

Molecular Mechanisms Underlying Mammalian Cytokinesis and Cellular Differentiation

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Molecular Mechanisms Underlying Mammalian Cytokinesis and Cellular Differentiation

Koç University
Graduate School of Sciences and Engineering

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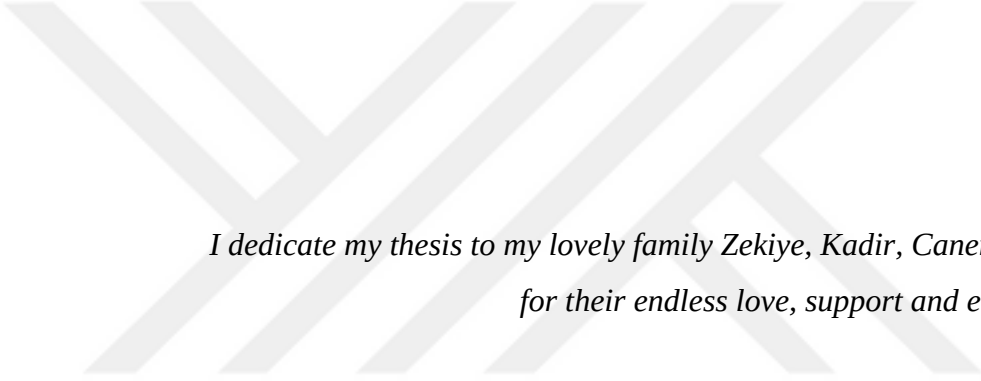
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*I dedicate my thesis to my lovely family Zekiye, Kadir, Caner, and Bechtset
for their endless love, support and encouragement.*

ABSTRACT

Cytokinesis is the last step of eukaryotic cell division, where actin cytoskeleton, plasma membrane and cell division machinery interact to physically separate newly forming daughter cells. Any mistake during this process may transform normal cells into cancer cells. However, as a highly complex mechanism, mammalian cytokinesis has still many fundamental questions that need to be answered. Our previous proteomic analysis suggested a role of chloride intracellular channel (CLIC) proteins during cytokinesis. In the first part of this study, a mass spectrometry based systematic analysis of proximity interactome revealed novel cytokinesis related interaction partners of CLIC4. We found that these interactions enhance the fidelity of cytokinesis.

In the second part, a quantitative proteomic approach was used to systematically analyze the biochemical changes that drive differentiation of human mammary epithelial cells into mesenchymal cells. Normally, the epithelial-mesenchymal transition (EMT) plays a central role in embryonic development; however, cells may use EMT as a way of cellular differentiation to facilitate the initiation and progression of metastasis and carcinogenesis. So far, several studies have been confined to the transcriptional regulation of EMT. However, transcriptome analysis may not thoroughly predict the regulation at the protein level due to post-transcriptional modifications. As a result of our proteomic analysis, several novel mRNA and/or protein level modulators of EMT were identified and validated by *in vitro* and *in vivo* experiments. Among these, we revealed DNAJB4 and CD81 as novel metastasis inducers in breast cancer. This work also provides a unique platform and resource for future studies focusing on metastasis and carcinogenesis.

ÖZET

Sitokinez, aktin hücre iskeletinin, plazma zarının ve hücre bölünmesi mekanizmasının yeni oluşan kardeş hücreleri fiziksel olarak ayırmak için etkileşimde bulunduğu, ökaryotik hücre bölünmesinin son evresidir. Bu süreç boyunca meydana gelecek herhangi bir hata normal hücreleri kanser hücrelerine dönüştürebilir. Fakat oldukça karmaşık bir mekanizma olan memeli sitokinezi hala cevaplanmaya ihtiyaç duyulan belli başlı sorular içermektedir. Daha önce gerçekleştirdiğimiz proteomik analizimiz, chloride intracellular channel (CLIC) proteinlerinin sitokinez sırasında bir rolü olabileceğini öne sürmüştür. Bu çalışmanın birinci kısmında, yakınlık bağımlı protein etkileşim ağının kütle spektrometresi temelli sistematik bir analizi, CLIC4'ün etkileşim gösterdiği sitokinez ile ilgili yeni proteinleri açığa çıkarmıştır. Bu etkileşimlerin sitokinezin doğru bir şekilde gerçekleşmesini desteklediğini bulduk.

İkinci kısımda, insan meme epiteli hücrelerinin mezenkimal hücrelere dönüşümünü sağlayan biyokimyasal değişikliklerin sistematik şekilde analizi için nicel proteomik bir yaklaşım kullanıldı. Normalde epitel-mezenkimal dönüşüm (EMT) embriyonik gelişimde temel bir rol oynamaktadır, fakat hücreler metastazın ve karsinogenezin başlamasını ve ilerlemesini hızlandırmak için EMT'yi hücrel farklılaşmanın bir yolu olarak kullanabilirler. Şimdiye kadar yapılan birçok çalışma EMT'nin transkripsiyonel düzenlemesi üzerinde sınırlandırılmıştır. Fakat, transkriptom analizi transkripsiyon sonrası değişikliklerden dolayı protein seviyesindeki düzenlemeyi tam olarak yansıtamayabilir. Bizim proteomik analizimizin sonucu olarak EMT'nin birçok yeni mRNA ve/veya protein seviyesindeki modülatörleri tespit edilmiş ve *in vitro* ve *in vivo* deneyler ile doğrulanmıştır. Bunların arasından DNAJB4'ü ve CD81'i meme kanserinde yeni metastaz indükleyicileri olarak açığa çıkardık. Bu çalışma aynı zamanda metastaza ve karsinogeneze odaklanacak çalışmalar için eşsiz bir platform ve kaynak sağlamaktadır.

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NOMENCLATURE

ALIX	Apoptosis-linked gene 2-interacting protein X
ANLN	Anillin Actin Binding Protein
APC/C	Anaphase-Promoting Complex/Cyclosome
BioID	Proximity dependent biotin identification
Cas9	CRISPR associated protein 9
CD81	Cluster of differentiation 81
Cdk	Cyclin dependent kinase
CLIC1	Chloride intracellular channel protein 1
CLIC4	Chloride intracellular channel protein 4
CRISPR	Clustered regularly interspaced palindromic repeats
DKO	Double knockout
DNAJB4	DnaJ heat shock protein family (Hsp40) member B4
ECT2	Epithelial cell transforming 2
EMT	Epithelial-mesenchymal transition
ESCRT	Endosomal sorting complex required for transport
EZR	Ezrin
ERM	Ezrin, Radixin, Moesin
FACS	Fluorescence-activated cell sorting
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GSTO1	Glutathione S-transferase omega 1
HMLE	Human mammary epithelial cells
KO	Knockout
LC	Liquid chromatography
MS	Mass spectrometry

PAM	Protospacer adjacent motif
PLA	Proximity ligation assay
RFP	Red fluorescent protein
RhoA	Ras homolog family member A
RTCA	Real time cell analyzer
SAC	Spindle assembly checkpoint
sgRNA	Single guide RNA
STC	S-trityl-L-cysteine
Twist	Twist family BHLH transcription factor 1
UPR	Unfolded protein response
WT	Wild type

Chapter 1

INTRODUCTION

CLIC4 is a member of well-conserved chloride intracellular channel (CLIC) proteins existing in both cytoplasmic and membrane-bound forms. Previously, we revealed the biochemical changes at the plasma membrane as mammalian cells undergo cell division and we identified that CLIC1 and CLIC4 are among the proteins significantly accumulating at the plasma membrane in a cell division dependent manner [1]. The cytoplasmic form of CLIC4 is structurally related to glutathione S-transferases and translocates to the plasma membrane upon RhoA activation. However, the role of CLIC4 in cytoplasmic and membrane-bound forms as well as its interaction partners remain elusive. In the first part of my thesis, the cell cycle dependent localization dynamics of CLIC4 and its function in mammalian cytokinesis were investigated. Moreover, a systematic comparison between proximity interactomes of wild type and mutant CLIC4 proteins revealed novel interaction partners of CLIC4 in cytokinesis. Our results revealed that CLIC4 enriches at the mitotic spindle microtubules and plasma membrane, accumulates at the cleavage furrow and later localizes to the midbody. Cleavage furrow localization of CLIC4 is abolished when the Cys35 and Phe37 residues of CLIC4 are mutated or RhoA activation is inhibited. We identified Ezrin, Anillin and ALIX as novel interaction partners of CLIC4 in cytokinesis. CRISPR/Cas9-based single and double deletions of *CLIC4* and *CLIC1* in HeLa S3 cells reveal a functional redundancy between CLIC4 and CLIC1 during cytokinesis resulting in the formation of multinucleated cells due to defects in the completion of late cytokinesis.

Epithelial-mesenchymal transition is driven by complex signaling events that induce dramatic biochemical and morphological changes whereby epithelial cells are converted into cancer cells. Previously, immortalized human mammary epithelial cells (HMLE) were induced to undergo EMT by expressing Goosecoid, Snail, Twist or TGF- β , or by knocking down the expression of E-cadherin gene and the transcriptome profiles of HMLE cells in epithelial and mesenchymal states were obtained as ‘EMT core signature’ [2]. Although transcriptome data represents gene regulation during EMT process, it may poorly predict the regulation at the proteome level; the mRNA levels may not correlate well with the translated proteins. In the second part of my thesis, a mass spectrometry-based quantitative proteomic approach was used to systematically analyze the biochemical changes that drive differentiation of human mammary epithelial cells into mesenchymal cells. We found that multiple alterations at the proteome level have not been represented by previous transcriptome studies highlighting the necessity of proteome level analysis during EMT. To validate several novel biochemical changes identified in this study, we first analyzed their mRNA expression levels in epithelial and mesenchymal breast cell lines, EMT score correlations and metastasis free survival rates using already published datasets and tested their effects on the migration ability of cells by using RNAi-mediated knockdown. Based on all these data, to further analyze their effects on EMT both *in vitro* and *in vivo* we selected two proteins, DNAJB4 and CD81, showing significantly increased protein expression levels in mesenchymal cells. We observed a significant inhibition of migration in mesenchymal cells after DNAJB4 and CD81 knockdown by real-time migration assays and scratch wound healing experiments. Flow cytometry analyses of HMLE cells revealed that surface CD81 expression highly increased in mesenchymal cells. Moreover, time-dependent induction of EMT by 4OHT treatment revealed that the protein expression levels of both DNAJB4 and CD81 gradually increase with the progression of EMT. Finally, we

showed that the knockdown of DNAJB4 and CD81 in invasive breast cancer cells significantly decreases primary tumor growth, spontaneous metastasis, extravasation and colonization by *in vivo* experiments.

Chapter 2 covers my studies on the role of chloride intracellular channel protein CLIC4 in cytokinesis. The detailed literature review is represented in Section 2.1. The materials and methods used for the experiments of this study are explained in Section 2.2. The results of the experiments and their detailed interpretations are represented in Section 2.3. The discussion of our results and the future directions of this study are represented in Section 2.4.

Chapter 3 covers my studies on the analysis of biochemical changes during epithelial-mesenchymal transition. The detailed literature review is represented in Section 3.1. The materials and methods used for the experiments of this study are explained in Section 3.2. The results of the experiments and their detailed interpretations are represented in Section 3.3. The discussion of our results and the future directions of this study are represented in Section 3.4.

Chapter 2

ROLE OF CHLORIDE INTRACELLULAR CHANNEL PROTEIN CLIC4 IN CYTOKINESIS

2.1 LITERATURE REVIEW

2.1.1 Cell Division

Most cells grow and divide to proliferate throughout their lifespan. Cells duplicate their chromosomes and split by dividing their cytoplasm to create progeny cells.

2.1.1.1 Eukaryotic Cell Division

Eukaryotic cells may divide via either mitosis or meiosis. Mitotic cell division is more common in eukaryotes since only sexually reproducing eukaryotes need meiotic cell division. The mitotic cell division is composed of two main stages, namely interphase and mitosis. Interphase consists of G1, S and G2 phases [3]. G1 and G2 phases are the intervening gap phases. During G1, cells grow and prepare for DNA replication. Within G1, there is a resting state called G0 in which cells stay when there is no commitment to DNA replication. Replication of DNA occurs in the S phase which is succeeded by G2. During G2, cells continue to grow and prepare for mitosis. In mitosis, separation of sister chromatids and their distribution to the newly forming daughter cells occur. Mitosis also consists of sub-stages called prophase, metaphase, anaphase and telophase. At the onset of

anaphase, cytokinesis starts and divides the parental cytoplasm into two newly forming daughter cells, which is the last step in cell division (**Figure 2.1**).

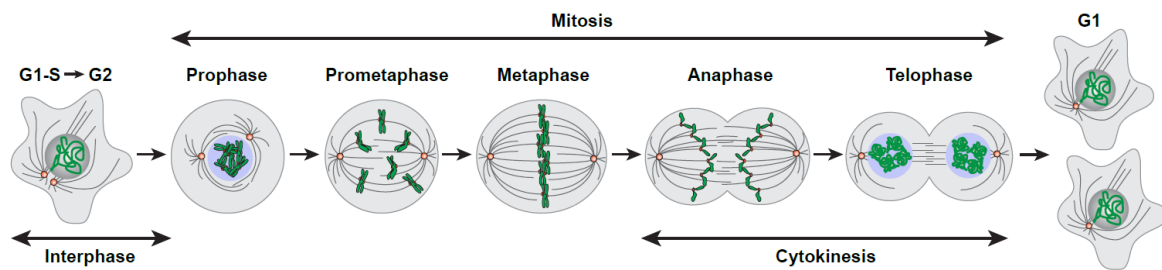


Figure 2.1: Ordered progression of cell division.

The eukaryotic cell cycle stages (Adapted from [4]).

2.1.1.2 Regulation of Cell Division

The cell cycle is driven by the expression of cyclin proteins and controlled at distinct checkpoints to ensure an orderly sequence of events. There are three major checkpoints throughout the cell cycle, namely G1-S checkpoint, G2-M checkpoint and spindle assembly checkpoint [5]. G1-S and G2-M checkpoints are positioned before entry into S phase and after DNA replication, respectively and control for the DNA damage. If DNA damage occurs during replication, in order to provide time for DNA repair, G1-S and G2-M checkpoints arrest the cell cycle. On the other hand, if the chromosomes align improperly on the mitotic spindle in metaphase, the spindle assembly checkpoint stops the cell cycle and controls progression into anaphase [6]. At G1-S and G2-M checkpoints, ATR and ATM kinases act as DNA damage sensors and they detect single strand breaks (SSBs) and double strand breaks (DSBs) in DNA, respectively. The signaling pathways that lead to the activation of DNA damage checkpoints in G1 and G2 are initiated by the checkpoint kinases ChK1 and ChK2. Following G1-S and G2-M checkpoints, in order to prevent

aneuploidy, the spindle assembly checkpoint (SAC) ensures that all chromosomes are correctly aligned on the mitotic spindles by delaying anaphase onset. Aurora kinase B (AurB) and Mps1 kinase are implicated in the activation of spindle assembly checkpoint at the onset of mitosis [7] (**Figure 2.2**).

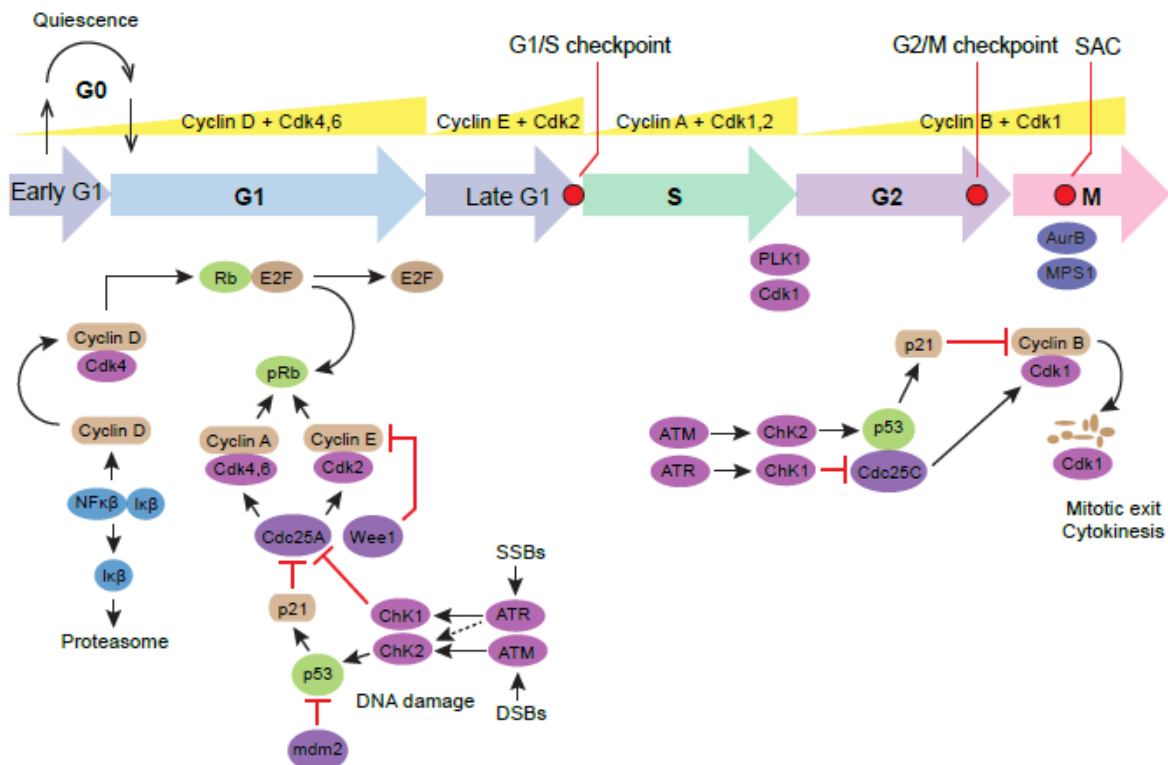


Figure 2.2: Cell cycle progression and checkpoints.

