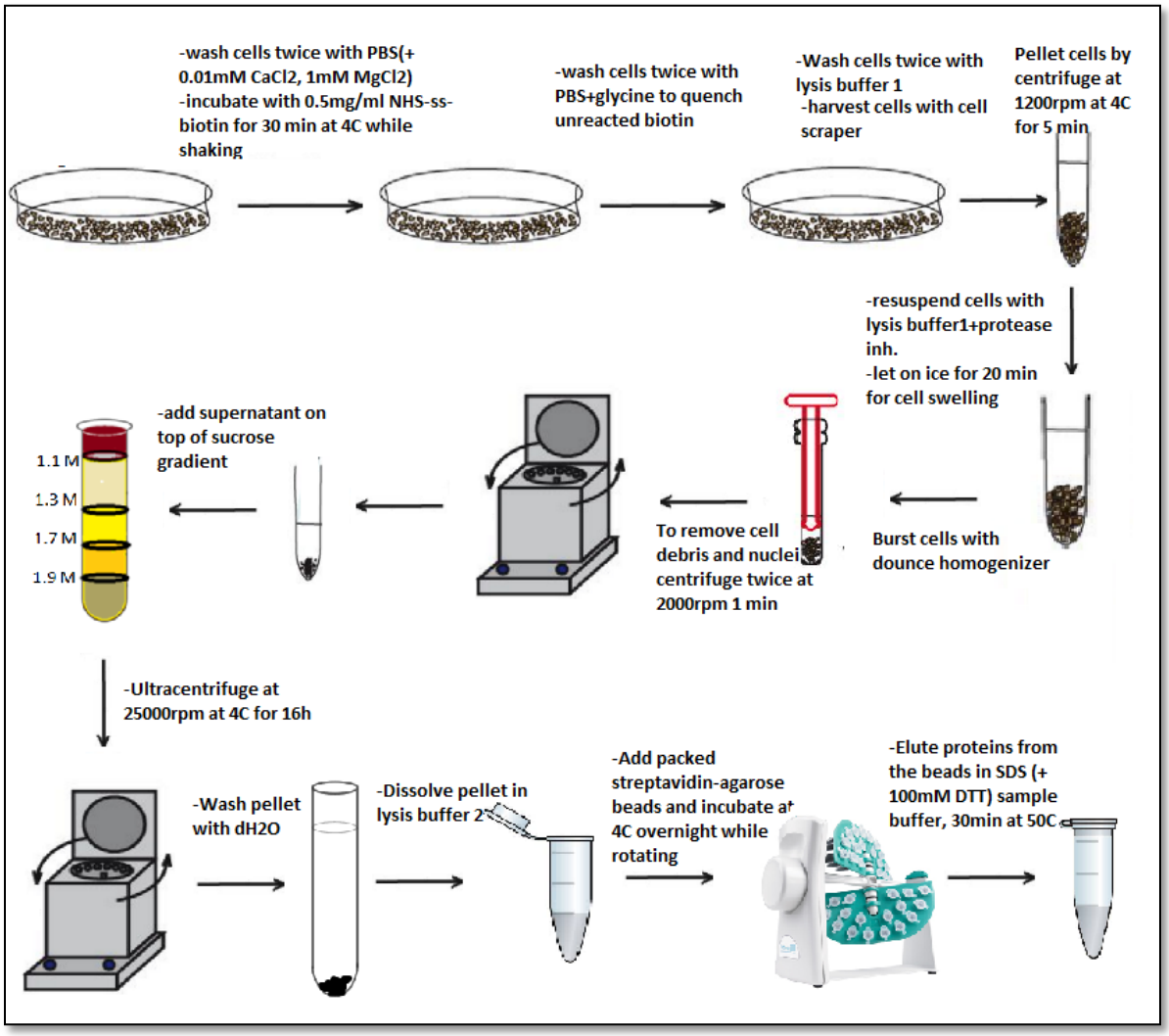


PCDH7 withMS



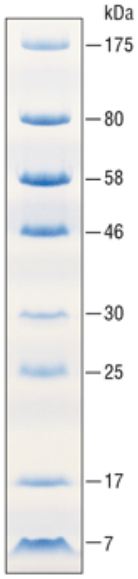
Appendix C: Experimental Workflow of Membrane Fractionation

Sucrose gradient membrane fractionation and biotinylation experimental workflow



Appendix D: Protein Marker

Protein molecular weight marker



10-20% SDS PAGE

Prestained Protein Marker, Broad Range (7-175 kDa), New England Biolabs (NEB)