The eukaryotic cell cycle with regulatory checkpoints and protein signaling pathways (Adapted from [6, 8] and Tocris Product Guide Series BR_CancerGuide_7165).

Cyclin-dependent kinases (Cdks), a family of serine/threonine kinases activated by cyclins, are regulatory proteins of the cell cycle. During cell cycle, cyclin levels increase and decrease whereas Cdk levels do not change. This results in temporally regulated

activation of Cdks during cell cycle. When they are activated, Cdks phosphorylate their key substrates and lead to downstream processes [9]. There are different cyclins involved in the different phases of the cell cycle. Until now, nine Cdks and sixteen cyclins have been identified but not all of them are cell cycle related [6]. In order to promote the transition from G0 and the entry in G1, Cdk4 and Cdk6 make complexes with D-type cyclins. Cdk2 makes complexes with cyclin E and cyclin A for the progression from G1 into S phase and during S phase, respectively. In late G2, Cdk1 makes complex with cyclin A to control entry into mitosis and it makes complex with cyclin B to control the progression through mitosis (**Figure 2.2**). The ubiquitin ligase called anaphase promoting complex/cyclosome (APC/C) marks mitotic proteins by poly-ubiquitin chains and leads to mitotic exit through dismantling of mitotic cyclin A and cyclin B by proteasomes. By this way, the APC-dependent proteolysis of cyclins induce entry into cytokinesis [10, 11].

2.1.1.3 Mammalian Cytokinesis

Cytokinesis is the succeeding stage of mitosis and the last stage of the cell cycle. Cytokinesis has been under investigation more than 40 years, but this highly complicated process still has many fundamental questions to be answered [12]. Recent research revealed that cytokinesis of mammalian cells includes six key stages: (1) determination of the cleavage plane, (2) assembly of the contractile ring, (3) ingression of the cleavage furrow, (4) plasma membrane deposition, (5) extracellular matrix formation, and (6) formation of the midbody and abscission [13-15] (**Figure 2.3**). As a result of these sequential and highly complex processes, cell division completes and two daughter cells are separated.

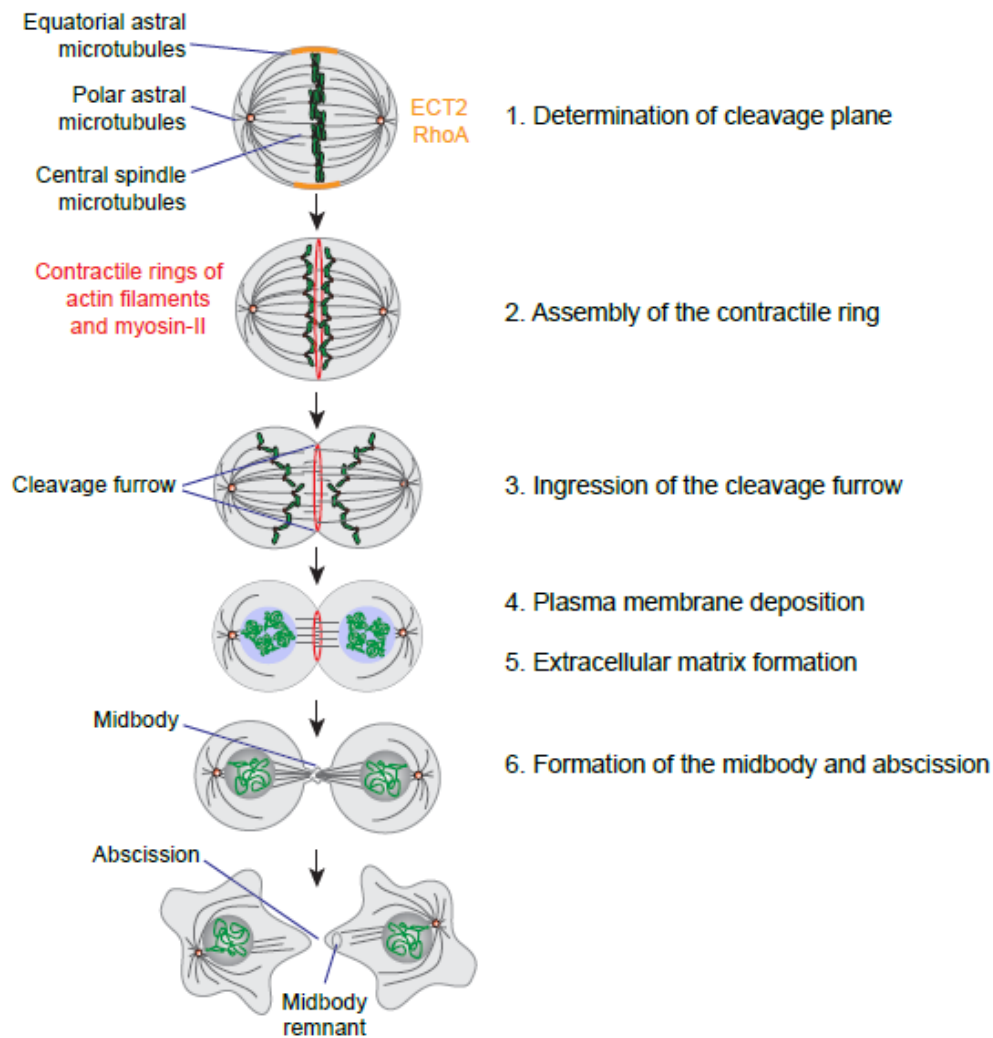


Figure 2.3: The six key stages of mammalian cytokinesis.

The mammalian cytokinesis starts with the determination of cleavage plane and ends with the completion of abscission (Adapted from [4, 14, 16]).

A cleavage plane in mammalian cells is determined by the mitotic apparatus. The mitotic apparatus includes three types of microtubules: The equatorial astral microtubules emanating radially from spindle poles to the site of cleavage, the polar astral microtubules

emanating from the spindle poles to the sites away from the site of cleavage, and the central spindle microtubules forming anti-parallel overlapping microtubules between sister chromatids after chromosomal segregation [14, 17]. In the first stage of cytokinesis, these distinct arrays of microtubules specify the cleavage plane by recruiting RhoA to the site of cleavage [17] (**Figure 2.3**).

RhoA is the central regulator of cytokinesis in mammalian cells and essential for the initiation of cytokinesis. RhoA is activated in a narrow zone within the cleavage furrow [18]. Activation of RhoA requires the guanine nucleotide exchange factor ECT2 [19]. Until late anaphase, ECT2 remains inactive due to its auto-inhibited conformation [20]. In late anaphase, ECT2 localizes to the central spindle where it interacts with the centralspindlin complex. The centralspindlin complex includes the kinesin protein MKLP1 and the GTPase activating protein MgcRacGAP [21]. The interaction between ECT2 and MgcRacGAP releases auto-inhibition of ECT2 and stabilizes ECT2 in an active conformation. Active ECT2 interacts with RhoA and then RhoA becomes activated by the exchange of GDP to GTP [14] (**Figure 2.4**). In the second stage of cytokinesis, RhoA activates formins for the nucleation of actin filaments, and promotes the phosphorylation and activation of myosin-II by activating ROCK and Citron kinases. By this way, the assembly of the contractile ring occurs at the cleavage plane [14] (**Figures 2.3 and 2.4**).

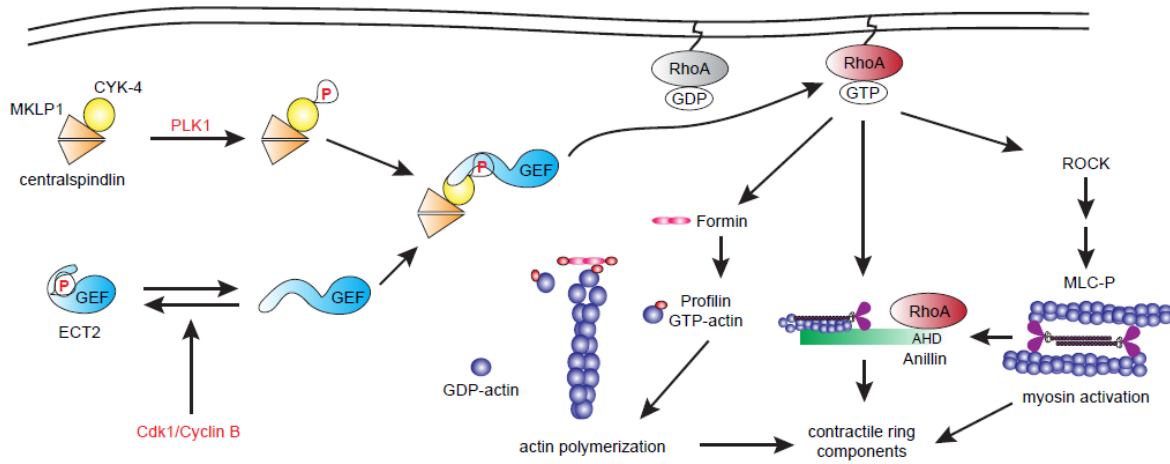


Figure 2.4: The central role of RhoA in controlling cortical contractility.

After release of its autoinhibition by centralspindlin complex, ECT2 activates RhoA by exchanging GDP on RhoA for GTP. Then, active RhoA stimulates actin polymerization and myosin activation. AHD, anillin homology domain; MLC-P, myosin II light chain phosphorylation (Adapted from [14, 16]).

In the third stage of cytokinesis, the cleavage furrow ingression starts with the formation of actomyosin ring and myosin-dependent motor activity. During cytokinesis, motor protein myosin works together with actin filaments to provide constriction. The furrow ingression continues until 1-1.5 microns in diameter and constricts the microtubule array. Together with the cleavage furrow ingression, in the fourth stage of cytokinesis, the need of plasma membrane expansion arises. Building a physical barrier to separate newly forming cells requires the plasma membrane addition and transport of specific proteins. During cytokinesis, new plasma membrane and cargoes are delivered to the plasma membrane from late Golgi by exocytosis whereas endocytosis removes excess plasma membrane and recycles exocytic proteins back from the cleavage furrow [13]. Therefore, the cleavage furrow ingression requires the coordinated activity of exocytosis and

endocytosis to expand the plasma membrane [22]. Extracellular matrix remodeling is also essential for proper cytokinesis. For instance, in mice and *Caenorhabditis elegans*, a large protein hemicentin secreted from the cells are important for stabilization and ingression of cleavage furrow [23].

In the final stage of cytokinesis, the constriction of the microtubule array results in the formation of electron dense structure called midbody. The separation of two newly formed daughter cells at the midbody region is called abscission. A variety of proteins is involved in the abscission. Secretory vesicles derived from the Golgi apparatus locate to the one side of the midbody. They release their contents and by fusing with each other and the plasma membrane they disappear at the time of abscission. Following abscission, the midbody remnant stays attached to one of the two daughter cells (**Figure 2.3**). In addition, the endocytic and the recycling endosome pathways are required for cytokinesis completion. The cell removes actin and microtubules from the midbody by endocytosis [24]. Especially, Rab11 and Rab35 marked endosomes are used for actin filament clearance in the midbody [13]. Moreover, ESCRT (endosomal sorting complex required for transport) pathway proteins are implicated in abscission [25-27].

There are four main ESCRT complexes in mammalian cells: ESCRT-0, -I, -II and -III. An AAA-ATPase protein VPS4 is responsible for the disassembly of the ESCRT complexes. During abscission stage, the homodimer of centrosomal protein Cep55 (Centrosomal protein of 55 kDa) first recruits ESCRT-I components TSG101 (tumour susceptibility gene 101) and ALIX (apoptosis-linked gene 2-interacting protein) to the midbody, which is followed by the recruitment of ESCRT-III components CHMPs and hIST1. ESCRT-III helices induce cortical constriction through formation of 17-nm filaments and help membrane scission. The cortical ingression occurs at only one side of

the midbody where the ESCRT-III components reside. The microtubule-severing enzyme spastin is also recruited to the midbody by ESCRT-III complex and it disassembles underlying microtubules in the midbody. Following microtubule severing, VPS4 as an ATPase catalyzes removal of the ESCRT-III subunits from endosomes [25] (**Figure 2.5**). Previous studies revealed that ALIX and TSG101 are the essential components of ESCRT-III mediated abscission machinery since their depletion results in inhibition of abscission stage during cytokinesis [27].

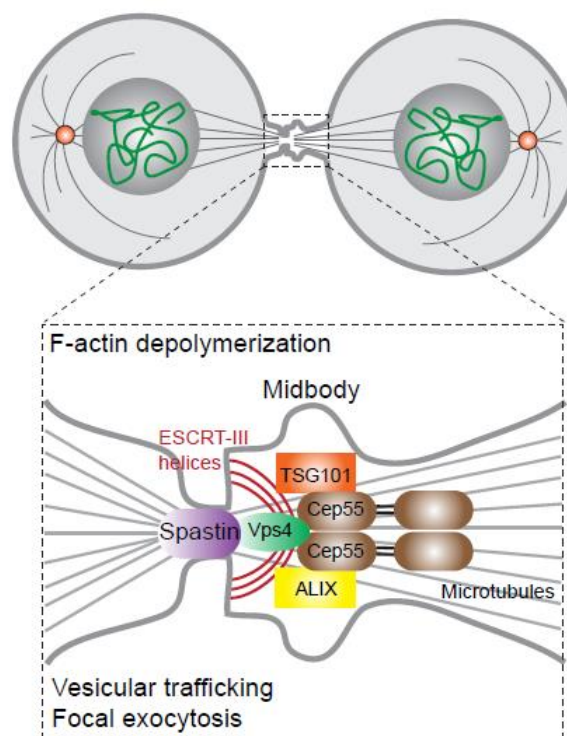


Figure 2.5: ESCRT mediated abscission model in mammalian cells.

Abscission is thought to be driven by the ESCRT-III/VPS4 complex (Adapted from [25, 28]).

2.1.2 The Chloride Intracellular Channel (CLIC) Family

The chloride intracellular channel (CLIC) family is composed of proteins encoded by seven different genes, p64 and CLIC1-6, in mammalian cells. Although, their N-termini are different in terms of both length and sequence of amino acids, all CLIC family proteins contain approximately 220 amino acid CLIC homology domain through the C-terminal together with two putative transmembrane domains (**Figure 2.6**). In addition to their putative transmembrane domains, CLIC proteins include nuclear localization signal (NLS) and putative phosphorylation sites [29].

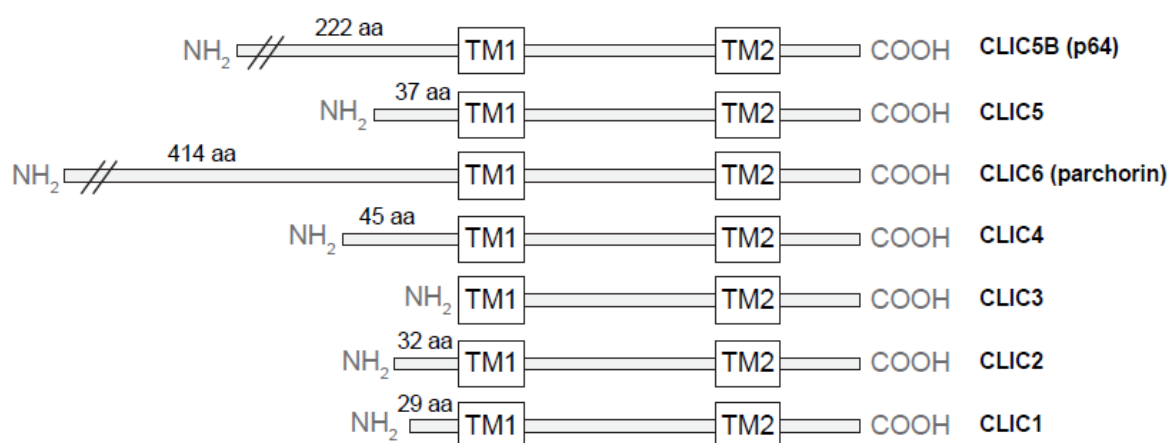


Figure 2.6: Seven members of chloride intracellular channel family.

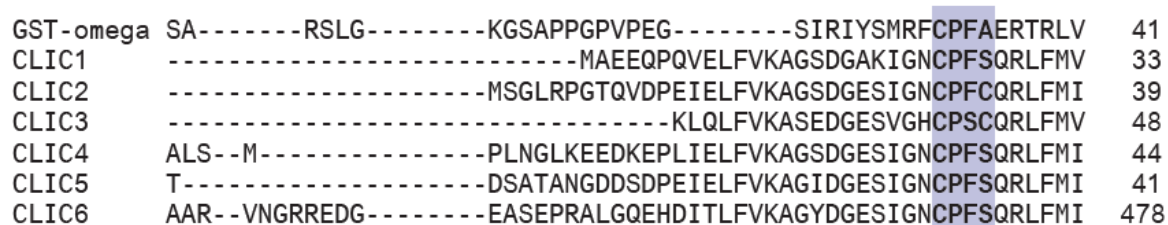
Alignment of CLIC proteins exhibits structure, transmembrane domain and size similarities among members of the family. CLIC5B and CLIC6 are also known as p64 and parchorin, respectively (Adapted from [29]).

In contrast to their original name, CLIC proteins do not act as traditional chloride channels. A growing evidence suggest that some proteins may have multiple three-dimensional configurations instead of a well-defined only one three-dimensional structure.

These type of proteins are called metamorphic proteins and CLIC proteins are classified in this group due to their unusual biochemical properties [30]. CLIC proteins can adopt multiple stable three-dimensional configurations which are reversible conformational transitions. They can be present as either membrane bound or soluble cytoplasmic proteins in cells. However, there is no assigned chloride channel function to membrane bound form of CLICs in cells. A limited number of studies revealed that CLIC proteins can form selective chloride ion channels in the artificial membrane systems, i.e. lipid bilayers [31]. Most of the CLIC proteins are present as soluble cytoplasmic proteins in cells and very little is known about the functions of the soluble form of CLIC proteins [32]. Therefore, the central parts of present studies mostly focus on the transition mechanism of CLIC proteins between membrane bound and soluble forms, how this transition mechanism is regulated and the functions of soluble CLIC proteins in cells.

Previous studies revealed that in their soluble form CLIC proteins are structurally related to the omega class of glutathione S-transferase (GST) family proteins [33, 34]. Glutathione S-transferases are the members of major phase II detoxification enzymes present mostly in the cytosol of cells [35]. They function in redox signaling pathways and act as a catalyzer for the conjugation of glutathione (GSH) to substrates such as exogenous toxins and xenobiotics which are harmful for cells metabolism [36]. The N-terminal domain of GST proteins binds to glutathione which is a ubiquitous cysteine-containing tripeptide (γ -Glu-Cys-Gly) while the C-terminal domain of GST proteins binds to the substrates [36]. The glutathione-substrate conjugation results in the formation of highly hydrophilic product which can either diffuse freely from the cell membrane or can be pumped out actively by a transmembrane ATPase [37].

The omega class GST proteins include a reactive cysteine in their glutathione binding site and this cysteine residue is conserved in all CLIC proteins [38] (**Figure 2.7**). Therefore, previous studies proposed that CLIC proteins may have intrinsic redox activities or they may be regulated by glutathione dependent redox reactions [38]. Alternatively, it has also been proposed that the conserved glutathione binding site found in CLIC proteins may target them to certain compartments in the cell or may affect their interactions with other proteins [39].



GST-omega	SA-----RSLG-----KGSAPPGPVPEG-----SIRIYSMRFCPFAERTRLV	41
CLIC1	-----MAEEQPQVELFVKAGSDGAKIGNCPFSQRLFMV	33
CLIC2	-----MSGLRPGTQVDPEIELFVKAGSDGESIGNCPFCQRLFMI	39
CLIC3	-----KLQLFVKASEDGESVGHCPSCQRLFMV	48
CLIC4	ALS--M-----PLNGLKEEDKEPLIELFVKAGSDGESIGNCPFSQRLFMI	44
CLIC5	T-----DSATANGDDSDPEIELFVKAGIDGESIGNCPFSQRLFMI	41
CLIC6	AAR--VNGRREDG-----EASEPRALGQEHDTL FVKAGYDGESIGNCPFSQRLFMI	478

Figure 2.7: GST-omega active site is conserved in CLIC proteins.

The reactive cysteine residue of GST-omega active site is conserved in all CLIC proteins. The highlighted residues in blue are the active site motif of GST-omega and the equivalents of this active site motif in CLIC proteins (Adapted from [38]).

2.1.2.1 Chloride Intracellular Channel Protein 4 (CLIC4)

Chloride intracellular channel protein 4 (CLIC4), also known as mtCLIC, p64H1, RS43 and HuH1, is a 253 amino acids protein. The protein structure of CLIC4 contains an N-terminal thioredoxin-like domain and a C-terminal domain which is composed of all α -helices. N-terminal domain of CLIC4 has a putative transmembrane region whereas its C-terminal domain includes a nuclear localization signal (NLS) [40] (**Figure 2.8**).

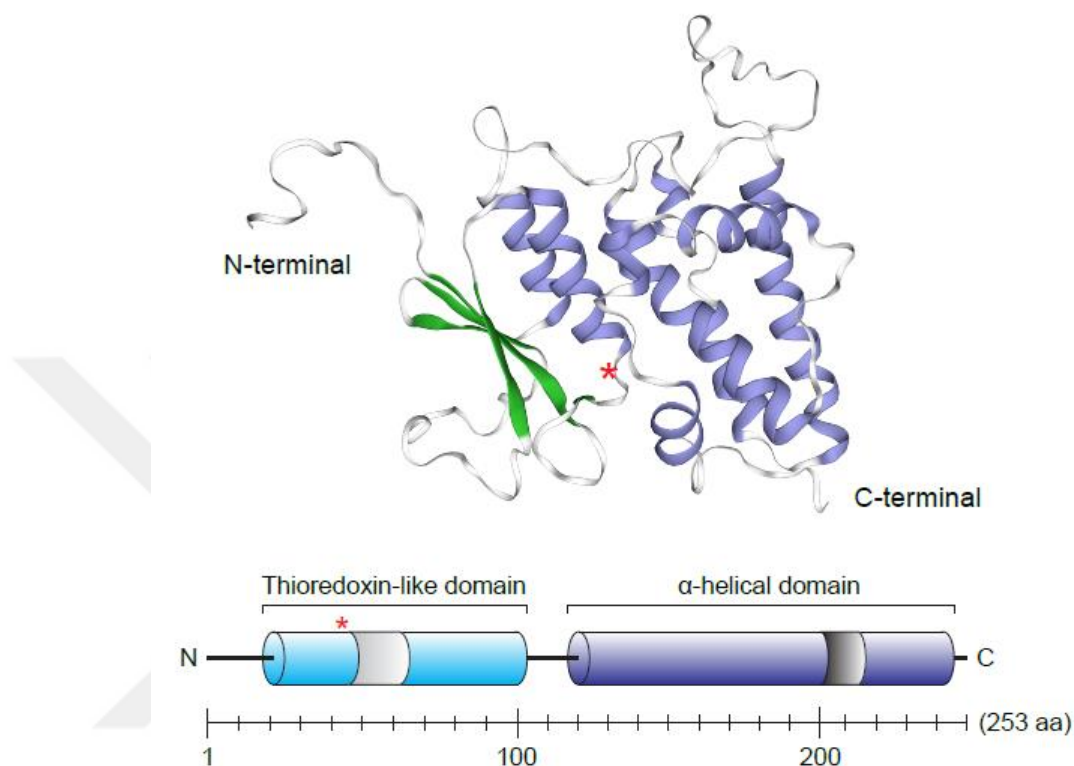


Figure 2.8: CLIC4 protein structure.

The three dimensional protein structure of CLIC4 (template: 2d2z.2.A) obtained from the SWISS-MODEL Repository [41] was shown at top panel. The two domains of CLIC4 including reactive cysteine residue, Cys35 (red asterisk), putative transmembrane region (light gray) and NLS (dark gray) were shown at the bottom panel (Adapted from [40]).

CLIC4 is the most studied member of the CLIC family and has been implicated in many actin-based cellular processes including G-protein coupled receptor signaling, cell differentiation, adhesion, spreading, migration, integrin signaling and protein trafficking [42-46]. However, how CLIC4 functions in these diverse cellular processes and the molecular mechanisms underlying the transition of CLIC4 protein between membrane

bound and soluble forms still remain elusive. So far, only a growing number of studies has revealed some clues about the transition mechanism of CLIC4 protein.

In 2009, Ponsioen *et al.* [45] discovered that the translocation of cytosolic CLIC4 to the plasma membrane occurs in N1E-115 neuroblastoma cells in response to $G\alpha_{13}$ -mediated RhoA activation by lysophosphatidic acid (LPA). As a phospholipid derivative signaling molecule, LPA induces rapid but transient translocation of cytosolic CLIC4 to the plasma membrane and this translocation depends on the presence of intact F-actin [45]. The structural homology between GST omega-1 (GSTO1) and CLIC4 revealed some essential residues of CLIC4 to be targeted to the plasma membrane. For instance, when the reactive cysteine equivalent of GSTO1 is mutated in CLIC4 protein (C35A), CLIC4 fails to translocate to the plasma membrane even in the presence of LPA stimulation. Moreover, mutations of the glutathione binding site equivalents of GSTO1 in CLIC4 protein (F37D, D87A, P76A) and the substrate binding site equivalents of GSTO1 in CLIC4 protein (F122R, Y244A) revealed the same result. However, other cysteine mutations in CLIC4 protein (C189S, C234S) did not affect the CLIC4 translocation to plasma membrane [45].

In the same study, Ponsioen *et al.* [45] also identified the colocalization of CLIC4 and NHERF2 at the plasma membrane. Having known NHERF2 is an ezrin, radixin and moesin (ERM) binding protein and ERM proteins are the linkers between membranes and the cortical actin cytoskeleton, it has been suggested that ERMs may directly or indirectly involve in the translocation of CLIC4 protein to the plasma membrane [30]. In line with this, another study revealed the conformation specific interaction partners of ERM member ezrin protein by a proteomic analysis in an epithelial cell line, Jeg-3 and discovered that CLIC4 is only present in the protein interaction network of activated ezrin protein [47].

Growing body of evidences highlights the actin-mediated role of CLIC4. CLIC4 has been shown to regulate the formation of branched actin networks on the early and recycling endosomes. Its depletion leads to massive accumulation of branched actin on the surface of early endosomes and interferes with cargo transport and vesicular trafficking [44]. Recently, CLIC4 was found to directly interact with a G-actin binding protein profilin-1 and identified in RhoA-mDia2 signaling, an inducer of actin polymerization [42]. mDia2 depletion led to a reduction in the plasma membrane translocation of CLIC4 while CLIC4 knockdown increased the formation of integrin-regulated long filopodium structures [42]. Collectively, these results suggest that CLIC4 functions in membrane trafficking in cooperation with actin cytoskeleton.

A very recent study revealed that a GXXXG motif in the putative transmembrane domain of CLIC proteins is conserved in vertebrates. Except CLIC3, this motif in CLIC proteins was actually a part of GXXGXXXG motif [48]. The GXXXG motif has an affinity to bind cholesterol and the mutational analysis of this motif revealed that G18A and G22A mutants of CLIC1 are not able to insert membranes in the presence of cholesterol. This suggests that GXXXG motif acts as a cholesterol-binding site within CLIC1 structure and it is essential for the cholesterol dependent membrane interactions [48].

CLIC1	AGSDGAKIGNCPFSQRLFMV	33
CLIC2	AGSDGESIGNCPFCQRLFMI	39
CLIC3	ASEDGESVGHCPSCQRLFMV	48
CLIC4	AGSDGESIGNCPFSQRLFMI	44
CLIC5	AGIDGESIGNCPFSQRLFMI	41
CLIC6	AGYDGESIGNCPFSQRLFMI	478

Figure 2.9: Multiple sequence alignment of vertebrate CLIC proteins.

The conserved glycine residues in cholesterol-binding sites were highlighted in red and the blue highlighted residues are the active site motif equivalents of GST-omega in CLIC proteins (Adapted from [38, 48]).

On the other hand, there are multiple studies implicating CLIC4 in cancer progression [49] and they revealed that CLIC4 has been regulated differently depending on the carcinoma cell line. CLIC4 expression was found to be gradually decreased in squamous cancer cells as they transformed from benign to malignant [50] while the high level of CLIC4 expression was found associated with the metastatic behavior of cancer-stem like cells of the colorectal cancer [51]. Moreover, increased CLIC4 expression was identified as a negative indicator of survival for epithelial ovarian cancer patients and CLIC4 has been suggested having a value as a cancer biomarker [52]. Although CLIC4 was highlighted as a novel target for cancer therapy, the underlying molecular mechanisms still remain to be elucidated.

In addition to studies mentioned above, a previous quantitative proteomics study from Ozlu *et al.* identified plasma membrane changes between interphase and mitosis cells in terms of protein quantity alterations [1]. Accordingly, several proteins were identified whose quantity differ at the plasma membrane of interphase and mitosis cells. CLIC family members, CLIC1 and CLIC4, were identified among these proteins and their quantity at the plasma membrane were significantly altered as cells progress from interphase to mitosis (**Table 2.1**).

Table 2.1: CLIC1 and CLIC4 enrich at the plasma membrane during mitosis.

Gene name	Protein	<i>p</i> -value	Ratio	Peptide	Coverage	Direction
FAS	Fas cell surface death receptor	Std	1.03	8	30	No change
CLIC4	Chloride intracellular channel protein 4	0.01	3.08	6	37	Mitosis
CLIC1	Chloride intracellular channel protein 1	0.01	2.35	8	39	Mitosis
ECE1	Endothelin-converting enzyme1	0.01	0.35	7	15	Interphase

For each protein, adjusted *p*-values, median ratio (mitosis/interphase), number of quantified peptides, percentage of coverage and significance direction are indicated (Adapted from [1]).

In the light of all studies mentioned above, this project focuses on the cell cycle dependent regulation of chloride intracellular channel proteins, especially CLIC4. Although it is known that CLIC4 increases on the plasma membrane during mitosis compared to interphase and RhoA activation, the key player of cytokinesis initiation, leads to plasma membrane translocation of CLIC4, the relationship between CLIC4 and cell division machinery has not yet been addressed. Therefore, investigating the role of CLIC4 during cell division would greatly help our understanding of CLIC4 involvement in cytoskeletal rearrangement and membrane remodeling events as well as its contribution to carcinogenesis.

2.2 MATERIALS AND METHODS

2.2.1 Cell culture conditions and synchronization methods

HeLa S3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) at 37°C, 5% CO₂ in a humidified incubator. For interphase arrest, cells were treated with 2 mM thymidine (296542, Santa Cruz) for 16 hours and released for 8 hours, twice. For mitosis arrest, following second thymidine release, cells were incubated with 10 ng/ml of nocodazole (487928, Calbiochem) for 5 hours. For cytokinesis arrest, cells were released for 1 hour after nocodazole treatment. To induce monopolar cytokinesis, following double thymidine blocking, cells were arrested in monopolar mitosis by 10 uM STC treatment (164739, Sigma-Aldrich) for 12 hours and forced into cytokinesis by 100 uM purvalanol A (1580, Tocris Bioscience) treatment for 15 min.

2.2.2 Cloning of CLIC1 and CLIC4 to pEGFP-N1 plasmids

Human chloride intracellular channel proteins CLIC1 (GenBank: AF109197.1) and CLIC4 (GenBank: AF097330.1) were cloned to pEGFP-N1 (Clontech #6085-1) plasmids. CLIC1 and CLIC4 were amplified from HeLa cDNA (20 ng/μl) with PCR (4.0 ul of 10X Phusion HF Buffer; 0.6 ul of DMSO 100%; 0.4 ul of 10 mM dNTP mix; 1.0 ul of 10 uM forward primer; 1.0 ul of 10 uM reverse primer; 11.7 ul of dH₂O; 1.0 ul of HeLa cDNA; 0.3 ul of Phusion HF DNA Polymerase (M0530S, NEB) – negative control without primers; primer pairs per each reaction (CLIC1-pEGFP-N1: 5'-GCGCAAGCTTATGGCTGAAGAACAACCGCAGG-3' and 5'-GCGCGGATCCGCTTTGAGGGCCTTTGCCACTTG-3'; CLIC4-pEGFP-N1: 5'-

GCGCCTCGAGATGGCGTTGTCGATGCCGCTGA-3' and 5'-GCGCGGATCCGCCTTGGTGAGTCTTTTGGCTACATCACTA-3'); 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 GenElute PCR Clean-Up Kit (NA1020, Sigma-Aldrich) according to the manufacturer's instructions.

PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primers (2.5 ul of 10X NEB4 Buffer; 0.25 ul of BSA; 5.0 ul of DNA; 1.0 ul of HindIII (R3104S, NEB) and 1.0 ul of BamHI (R3136S, NEB) for CLIC1 / 1.0 ul of BamHI (R3136S, NEB) and 1.0 ul of XhoI (R0146S, NEB) for CLIC4; 15.25 ul of dH₂O; Reaction conditions: 37°C-1 h). Digestions products were also purified with PCR Clean-Up Kit. pEGFP-N1 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 Quick Gel Extraction Kit (K2100-12, Invitrogen). Digested vectors were dephosphorylated with alkaline phosphatase enzyme (10 ul of 10X NEB3 Buffer; 30.0 ul of DNA; 10.0 ul of dH₂O; 5.0 ul of CIP (M0290S, NEB); Reaction conditions: 37°C-1 h). Then, plasmids were purified from the reaction with PCR Clean-Up Kit. Digested inserts and plasmids were used in the ligation reaction with vector to insert ratio of 1:3. Concentration measurements of prepared DNA molecules were made with Nanodrop Spectrophotometer (Thermo Scientific) and ligation calculation was done with Ligation Calculator online program (NEB).

Ligation reaction was performed (1.0 ul of 10X T4 Buffer; 1.0 ul of T4 Ligase (M0202S, NEB); 1.0 ul of vector; 2 ul of insert; 5 ul of dH₂O) – negative control without 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 ul DNA + 100 ul bacteria; 30 min incubation on ice; 1 min heat shock at 42°C; 2 min incubation on ice; addition of 900 ul LB and incubation at 37°C with 700 rpm shaking for 1.5 h; centrifugation at 8,000 rpm for 10 min; discard 800 ul of supernatant; resuspend the pellet; plate 100 ul onto LB agar plate with selective antibiotic – pEGFP plasmid has 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 ul of dH₂O and 3 ul of them were used as DNA template in PCR (2 ul of 10X Taq Buffer; 1.0 ul of DMSO 100%; 0.5 ul of 10 mM dNTP mix; 1.5 ul of 25 mM MgCl₂; 1.0 ul of 10 uM forward primer; 1.0 ul of 10 uM reverse primer; 15.7 ul of dH₂O; 2.0 ul of template DNA; 0.3 ul of Taq DNA Polymerase (EP0402, NEB); primer pairs per each reaction (CLIC1: 5'-GCGCAAGCTTATGGCTGAAGAACAACCGCAGG-3' and 5'-GCGCGGATCCGCTTTGAGGGCCTTTGCCACTTG-3'; CLIC4: 5'-GCGCCTCGAGATGGCGTTGTCGATGCCGCTGA-3' and 5'-GCGCGGATCCGCTTGGTGAGTCTTTTGGCTACATCACTA-3'); 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 of LB agar) and 5 ml LB (50 ug/ml kanamycin) at 37°C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Miniprep

Kit (K0503, Fermentas) according to the manufacturer's instructions. Purified plasmids were sequenced with pEGFP-N1 forward and reverse sequencing primers (5'-TTTAGTGAACCGTCAGATC-3' and 5'-AACAGCTCCTCGCCCTTG-3') by MacroGen Company, The Netherlands. After confirmation by sequencing, stored selected colonies were inoculated into 40 ml LB liquid (50 ug/ml kanamycin) and incubated at 37°C overnight with 170 rpm rotational shaking. Then, plasmid DNAs were isolated in large amounts from bacteria with GenElute Plasmid Midiprep Kit (NA0200-1KT, Sigma-Aldrich).

*** *Cloning of CLIC1 and CLIC4 to pEGFP-N1 plasmids was performed by **Gürkan Mollaoglu**. ****

2.2.3 Site-directed mutagenesis of CLIC4 to generate CLIC4-C35A and CLIC4-F37D in pEGFP-N1 plasmids

Two single amino acid changes (C35A and F37D [C: cysteine; A: alanine; F: phenylalanine; D: aspartic acid]) to CLIC4 were introduced by site-directed mutagenesis using CLIC4 in pEGFP-N1 (40 ng/μl) as template in PCR (2.5 ul of 10X PfuTurbo Buffer; 0.5 ul of 10 mM dNTP mix; 1.0 ul of 10 uM forward primer; 1.0 ul of 10 uM reverse primer; 18.5 ul of dH₂O; 1.0 ul of DNA; 0.5 ul of PfuTurbo DNA Polymerase (600252, Stratagene) – negative control without 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). CLIC4-C35A and CLIC4-F37D mutations were obtained using following forward/reverse primer pairs: For C35A mutation, 5'-GAAAGCATAGGAAACGCCCCCTTTTCCCAGAGGCTC-3' and 5'-GAGCCTCTGGGAAAAGGGGGCGTTTCCTATGCTTTC-3'; for F37D mutation, 5'-

GAAAGCATAGGAAACTGCCCCGACTCCCAGAGGCTCTTC-3' and 5'-
GAAGAGCCTCTGGGAGTCGGGGCAGTTTCCTATGCTTTC-3'.

After the PCR reaction, samples were cooled to 4°C and 1.0 ul of DpnI enzyme (R0176S, NEB) was added to each reaction 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 h. 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 ul DNA + 100 ul bacteria; 30 min incubation on ice; 1 min heat shock at 42°C; 2 min incubation on ice; addition of 900 ul LB and incubation at 37°C with 700 rpm shaking for 1.5 h; centrifugation at 8,000 rpm for 10 min; discard 800 ul of supernatant; resuspend the pellet; plate 100 ul onto LB agar plate with selective antibiotic – pEGFP plasmid has kanamycin resistance). Plates were incubated at 37°C overnight. Single colonies were chosen from sample plate and incubated in 5 ml LB (50 ug/ml kanamycin) at 37°C overnight (liquid cultures were incubated with 170 rpm rotational shaking). DNA isolation from overnight bacteria cultures were performed with Miniprep Kit (K0503, Fermentas) according to the manufacturer's instructions. Purified plasmids were sequenced with pEGFP-N1 forward sequencing primer (5'-TTTAGTGAACCGTCAGATC-3') by Macrogen Company, The Netherlands. After confirmation by sequencing, mutant plasmids were produced in large quantity from bacteria with GenElute Plasmid Midiprep Kit (NA0200-1KT, Sigma-Aldrich).

*** Site-directed mutagenesis of *CLIC4* to generate *CLIC4-C35A* and *CLIC4-F37D* in *pEGFP-N1* plasmids was performed by **Gürkan Mollaoğlu**. ***

2.2.4 Cloning of CLIC4-WT, CLIC4-C35A and CLIC4-F37D to pcDNA3.1 mycBioID plasmids

CLIC4-WT, CLIC4-C35A and CLIC4-F37D inserts were cloned to pcDNA3.1 mycBioID plasmids. pcDNA3.1 mycBioID was a gift from Kyle Roux (Addgene plasmid # 35700 ; <http://n2t.net/addgene:35700> ; RRID:Addgene_35700) [53]. CLIC4-WT, CLIC4-C35A and CLIC4-F37D inserts were amplified respectively from CLIC4-WT, CLIC4-C35A and CLIC4-F37D in pEGFP-N1 (Clontech 6085-1) (30 ng/μl) with same primer pair in PCR (4.0 ul of 5X GC Buffer; 0.6 ul of 100% DMSO; 0.4 ul of 10mM dNTPs; 1 ul of 10 uM forward primer (5'-GCGCCTCGAGGCGTTGTCGATGCCGCTGAA-3'); 1 ul of 10 uM reverse primer (5'-GCGCGGATCCTTACTTGGTGAGTCTTTTGGCTACA-3'); 11.7 ul of dH₂O; 1.0 ul of template DNA; 0.3 ul of Phusion HF DNA Polymerase (M0530S, NEB); PCR conditions: Hot-Start 98°C, Initial denaturation 98°C-30 sec, Denaturation 98°C-15 sec, Annealing 72°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 check the success of PCR reaction. PCR products were purified with PureLink PCR Purification Kit (K3100-02, Thermo Fischer Scientific) according to the manufacturer's instructions.

PCR products were digested with restriction endonucleases which have recognition sites embedded in designed PCR primer pair (2.5 ul of 10X NEB4 Buffer; 0.25 ul of BSA; 5.0 ul of DNA; 1.0 ul of XhoI (R0146S, NEB); 1.0 ul of BamHI (R3136S, NEB); 15.25 ul of dH₂O; Reaction conditions: 37°C-1 h). Digestion products were also purified with PureLink PCR Purification Kit. pcDNA3.1 mycBioID plasmids were also digested with the same restriction endonucleases with the same reaction conditions. Digested plasmids were run in 1% agarose gel electrophoresis and then purified from the gel with Quick Gel Extraction Kit (K2100-12, Invitrogen). Then, digested plasmids were dephosphorylated

with alkaline phosphatase enzyme (5.0 ul of 10X NEB3 Buffer; 30.0 ul of DNA; 10.0 ul of dH₂O; 5.0 ul of CIP (M0290S, NEB); Reaction conditions: 37°C-1 h).

Plasmids were purified from the reaction with PureLink PCR Purification Kit. Digested inserts and plasmids were used in the ligation reaction with vector to insert ratio of 1:6. Concentrations of prepared DNA molecules were measured with Nanodrop Spectrophotometer (Thermo Scientific) and ligation calculation was done with Ligation Calculator online program (NEB). Ligation reaction was performed (1.0 ul of 10X T4 Buffer; 1.0 ul of T4 Ligase (M0202S, NEB); 1.0 ul of vector; n ul of insert; 7-n ul of dH₂O; insert amounts (n) were different for all three inserts - negative control without insert; Reaction conditions: 16°C-16 h). Ligation products were used to transform chemical competent *E. coli* DH5 α bacteria according to the standard transformation protocol (5 ul DNA + 100 ul bacteria; 30 min incubation on ice; 1 min heat shock at 42°C; 2 min incubation on ice; addition of 900 ul LB and incubation at 37°C with 700 rpm shaking for 1.5 h; centrifugation at 8,000 rpm for 10 min; discard 800 ul of supernatant; resuspend the pellet; plate 100 ul onto LB agar plate with selective antibiotic – pcDNA3.1 mycBioID plasmid has ampicillin resistance). Plates were incubated at 37°C overnight. Next day, single colonies from sample plates were picked and inoculated into liquid LB cultures. LB cultures were incubated at 37°C overnight with 200 rpm rotational shaking. DNA isolation from overnight bacteria cultures were performed with GeneJET Plasmid MidiPrep Kit (K0482, Thermo Fischer Scientific) according to the manufacturer's instructions. Purified plasmids were sequenced with BGH-R reverse primer (5'-TAGAAGGCACAGTCGAGG-3') by MacroGen Company, The Netherlands.

2.2.5 Cloning of lentiCRISPR v1 plasmids targeting *CLIC1* and *CLIC4* genes

CRISPR/Cas9-based *CLIC1* and *CLIC4* gene knockouts were performed using following oligonucleotide sequences: Human *CLIC1* sgRNAs as top/bottom pairs, sg1 5'-CACCGGCAGGTCGAATTGTTCTGTGA-3'/5'-AAACTCACGAACAATTCGACCTGCC-3', sg2 5'-CACCGATTCTCCCAGAGACTGTTCA-3'/5'-AAACTGAACAGTCTCTGGGAGAATC-3', sg4 5'-CACCGGTCGAATTGTTCTGTGAAGGC-3'/5'-AAACGCCTTCACGAACAATTCGACC-3', sg5 5'-CACCGGTTTCATGGTACTGTGGCTCA-3'/5'-AAACTGAGCCACAGTACCATGAACC-3'; human *CLIC4* sgRNAs as top/bottom pairs, sg1 5'-CACCGGATGCCGCTGAATGGGCTGA-3'/5'-AAACTCAGCCCATTTCAGCGGCATCC-3', sg2 5'-CACCGTTTCATGATTCTTTGGCTCAA-3'/5'-AAACTTGAGCCAAAGAATCATGAAC-3', sg3 5'-CACCGAACGGATGTAAATAAGATTG-3'/5'-AAACCAATCTTATTTACATCCGTTTC-3'; non-targeting sgRNA as top/bottom pair, 5'-CACCGACGGAGGCTAAGCGTCGCAA-3'/5'-AAACTTGCGACGCTTAGCCTCCGTC-3'. All sgRNAs were cloned into lentiCRISPR v1 (Addgene plasmid # 49535) as described previously [54]. Firstly, lentiCRISPR v1 plasmid was digested with BsmBI (Esp3I) restriction enzyme (5 ug of lentiCRISPR plasmid; 3 ul of BsmBI (R0580S, NEB); 3 ul of FastAP Thermosensitive Alkaline Phosphatase (EF0651, Thermo Scientific); 6 ul of 10X FastDigest Buffer; add dH₂O to total 60 ul volume; Reaction conditions: 37°C-30 min). Digested plasmids were run in 1% agarose gel electrophoresis and then the large band (about 11 kb) was purified from the gel with NucleoSpin Gel and PCR Clean-up Kit (740609, Machare-Nagel).

Each pair of oligos (top/bottom) was phosphorylated and annealed to each other (1 ul of 100 uM top oligo; 1 ul of 100 uM bottom oligo; 1 ul of 10X T4 Ligation Buffer; 0.5 ul of T4 PNK (M0201S, NEB); 6.5 ul of dH₂O; Reaction conditions: Hot Start 100°C, 37°C-30 min, 95°C-5 min and then ramp down to 25°C as 5°C/min). Annealed and phosphorylated oligos were diluted to a ratio of 1:20 in dH₂O and kept at 4°C. Ligation of oligo duplexes to digested lentiCRISPR v1 plasmid was performed (50 ng of digested lentiCRISPR v1 plasmid; 1 ul of diluted oligo duplex; 5 ul of 2X Quick Ligase Buffer; 1 ul of Quick Ligase (M2200L, NEB); dH₂O to total 11 ul volume - negative control without oligo duplex; Reaction conditions: 25°C-15 min). After reaction, ligation products were kept at 4°C.

Ligation products were used to transform chemical competent *E. coli* Stbl3 bacteria according to the standard transformation protocol (5 ul DNA + 100 ul bacteria; 30 min incubation on ice; 1 min heat shock at 42°C; 2 min incubation on ice; addition of 900 ul LB and incubation at 37°C with 700 rpm shaking for 1.5 h; centrifugation at 8,000 rpm for 10 min; discard 800 ul of supernatant; resuspend the pellet; plate 100 ul onto LB agar plate with selective antibiotic - lentiCRISPR plasmid has ampicillin resistance). Plates were incubated at 37°C overnight. Next day, single colonies from sample plates were picked and inoculated into liquid LB cultures. LB cultures were incubated at 37°C overnight with 200 rpm rotational shaking. DNA isolation from overnight bacteria cultures were performed using NucleoSpin Plasmid kit (740588, Macharey-Nagel) according to the manufacturer's instructions. Purified plasmids were sequenced with Human U6 Seq F_Insert primer (5'-ACTATCATATGCTTACCGTAAC-3') by MacroGen Company, The Netherlands.

2.2.6 Cloning of CLIC1-GFP, CLIC4-WT-GFP, CLIC4-C35A-GFP and CLIC4-F37D-GFP to pLenti CMV Hygro DEST plasmids

For the generation of stable cell lines expressing CLIC1-GFP, CLIC4-WT-GFP, CLIC4-C35A-GFP and CLIC4-F37D-GFP, inserts were amplified from corresponding pEGFP-N1 plasmids using attB1/attB2 flanking primer pairs (attB1_CLIC4: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAACCATGGCGTTGTCGATGCCG-3' and GFP_attB2: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTTGTACAGCTCGTCCATGCCG-3'). Entry clones were obtained via Gateway BP reaction of PCR products and pDONR221. The inserts were then transferred to lentiviral Gateway destination plasmid pLenti CMV Hygro DEST through LR reaction to create expression clones. pLenti CMV Hygro DEST (w117-1) was a gift from Eric Campeau & Paul Kaufman (Addgene plasmid # 17454 ; <http://n2t.net/addgene:17454> ; RRID:Addgene_17454) [55]. Inserts were confirmed by sequencing with M13 forward primer (5'-GTAAAACGACGGCCAGT-3') and T7 reverse primer (5'-GCTAGTTATTGCTCAGCGG-3') after BP reaction.

*** *Cloning of CLIC1-GFP, CLIC4-WT-GFP, CLIC4-C35A-GFP and CLIC4-F37D-GFP to pLenti CMV Hygro DEST plasmids was performed by Nazan Saner.* ***

2.2.7 Transfection and lentiviral transduction

Cells were transfected using Lipofectamine™ 2000 transfection reagent (11668027, Invitrogen) according to manufacturer's instructions. To produce lentiviruses, HEK293T cell line was used for packaging of lentiviral plasmids. Cells were seeded on 6 cm cell culture plates to be 25% confluent on the day of transfection. For the lentiviral packaging, psPAX2 and pCMV-VSV-G plasmids were used. psPAX2 was a gift from Didier Trono

(Addgene plasmid # 12260 ; <http://n2t.net/addgene:12260> ; RRID:Addgene_12260) and pCMV-VSV-G was a gift from Bob Weinberg (Addgene plasmid # 8454 ; <http://n2t.net/addgene:8454> ; RRID:Addgene_8454) [56]. Transfection of cells was performed in the afternoon as follows: 1000 ng transfer plasmid, 1000 ng psPAX2 and 100 ng pCMV-VSV-G were mixed in 90 μ l Opti-MEM (31985062, Thermo Fisher Scientific) for simultaneous transfection of cells. In a separate Eppendorf tube, 93 μ l Opti-MEM and 7 μ l FuGene transfection reagent (E2311, Promega) were mixed and incubated for 5 min at room temperature. Then, plasmid mix was added dropwise to transfection reagent solution and gently mixed. Final mix was incubated for 30 min at room temperature and then added directly to cells by dripping on top. Transfection mix was evenly distributed in cell growth medium by gently swirling the cell culture plate. Next day in the morning, transfection medium was replaced with 3 ml fresh cell growth medium. First viral supernatant was harvested at 48 hours post-transfection and stored at 4°C. Again, cells were given 3 ml fresh cell growth medium. Second viral supernatant was harvested at 72 hours post-transfection and pooled with the first one. Packaging cells were discarded after bleach sterilization. The viral supernatant was passed through 0.45 μ m filter and aliquoted as 1 ml into Eppendorf tubes. Viruses were stored at -80°C for long-term storage.

For viral transduction, HeLa S3 cells were seeded on 12 well cell culture plate to be 25% confluent on the day of transduction. During viral transduction, protamine sulfate was used as adjuvant. Protamine sulfate (P4020, Sigma-Aldrich) was prepared as 8 mg/ml stock solution in water and used at 8 μ g/ml final concentration during transduction. Two successive transductions were performed to increase transduction efficiencies. First transduction of cells was performed on day 1 as follows: 200 μ l HeLa S3 growth medium supplemented with 0.7 μ l protamine sulfate was given to cells in each well. Then, 500 μ l of corresponding viral supernatant for each well was added directly to cells by dripping on

top. For control well, 500 μ l growth medium was given to cells instead of viral supernatant. Next day, GFP expression level was checked to evaluate transduction efficiency of HeLa S3 cells. On day 3, first transduction medium was removed and second viral transduction was performed as on day 1. Depending on GFP expression level on day 4, cells were either kept in transduction medium one day more or given antibiotic selection medium. For selection of transduced HeLa S3 cells, 2 μ g/ml puromycin (for lentiCRISPR transduction) for 2 days or 300 μ g/ml hygromycin (for pLenti CMV Hygro DEST transduction) for a week was supplemented in growth medium.

2.2.8 Generation of single and double knockout cell lines

For the generation of CLIC4 knockout (CLIC4 KO) cell lines, HeLa S3 cells were seeded on 6 well plate to be 50% confluent on the day of transfection. The transient transfection of *CLIC4* targeting lentiCRISPR plasmids described in **Section 2.2.5** was performed by LipofectamineTM 2000 transfection reagent (11668027, Invitrogen) according to manufacturer's instructions. At 24 hours post-transfection, the transfection medium was discarded and cells were selected in growth medium supplemented with 2 μ g/ml puromycin for 1 day. Next day, selection medium was discarded and cells were cultured in fresh growth medium for a week. Cell pellets were obtained for Western blotting analysis of CLIC4 expression and frozen stocks of each cell line were prepared for single cell clone isolation experiments. For the generation of CLIC1 knockout (CLIC1 KO) cell lines, HeLa S3 cells were seeded on 12 well plate to be 25% confluent on the day of transduction. Lentiviral transduction of HeLa S3 cells with *CLIC1* targeting lentiCRISPR plasmids described in **Section 2.2.5** was performed as explained in **Section 2.2.7**. Transduced cells were selected in growth medium supplemented with 2 μ g/ml puromycin for 2 days. Then, cells were cultured in fresh growth medium for a week. Cell pellets were obtained for Western blotting analysis of CLIC1 expression and frozen stocks of each cell line were

prepared for single cell clone isolation experiments. To generate CLIC1 and CLIC4 double knockout (CLIC1/4 DKO) cell line, lentiviral transduction of the CLIC4 knockout sg3-c7 cell line (puromycin-sensitive generated by transient transfection) was performed with CLIC1-sg1 lentiviruses. In parallel, lentiviral transduction of the nontargeting sgRNA treated control cell line (puromycin-sensitive generated by transient transfection) was performed with nontargeting sgRNA lentiviruses. Lentiviral transduction was performed as explained in **Section 2.2.7** and in the generation of CLIC1 knockout cell lines. Following puromycin selection, puromycin-resistant control and double knockout cell lines were cultured in fresh growth medium for a week. Cell pellets were obtained for Western blotting analysis and frozen stocks of each cell line were prepared for single cell clone isolation experiments.

2.2.9 Single cell clone isolation and multinucleation quantification experiments

To obtain single cell clones of CRISPR cell populations, a cell solution at a concentration of 8 cells/ml was prepared. Approximately 10 ml of this solution was prepared for 96-well plate. 100 μ L of the 8 cells/mL solution was transferred into each well of a 96-well plate. Cells were incubated at 37°C, 5% CO₂ in a humidified incubator for 7 days and then the plate was scanned for cell growth. When the cells expanded, they were trypsinized and seeded onto larger culture dishes. After the monoclonal cell lines were sufficiently expanded, each cell line was screened for the expression of CLIC1 and/or CLIC4 by Western blotting. To analyze the multinucleation percentage of each monoclonal cell line, cells were plated on glass coverslips. When the cells reached 70% confluency, they were fixed with 3.2% PFA and prepared for microscopy analysis by staining with anti-Tubulin primary antibody, Alexa Fluor® 488- or 594-conjugated secondary antibody and DAPI. The number of multinucleated cells were quantified using ImageJ software [57].

*** *Single cell clone isolation from CLIC4-sg3 treated HeLa S3 cells was performed by Nazan Saner. Single cell clone isolations from CLIC1-sg1 and CLIC4-sg1 treated HeLa S3 cells were performed by Mehmet Akdağ. Multinucleation quantifications of single CLIC1 or CLIC4 knockout cell lines were performed with the help of Mehmet Akdağ. ****

2.2.10 Detection of mutations caused by CRISPR/Cas9 genome editing

For the detection of mutations caused by the sgRNAs targeting *CLIC1* and *CLIC4* in the HeLa S3 genome, genomic DNA isolations of CLIC1 KO sg1-c1, CLIC1 KO sg5-c1, CLIC4 KO sg1-c32 and CLIC4 KO sg3-c7 cell lines were performed using genomic DNA isolation kit (740952 Genomic DNA from tissue kit, Macherey-Nagel) according to manufacturer's instructions. For each cell line, approximately 1×10^7 cultured cells were used for genomic DNA isolation. Concentration measurements of prepared genomic DNA molecules were made with Nanodrop Spectrophotometer (Thermo Scientific). Each genomic DNA was used for the amplification of sgRNA targeted genomic regions by specific primer pairs. Each region was amplified from HeLa S3 gDNA (250 ng) with PCR (4.0 ul of 5X Phusion GC Buffer; 0.6 ul of DMSO 100%; 0.4 ul of 10 mM dNTP mix; 1.0 ul of 10 uM forward primer; 1.0 ul of 10 uM reverse primer; 12 ul of dH₂O; 0.8 ul of HeLa S3 gDNA; 0.2 ul of Phusion DNA Polymerase (M0530S, NEB). For each reaction specific primer pairs were used (CLIC1-exon1, forward: 5'-GAGTCCCTGAGTGTGAGACCC-3', reverse: 5'-CAAAGAGTGCACGTGGGATT-3', annealing temperature 65°C, product size: 256 bp; CLIC1-exon2, forward: 5'-CAGTGATGGGGCCAAGATT-3', reverse: 5'-TTTTGGTGTCAACGGTGTA-3', annealing temperature 64°C, product size: 104 bp ; CLIC4-exon1, forward: 5'-GCAGTCCCGGAGCAGAAG-3', reverse: 5'-GCGCTCACCTTGACGAAG-3', annealing temperature 65°C, product size: 150 bp; CLIC4-exon3, forward: 5'-GGAGACAGGATCTTACCATGTTG-3', reverse: 5'-

GGAGGGCATAAGACTTCTTCAA-3', annealing temperature 66°C, product size: 316 bp). PCR conditions: Initial denaturation 98°C-30 sec, Denaturation 98°C-10 sec, Annealing gradient-30 sec, Extension 72°C-30 sec, Final extension 72°C-10 min, 35 cycles). PCR products were run on 2% agarose gel at 100 V for 50 min. Then, PCR products were gel extracted using NucleoSpin® Gel and PCR Clean-up kit (740609, Macherey-Nagel) according to the manufacturer's instructions.

Following gel extraction, NEB® PCR Cloning Kit (E1202S) was used to ligate each PCR product into linearized pMiniT 2.0 plasmid. Normally, this *E. coli* plasmid includes a constitutively active two amino acids (Met-Ile) toxic minigene which is interrupted by linearization. Therefore, in the absence of an insert, this toxic gene causes lethality and no colony formation. However, if it carries an insert, *E. coli* colony starts to grow. Based on the manufacturer's instructions, 25 ng linearized pMiniT 2.0 plasmid was used in the ligation reaction of each PCR product with an insert-to vector ratio of 3:1. Transformation of ligation products into *E. coli* DH5α bacteria and their plating onto ampicillin containing agar plates were performed as explained in Section 2.2.4. Plates were incubated at 37°C overnight. Next day, five colonies from each plate were picked and inoculated into ampicillin containing liquid LB cultures. LB cultures were incubated at 37°C overnight with 170 rpm rotational shaking. Plasmid DNA isolation from overnight bacteria cultures were performed using NucleoSpin® Plasmid kit (740588, Macherey-Nagel) according to the manufacturer's instructions. Purified plasmids were sequenced with Cloning Analysis Forward Primer (5'-ACCTGCCAACCAGCGAGAAC-3') present in the NEB® PCR Cloning Kit (E1202S) by Macrogen Company, The Netherlands.

2.2.11 Generation of CLIC1-GFP, CLIC4-WT-GFP, CLIC4-C35A-GFP and CLIC4-F37D-GFP expressing stable HeLa S3 and U2OS-mCherry- α -Tubulin cell lines

CLIC1-GFP, CLIC4-WT-GFP, CLIC4-C35A-GFP and CLIC4-F37D-GFP sub-cloned pLenti CMV Hygro DEST lentiviral mammalian expression plasmids were packaged and transduction of HeLa S3 and U2OS-mCherry-Tubulin cells to generate stable cell lines were performed as explained in **Section 2.2.7**. Only for U2OS-mCherry- α -Tubulin cells, instead of two lentiviral infections, only one infection was performed due to high viral infection efficiency of the cells. Approximately 90% transduction efficiency was obtained and cells were selected in 150 ug/ml hygromycin supplemented growth medium for a week following first viral infection. On the other hand, HeLa S3 cells were selected in 300 ug/ml hygromycin supplemented growth medium for a week following second viral infection as explained in **Section 2.2.7**.

2.2.12 Generation of CLIC4-GFP and Ezrin-RFP expressing stable HeLa S3 cell line

Ezrin-RFP which is sub-cloned into a pLVX-puro vector (Takara Bio Inc.) was a kind gift from Dr. Michael Way (The Francis Crick Institute, Lincoln's Inn Fields Laboratories, London WC2A 3LY, England, UK) [58]. Since pLVX-puro is a lentiviral expression system, all packaging steps of lentiviral Ezrin-RFP plasmids were carried out as explained in **Section 2.2.7**. Viral transduction of CLIC4-GFP expressing stable HeLa S3 cells explained in **Section 2.2.10** was performed as explained in **Section 2.2.7**. For selection of transduced HeLa S3 cells, 2 ug/ml puromycin for 2 days was supplemented in growth medium.

2.2.13 Generation of CLIC1/4 DKO cell lines stably expressing Ezrin-RFP, Cortactin-RFP, LifeAct-RFP and H2B-mCherry

Ezrin-RFP, Cortactin-RFP and LifeAct-RFP sub-cloned pLVX-puro lentiviral expression vectors were kind gifts from Dr. Michael Way ((The Francis Crick Institute, Lincoln's Inn Fields Laboratories, London WC2A 3LY, England, UK) [58]. Histone H2B-mCherry containing PGK-H2BmCherry lentiviral expression vector was a gift from Mark Mercola (Addgene plasmid # 21217 ; <http://n2t.net/addgene:21217> ; RRID:Addgene_21217) [59]. The lentiviral packaging and transduction of viruses to control and CLIC1/4 double knockout cell lines were performed as explained in **Section 2.2.7**. Since control and double knockout cells were already resistant to puromycin, we could not enrich the cells transduced with Ezrin-RFP, Cortactin-RFP and LifeAct-RFP although they were kept in growth medium containing 2 ug/ml puromycin. On the other hand, PGK-H2BmCherry vector does not contain any mammalian selection marker. At the end, we obtained a mix population of cells for each cell line with a 30-50% transduction efficiency in terms of fluorescent protein expression. These cell lines were generated for future live cell fluorescence microscopy analysis of CLIC1/4 DKO cells.

2.2.14 Proximity-dependent biotin identification (BioID) and affinity capture of biotinylated proteins

The expression of BirA* tagged CLIC4 in cells leads to biotinylation of proteins which are proximal to CLIC4. R118G point mutation on BirA*, a promiscuous *Escherichia coli* biotin ligase, weakens its substrate selectivity enabling it to biotinylate any protein in close proximity. This method requires both ATP and biotin to form active biotin (biotinoyl-5'-AMP or bioAMP) [53]. HeLa S3 cells were seeded onto 15 cm dishes and transfected with 60 ug of mycBirA*-CLIC4 (pcDNA3.1 mycBioID) plasmids. Cells were cultured in

complete DMEM supplemented with 50 μ M D-Biotin (B-1595, Life Technologies) and 1 mM ATP after second thymidine block with the addition of 10 μ M STC for 12 hours. Cells were synchronized at monopolar cytokinesis by 100 μ M purvalanol-A treatment for 15 min and then lysed at room temperature in a Lysis Buffer (50 mM Tris pH 7.4, 500 mM NaCl, 0.4% SDS, 5 mM EDTA, 2% TritonX, 1 mM DTT, Protease Inhibitor) and sonicated. Then, cell lysates were centrifuged at 4°C, 7,500 rpm for 10 min and supernatants were incubated with streptavidin beads overnight. Beads were collected and washed twice with Wash Buffer 1 (2% SDS in dH₂O) for 10 min. Then, beads were washed in sequence once with Wash Buffer 2 (2% deoxycholate; 1% TritonX; 50 mM NaCl; 50 mM Hepes, pH 7.5; 1 mM EDTA) for 10 min, once with Wash Buffer 3 (0.5% NP-40; 0.5% deoxycholate; 1% TritonX; 500 mM NaCl; 1 mM EDTA; 10 mM Tris, pH 8.1) for 10 min, and once with Wash Buffer 4 (50 mM Tris, pH 7.4; 50 mM NaCl) for 30 min. Bound proteins were eluted from the streptavidin beads with Laemmli-DTT buffer containing D-Biotin at 98°C for 10 min with gentle agitation for Western blotting analysis.

2.2.15 Proteomic profiling of proximity interaction partners of CLIC4

To identify biotinylated proteins by mass spectrometry, on-bead tryptic digestion was performed for each sample. Briefly, protein bound streptavidin beads were washed with 50 mM NH₄HCO₃ and proteins were reduced by 100 mM DTT in 50 mM NH₄HCO₃ at 56°C, followed by alkylation using iodoacetamide. Equal bead volume of 1 ng/ μ l trypsin (MS Grade Trypsin Protease, Pierce) was added onto the beads for overnight digestion at 37°C. The resulting peptides were purified using C18 StageTips. Peptides were analyzed by online C18 nanoflow reversed-phase HPLC (2D nanoLC; Eksigent) linked to a Q-ExactiveOrbitrap mass spectrometer (Thermo Scientific). The data sets were searched against the human Swissprot/Uniprot database. Proteome Discoverer 1.4 software was used to identify proteins. The STRING v10.5 database [60] and GO cellular component [61]

analyses of the final protein lists were performed to observe already known protein interactions within the interactomes and the distribution of the protein hits in terms of sub-cellular localization, respectively. The protein interaction network was visualized using Cytoscape 3.6.0 [62].

*** *The preliminary analysis of the protein lists was performed by **Erdem Şanal**.* ***

2.2.16 Sensitive GFP-trap pulldown for protein interaction analysis

GFP-trap pulldown experiment was performed under low detergent conditions, which is referred as sensitive pulldown as described previously [63]. Stably GFP and wild type CLIC4-GFP expressing HeLa S3 cells were used as control and target samples, respectively. These cells were synchronized to monopolar cytokinesis and for each condition one confluent 10 cm dish approximately 1×10^7 cells were pelleted. Cells were resuspended in 500 μ l of 1X PBS containing protease and phosphatase inhibitors. Cell suspensions were transferred into a Dounce homogenizer and homogenized with 20 strokes with the tight pestle. Then, cell lysates were transferred into 1.5 ml Eppendorf tubes and passed through needle for five times. Triton-X100 was added into cell lysates to a final concentration of 0.01%, and cell lysates were incubated at 4°C for 20 min with mild rotation. Cell lysates were cleared by centrifugation at 4,000 g for 10 min. Protein lysates were transferred to fresh tubes and 50 μ l of them were stored as input samples. 25 μ l of GFP-Trap®_A beads slurry (gta-20, Chromotek) were placed into tubes and 500 μ l of ice cold dilution buffer (10 mM Tris HCl, pH 7.5; 150 mM NaCl; 0.5 mM EDTA) was added onto beads. Then, bead suspensions were centrifuged at 2,500 g for 2 min at 4°C. Supernatants were discarded and bead washing step was performed second time. Protein lysates were added onto equilibrated GFP-Trap®_A beads and protein lysate-bead mixtures were incubated 4°C for 3 h with mild rotation. Mixtures were centrifuged at 2,500 g for 2

min at 4°C and supernatants were stored as unbound samples. Beads were washed two times in 500 ul of ice cold dilution buffer by centrifugation at 2,500 g for 2 min at 4°C. Supernatants from washing steps were also stored as wash samples. Finally, GFP-Trap®_A beads were resuspended in 100 ul of SDS-sample buffer and boiled at 95°C for 10 min. Eluates were collected by centrifugation at 2,500 g for 2 min at 4°C. Input and eluate samples were analyzed by Western blotting to find interactions partners of CLIC4 protein.

2.2.17 Proximity ligation assay (PLA)

HeLa S3 cells were plated on glass coverslips and arrested in bipolar cytokinesis. Spatial associations of CLIC4 with Anillin, ALIX and Ezrin in cytokinesis were studied using Duolink® In Situ Red Starter Kit Mouse/Rabbit (DUO92101, Sigma-Aldrich) according to manufacturer's instructions. Briefly, cells were fixed with 3.2% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100, blocked with blocking solution for 30 min at 37°C, and then incubated with primary antibody pairs in antibody diluent overnight at 4°C. At this step, cells were also incubated with only anti-CLIC4 antibody as a technical control. Cells were washed in wash buffer A for 10 min at RT and incubated with secondary antibodies conjugated to plus and minus PLA probes in antibody diluent for 1 hour at 37°C. Cells were again washed in wash buffer A for 10 min at RT and then incubated with the ligase in ligation buffer for 30 min at 37°C. Then, cells were washed in wash buffer A for 10 min at RT and incubated with the polymerase in amplification buffer for 100 min at 37°C. Finally, cells were washed in wash buffer B for 20 min at RT and then 0.01X wash buffer B for 1 min at RT. Cells were incubated with mounting medium with DAPI for 15 min and coverslips were sealed to the slides. Leica DMI8/SP8 TCS-DLS confocal microscope were used for image acquisition of the fixed samples. For each condition, 20 different image frames were obtained in DAPI (to observe nuclei) and red (λ_{ex} 594 nm; λ_{em} 624 nm) (to observe red PLA dots) channels. Then, the fluorescent particle analysis of each

image frame from each channel was performed and automated with ImageJ software as explained in detail by Debaize *et al.* [64]. **Figure 2.10** displays a representative image of the automated quantification of total number of nuclei and PLA dots.

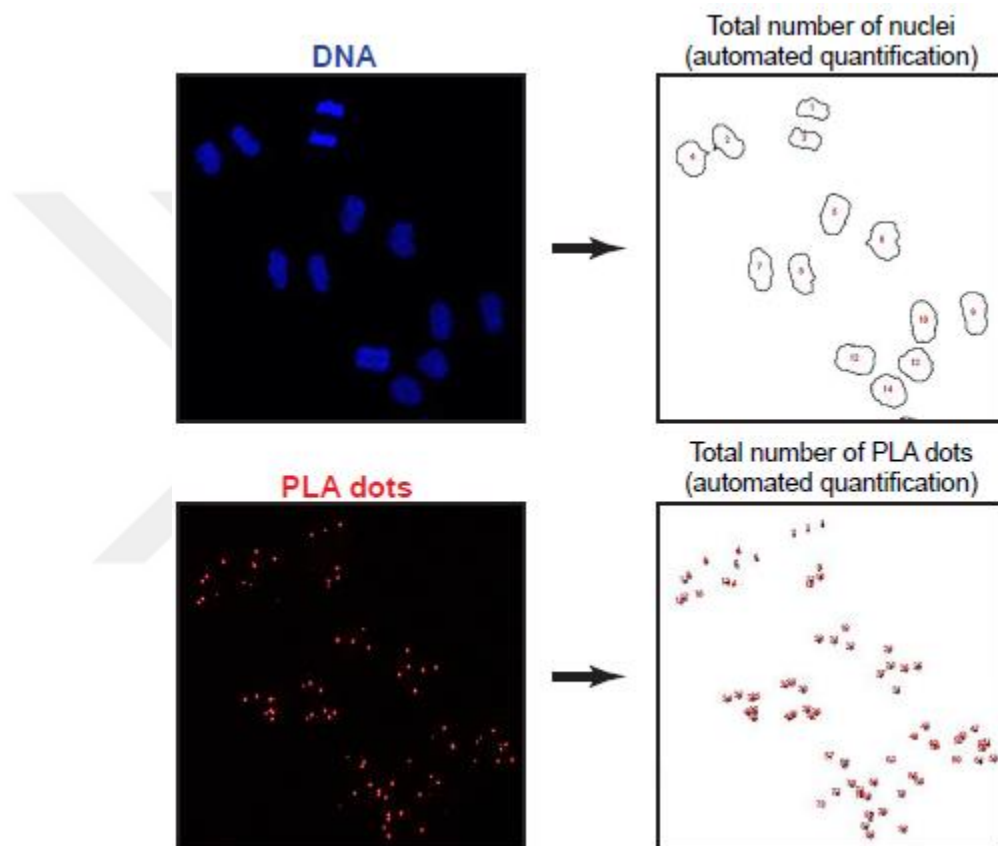


Figure 2.10: The automated quantification of total number of nuclei and PLA dots with ImageJ software.

2.2.18 Rhosin treatment

RhoA inhibitor, Rhosin (555460, Calbiochem) was dissolved in dimethylsulfoxide (DMSO) as 25 mM stock solution and stored at -20°C . Following nocodazole treatment,

cells were released in Rhosin containing medium for 1 hour. Different concentrations of Rhosin (25, 50, 100 and 150 μ M) were used to decrease the activity of intracellular RhoA during cytokinesis. Control cells were treated with DMSO.

2.2.19 Ezrin inhibitor treatment

Ezrin inhibitor, compound NSC305787, was a kind gift from Dr. Aykut Üren (Department of Oncology, Georgetown University Medical Center, Washington, DC, USA) [65, 66]. NSC305787 was dissolved in dimethylsulfoxide (DMSO) as 5 mM stock solution and stored at 4°C. During nocodazole treatment and nocodazole release, cells were treated with different concentrations of NSC305787 (8 and 10 μ M) for 6 hours in total to inhibit Ezrin activation. Control cells were treated with DMSO.

2.2.20 Western blotting and immunostaining

For Western blotting, cells were lysed in 1X PBS buffer containing 0.1% TritonX-100, cOmplete EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor mixture (Roche). Inhibitor cocktail tablets were used as 1 tablet for 10 ml of lysis buffer. Cell debris was removed by centrifugation at 14,000 rpm for 5 min at 4°C. Protein concentration was measured using BCA protein assay kit (23227, Pierce). Protein samples were prepared in 2X Laemmli sample buffer containing 4% (w/v) SDS, 20% glycerol, 120 mM Tris-Cl (pH 6.8), 0.02% (w/v) bromophenol blue and 100 mM dithiothreitol (DTT) as protein sample to 2X Laemmli sample buffer ratio of 1:1 (v/v). Samples were heated at 95°C for 5 min, cooled prior to loading onto SDS-PAGE gel. 6-12% SDS-PAGE gels were used for the separation of proteins according to their molecular weights. Protein samples were electrophoresed at 120 V for 100 min. Proteins were transferred to nitrocellulose membranes (BA85, Whatman Protran) by the Trans-Blot Turbo transfer system (Bio-Rad). After 45 min blocking with 4% milk in 0.1% Tween20 containing 1X TBS, the membrane

was incubated with primary antibodies overnight at 4°C or 3 hours at room temperature. The membrane was rinsed with 0.1% Tween20 containing 1X TBS Buffer for 5 min three times and incubated with secondary antibodies at room temperature for 1.5 hours. Finally, membranes were rinsed with 0.1% Tween20 containing 1X TBS Buffer for 5 min three times and proteins were visualized with the ECL Western Blotting Substrate system (32106, Pierce).

For immunostaining, cells seeded on glass coverslips were fixed in 3.2% paraformaldehyde in 1X PBS for 15 min. Coverslips were washed with TBS-0.1% Triton X-100 Buffer for 3 min three times. After 45 min blocking with 2% BSA in TBS-0.1% Triton X-100, coverslips were incubated with primary antibodies in 2% BSA in TBS-0.1% Triton X-100 overnight at 4°C or 3 hours at room temperature. Then, coverslips were washed with TBS-0.1% Triton X-100 Buffer for 3 min three times and incubated with secondary antibodies at room temperature for 1.5 hours. Coverslips were washed as previous and incubated with 1:500 DAPI in 2% BSA in TBS-0.1% Triton X-100 for 15 min. Following final washing step, coverslips were embedded in Mowiol® mounting medium (81381-Sigma-Aldrich) and sealed by nail polish onto microscope slides.

For Western blotting and immunostaining, the following primary antibodies were used: 1:200 anti-CLIC4 (sc-135739, Santa Cruz), 1:200 anti-CLIC1 (sc-81873, Santa Cruz), 1:1000 anti- α -Tubulin (2144, Cell Signaling), 1:200 anti- β -Tubulin (2128, Cell Signaling), 1:5000 anti-GFP (11814460001, Roche), 1:20000 anti-Biotin rabbit (gift from Dr. Timothy J. Mitchison, Harvard Medical School, Blavatnik Institute, Systems Biology, Boston, MA), 1:500 anti-phospho-Histone H3 (ser10) (32219, Upstate), 1:400 anti-Myc (2278, Cell Signaling), 1:500 anti-Anillin (ab99352, Abcam), 1:1000 anti-Ezrin (3145, Cell Signaling), 1:1000 anti-Phospho-ERM (3726, Cell Signaling) and 1:1000 anti-ALIX (ab88388,

Abcam). 1:2000 HRP-conjugated or 1:1000 Alexa Fluor® 488-, 555- and 594-conjugated anti-mouse and anti-rabbit IgGs (Invitrogen) were used as secondary antibodies.

2.2.21 Microscopy and quantification

The images of all fixed samples were acquired with Nikon Eclipse 90i (EZ-C1 software), Leica DMI8/SP8 TCS-DLS (LAS-X Software) laser scanning confocal microscopes and Leica DMI8 widefield microscope. Plan Apo VC 20X/0.75 DIC N2 OFN25 WD 1.0, Plan Apo λ 60X/1.40 Oil OFN25 DIC N2 and Plan Apo VC 100X/1.40 Oil OFN25 DIC N2 objectives of Nikon Eclipse 90i and HC PL APO 63x/1.40 Oil CS2 objective of Leica DMI8/SP8 TCS-DLS were used for image acquisition of fixed samples. Leica DMI8 widefield microscope equipped with HC PL FL L 20x/0.40 CORR and HC PL APO 63x/1.40-0.60 OIL objectives was used for live imaging of cells. All image quantifications were performed using ImageJ software [57]. To quantify the signal intensities at the cleavage furrow, the integrated densities were obtained for the ROIs as shown in figures. Unpaired two-tailed Student's t-test was used for significance analysis of the results. All graphs were created by GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

*** *Live cell fluorescence imaging of CLIC1-GFP, CLIC4-WT-GFP, CLIC4-C35A-GFP and CLIC4-F37D-GFP expressing HeLa S3 stable cell lines was performed by Nazan Saner.* ***

2.3 RESULTS

2.3.1 CLIC4 localizes to the cleavage furrow and the midbody during cytokinesis

To examine the spatiotemporal regulation of CLIC4 during cell division, HeLa S3 cells were synchronized to interphase, mitosis and cytokinesis. Then, cells were fixed and stained for endogenous CLIC4 protein (**Figure 2.11**). In parallel, CLIC4-GFP expressing HeLa S3 cells were visualized by live cell fluorescence imaging (**Figure 2.12**).

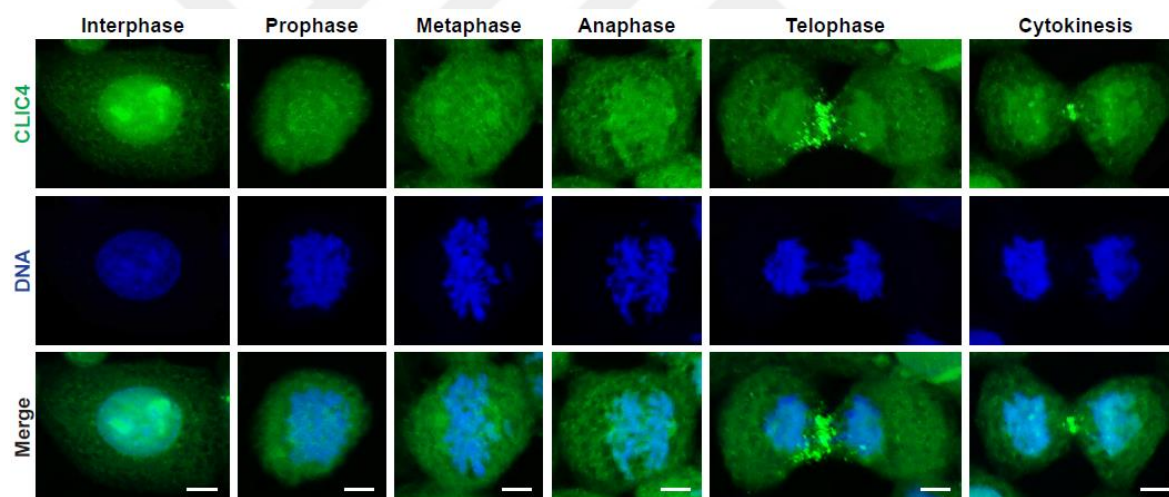


Figure 2.11: Endogenous CLIC4 localization during cell cycle.

Scale bars: 5 μm .

In interphase, in addition to a diffused cytosolic pool, CLIC4 has a nuclear localization. During mitosis, consistent with the previous proteomics data [1], CLIC4 decorates the cell surface as the cell rounds up at metaphase and is visible at the mitotic spindle poles and microtubules. Strikingly, as the sister chromatids separate at the onset of anaphase, CLIC4 moves away from the cell poles towards to future cleavage furrow region generating a

gradient. Once the ingression starts, CLIC4 accumulates at the cleavage furrow and finally concentrates at the midbody.

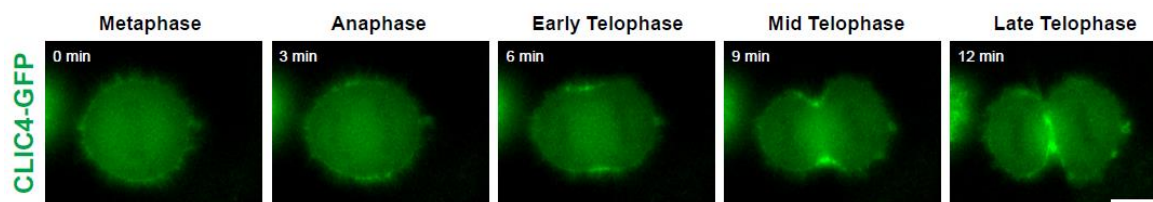


Figure 2.12: Live cell fluorescence imaging of CLIC4-GFP expressing cells.

Scale bar: 10 μm .

2.3.2 Cleavage furrow localization of CLIC4 is RhoA dependent

RhoA activation is one of the key regulatory steps of cytokinesis, which mediates the furrow formation and ingression [67]. To further assess whether the cleavage furrow localization of CLIC4 is specifically dependent on RhoA activation, HeLa S3 cells were synchronized to cytokinesis and treated with increasing concentrations of Rhosin, which inhibits the GEF activation domain of RhoA [68]. It has been shown that Rhosin dose-dependently reduces RhoA activity of MCF7 cells with an EC_{50} $\sim$ 30-50 μM [68]; thus, we used 0, 25, 50, 100 and 150 μM concentrations to treat HeLa S3 cells. Endogenous CLIC4 staining in cytokinesis cells revealed that increasing concentrations of the drug resulted in a progressive inhibition of CLIC4 accumulation at the cleavage furrow (**Figure 2.13**). Quantification of endogenous CLIC4 intensities at the cleavage furrow displayed a significant inhibition of CLIC4 localization to the cleavage furrow following RhoA inhibition (**Figure 2.14**). From these results, we conclude that CLIC4 localizes to the cleavage furrow and the midbody during cytokinesis in response to RhoA activation.

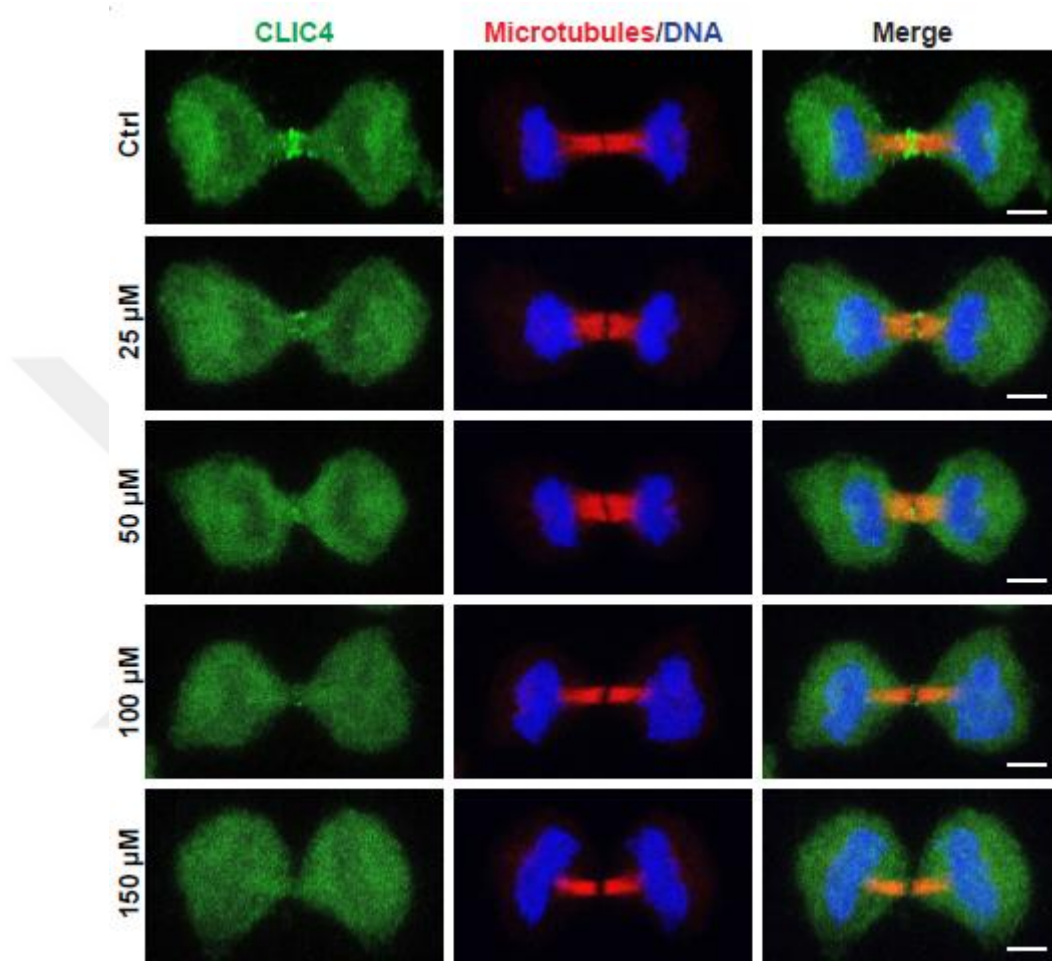


Figure 2.13: Localization of endogenous CLIC4 at the cleavage furrow after RhoA inhibition.

Scale bars: 5 μ m.

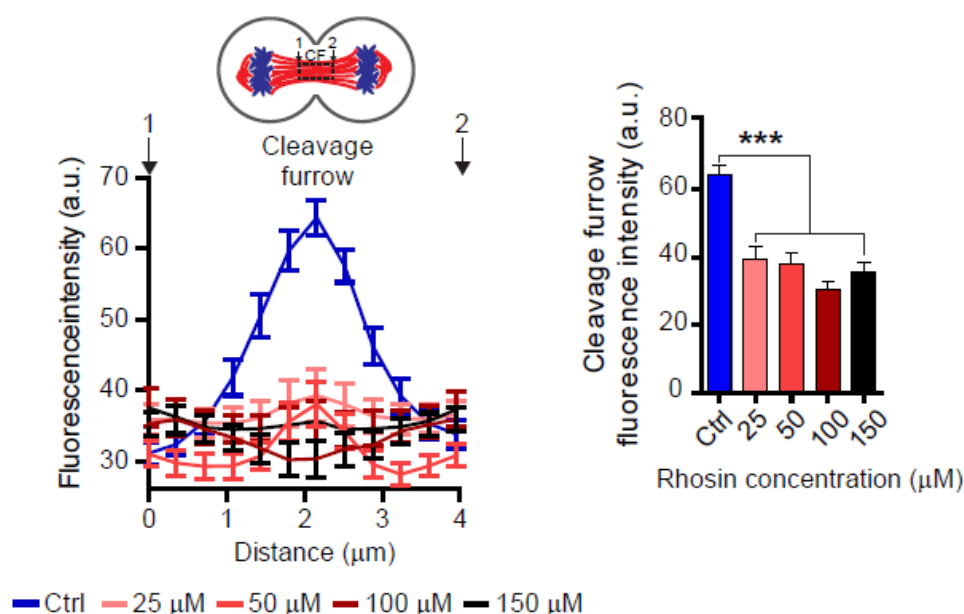


Figure 2.14: Quantification of endogenous CLIC4 signals at the cleavage furrow after RhoA inhibition.

n=15-17 cells for each condition. Unpaired two-tailed Student's t-test was used for significance analysis, *** $p < 0.0001$.

2.3.3 Cys35 and Phe37 residues of CLIC4 are essential for the cleavage furrow localization

Previously, six conserved residues of CLIC4, including Cys35 and Phe37, were identified as essential for the translocation of cytosolic CLIC4 to the plasma membrane as a response to RhoA activation [45]. Structurally both Cys35 and Phe37 residues are critical since their equivalents in GSTO1 are located at the enzymatically active cleft and the glutathione-binding site, respectively. To address the importance of Cys35 and Phe37 residues in RhoA-dependent cleavage furrow localization of CLIC4, the sub-cellular

localizations of CLIC4-WT-GFP, CLIC4-C35A-GFP and CLIC4-F37D-GFP were analyzed during cytokinesis. The expression levels of wild type and mutant CLIC4-GFP proteins were similar to the expression level of endogenous CLIC4 protein (**Figure 2.15**).

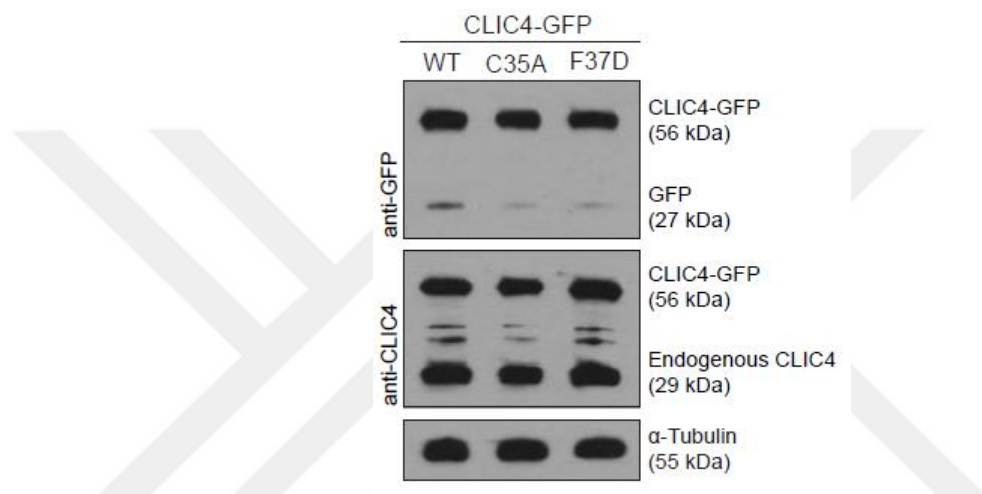


Figure 2.15: Analysis of transfection efficiencies of wild type and mutant CLIC4-GFP plasmids by Western blotting.

Anti-GFP blotting was performed to observe wild type and mutant CLIC4-GFP levels while anti-CLIC4 blotting was performed to observe both endogenous CLIC4 and CLIC4-GFP levels. Anti- α -Tubulin blotting was performed as loading control.

Only the wild type CLIC4 protein accumulated at the cleavage furrow while the mutant CLIC4 proteins were not detectable (**Figure 2.16**). Quantification of GFP intensities at the cleavage furrow clearly revealed that wild type CLIC4 protein accumulated at the cleavage furrow during cytokinesis whereas the mutant CLIC4 proteins could not (**Figure 2.17**). Live cell imaging of CLIC4-C35A-GFP and CLIC4-F37D-GFP expressing HeLa S3 cells revealed that not only the cleavage furrow localization but also the mitotic plasma membrane localizations of mutant CLIC4 proteins were altered (**Figure 2.18**). Based on

these results, we conclude that CLIC4 localizes to the cleavage furrow and the midbody during cytokinesis in response to RhoA activation via its conserved residues Cys35 and Phe37.

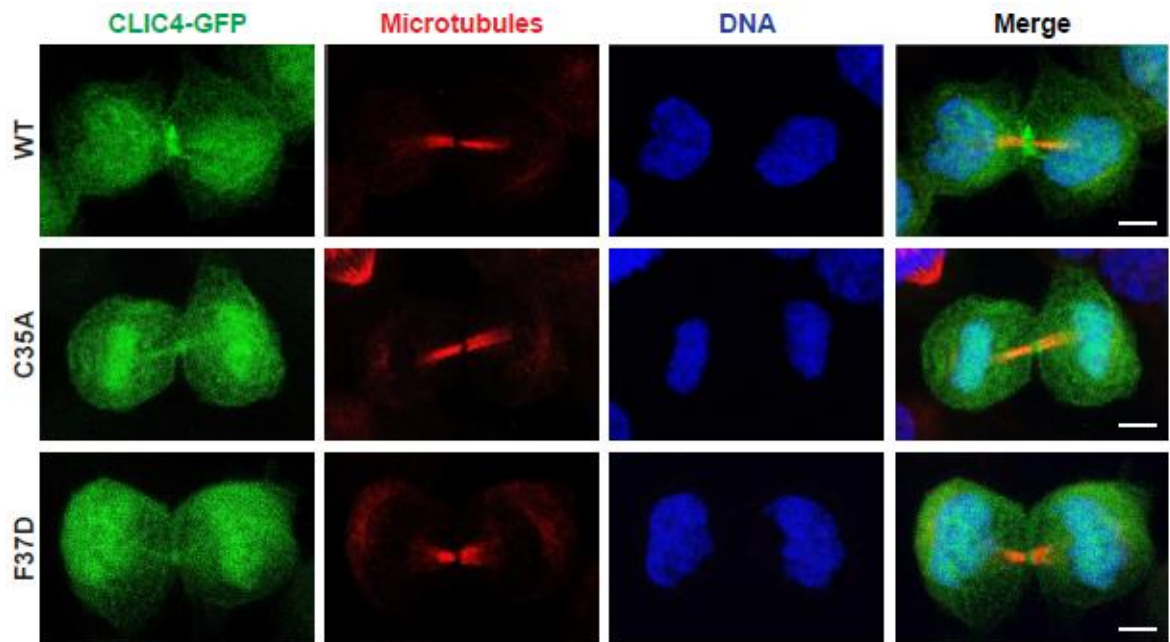


Figure 2.16: Localization of wild type and mutant CLIC4-GFP proteins at the cleavage furrow.

Scale bars: 5 μm .

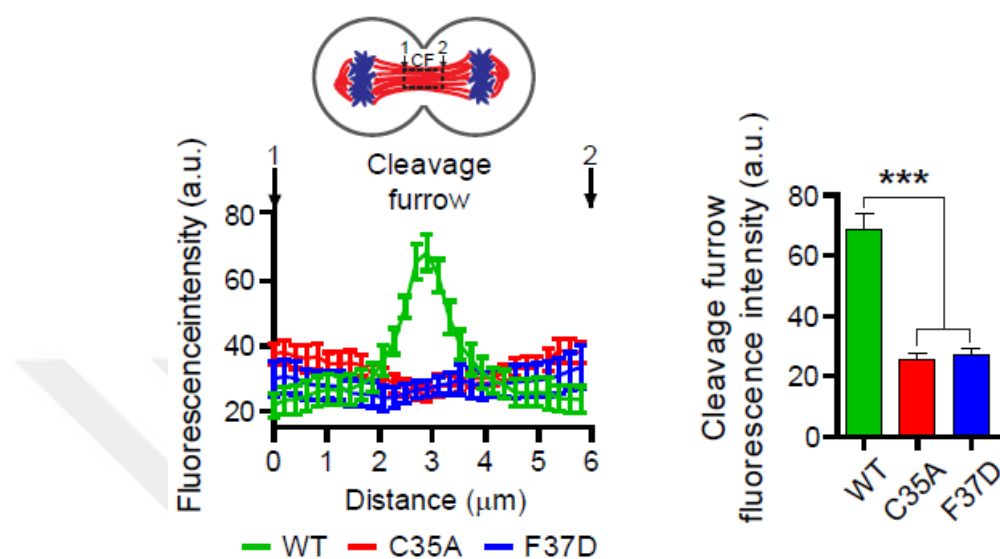


Figure 2.17: Quantification of wild type and mutant CLIC4-GFP signals at the cleavage furrow.

n=7 cells for each condition. Unpaired two-tailed Student's t-test was used for significance analysis, *** $p < 0.0001$.

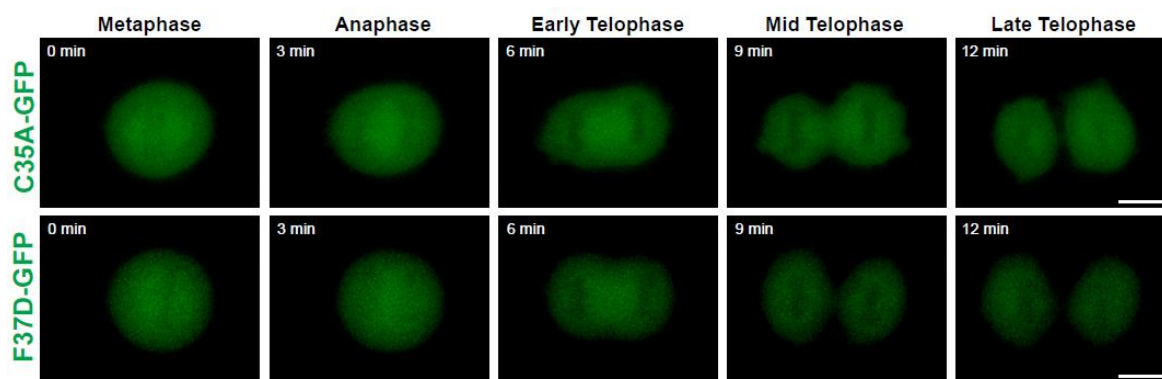


Figure 2.18: Live cell fluorescence imaging of CLIC4-C35A-GFP and CLIC4-F37D-GFP expressing cells.

Scale bar: 10 μm.

2.3.4 A systematic comparison between proximity interactomes of wild type and mutant CLIC4 proteins reveals novel interactors of CLIC4 in cytokinesis

To yield insights for the regulatory mechanisms behind the RhoA activation dependent plasma membrane targeting and cleavage furrow localization of CLIC4, we investigated interaction partner changes of wild type versus mutant CLIC4 proteins during cytokinesis. For this purpose, due to its higher synchronization efficiency and homogeneity, we used a well established monopolar cytokinesis synchronization method. Monopolar cytokinesis has been shown to be a good surrogate for the biochemical analysis of cytokinesis [69-71]. Cells undergoing monopolar cytokinesis possess a broader cleavage furrow region than cells undergoing bipolar cytokinesis (**Figure 2.19**). Therefore, recruitment to the cleavage furrow can be examined more clearly in monopolar cytokinesis cells. Same as in bipolar cytokinesis, only CLIC4-WT-GFP highly accumulated at the cleavage furrow in monopolar cytokinesis cells, but not the CLIC4-C35A-GFP and CLIC4-F37D-GFP. In addition, monopolar cytokinesis provided a wider cleavage furrow recruitment area for CLIC4-WT-GFP (**Figure 2.20**). This confirms that monopolar cytokinesis recapitulates the CLIC4 localization pattern of bipolar cytokinesis and provides a better synchrony for the following biochemical experiments.

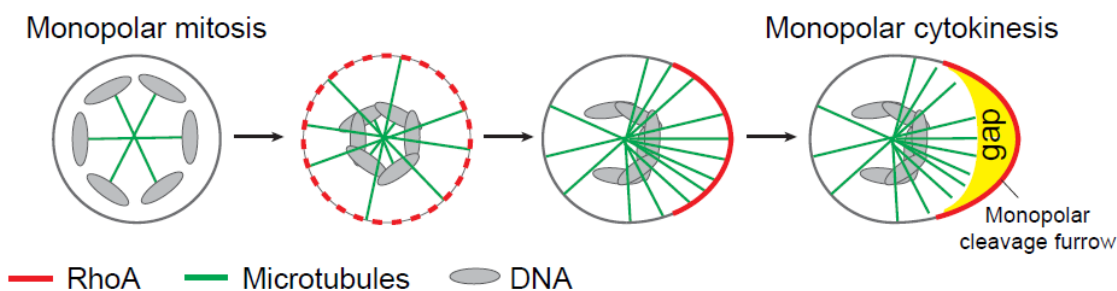


Figure 2.19: Monopolar cytokinesis following exit from monopolar mitosis.

Morphological changes observed in monopolar cytokinesis (Adapted from [69]).

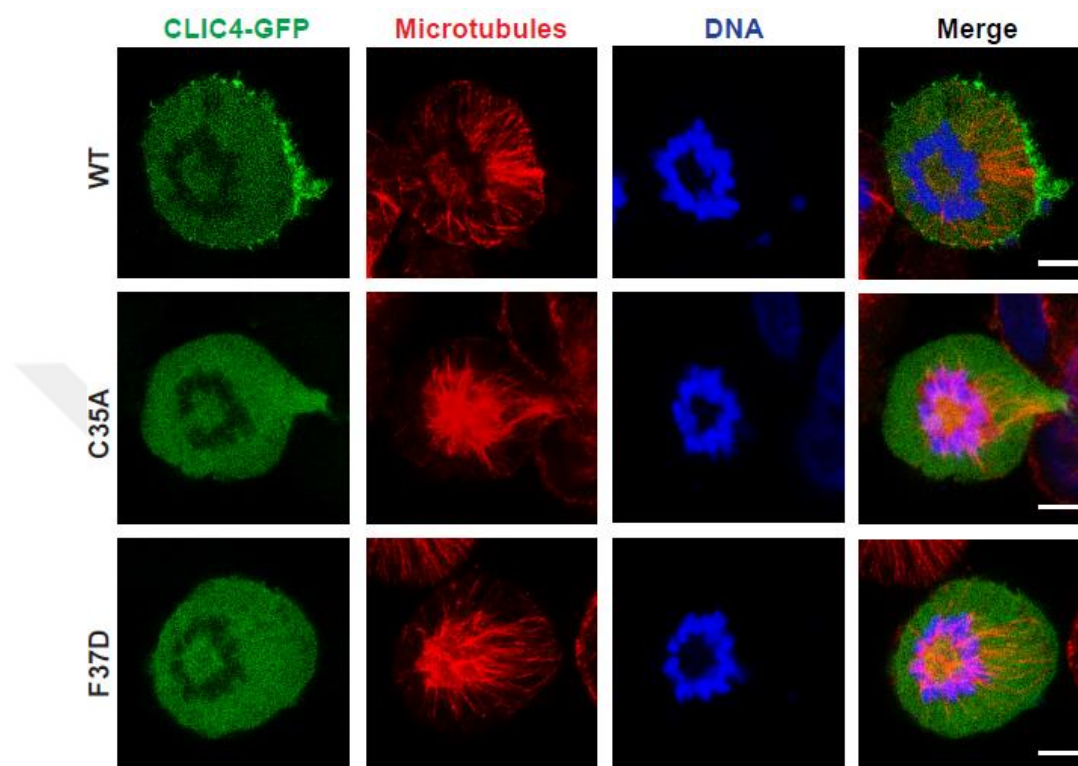


Figure 2.20: Localization of wild type and mutant CLIC4-GFP proteins in monopolar cytokinesis.

Scale bars: 5 μ m.

Given the transient and dynamic nature of CLIC4 interactions, traditional immunoprecipitations were not efficient in identifying its interacting partners [43]. To overcome the limitation in identifying transient and dynamic interactions of CLIC4 during cytokinesis, we performed the biotin identification (BioID) proximity labeling approach in live cells [53]. We generated mycBirA*-CLIC4-WT and mycBirA*-CLIC4-C35A expressing cells and confirmed their proper cleavage furrow localization at monopolar cytokinesis using anti-myc antibody (**Figure 2.21**). As expected, only mycBirA*-CLIC4-WT protein was able to localize to the cleavage furrow in monopolar cytokinesis. Since the

mutant CLIC4 proteins were not able to localize to the cleavage furrow, we took the advantage of a systematic comparison between proximity interactors of the wild type and mutant CLIC4 proteins during cytokinesis.

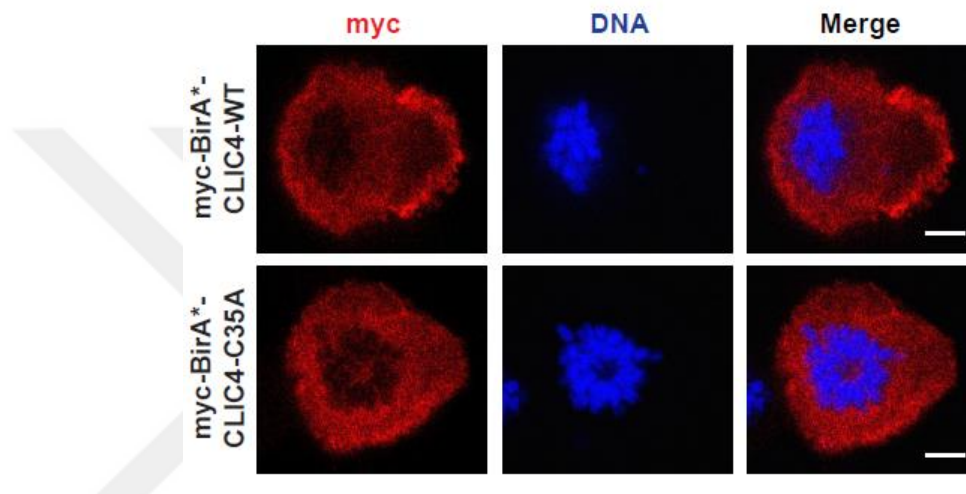


Figure 2.21: Localization of wild type and mutant mycBirA*-CLIC4 proteins in monopolar cytokinesis.

Scale bars: 5 μ m.

Western blotting analysis showed that wild type and mutant mycBirA*-CLIC4 proteins were able to biotinylate multiple proteins only in the presence of biotin and to pull down CLIC4 to a similar extent (**Figure 2.22**). To identify proximal interactors of CLIC4, the biotinylated proteins were isolated by performing streptavidin pulldown, which were then subjected to the on bead tryptic digestion followed by LC-MS/MS analysis. Untransfected monopolar cytokinesis cells incubated with biotin were used as control. The experiment was conducted twice and only the proteins identified in both biological replicates, but not in the control, were listed (**Appendix A**).

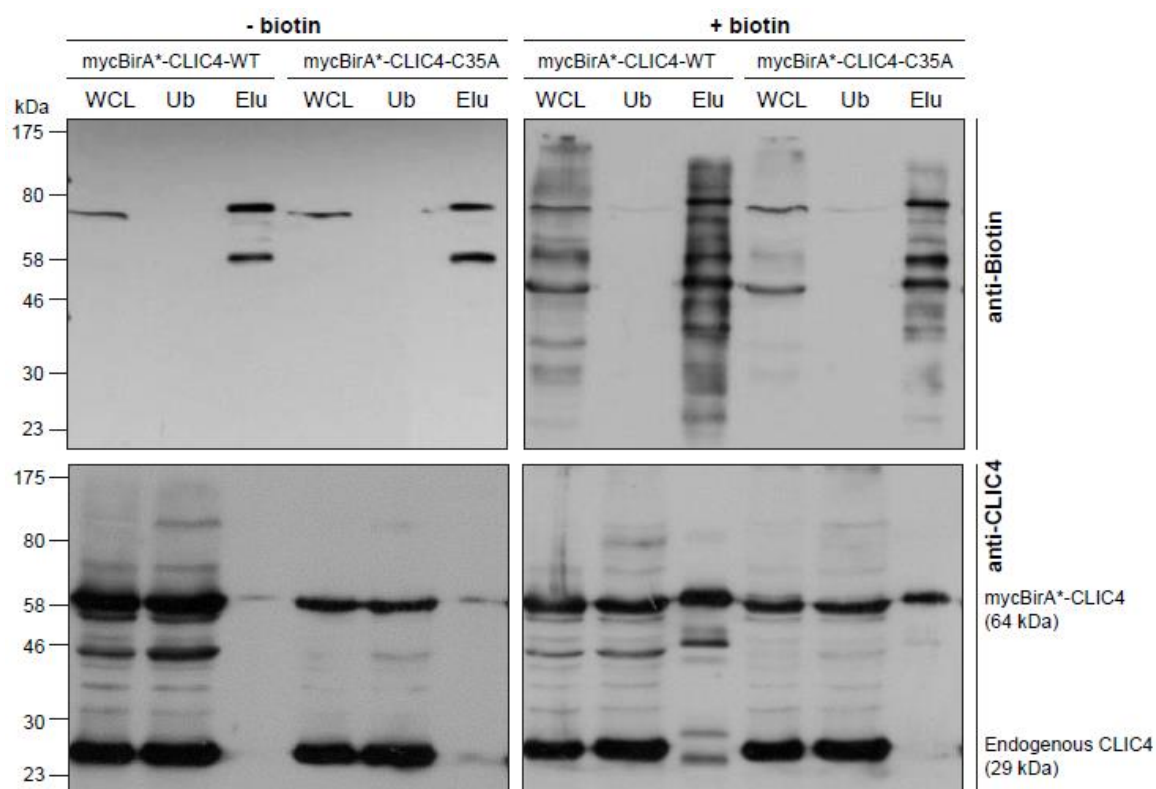


Figure 2.22: Western blotting of affinity captured proteins from wild type and mutant mycBirA*-CLIC4 transfected cells, incubated with or without biotin.

The top and bottom panels show Western blotting results with anti-Biotin and anti-CLIC4 antibodies, respectively. WCL: whole cell lysate, Ub: unbound fraction, Elu: eluate.

CLIC4 was found as the top hit with 67% and 61% coverages for WT and C35A mutant, respectively. 85 proteins were identified in the wild type CLIC4 interactome and 54 of them were specific to the wild type protein (**Figure 2.23** and **Appendix A-C**). The mutant CLIC4 interactome comprises less plasma membrane proteins compared to the wild type, most probably due to the abolished plasma membrane and cleavage furrow localization of CLIC4 C35A. Protocadherin alpha-9 (PCDHA9), SLC7A5, SLC13A3, SLC25A3, GP1BB, Ezrin (EZR) and ALIX were among the many plasma membrane proteins that were

identified as wild type CLIC4 specific proximity interactors in cytokinesis (**Figure 2.23**). A previous study has shown the interaction of active form of EZR with CLIC4 [47] validating our MS results.

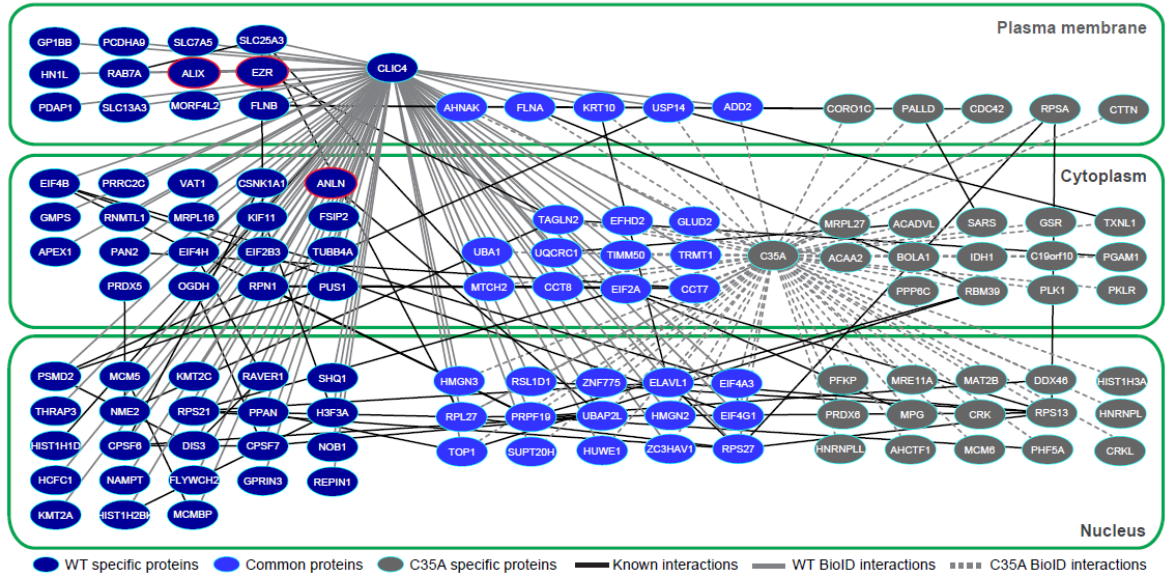


Figure 2.23: Comparison of wild type CLIC4 and CLIC4-C35A proximity interactomes in monopolar cytokinesis.

STRING v10.5 database [60] analysis and GO cellular component [61] analysis of the final protein lists were performed to see already known protein interactions in the interactomes and the distribution of the protein in terms of sub-cellular localization, respectively. The protein interaction network was visualized using Cytoscape 3.6.0 [62]. Selected candidate proteins (EZR, ANLN and ALIX) for further validation are marked in red.

2.3.5 CLIC4 associates with Ezrin, Anillin and ALIX during cell division

Following the identification of CLIC4 proximity interactors in monopolar cytokinesis cells by MS analysis, we focused on three cytokinesis proteins that were listed in the wild

type CLIC4 specific interactome: Ezrin, Anillin and ALIX. Activated Ezrin anchors the cortical actomyosin network to the plasma membrane as cell progresses into mitosis and accumulates at cleavage furrow upon chromosome segregation [72]. Anillin directly interacts with RhoA and shows a similar localization pattern with RhoA at cleavage furrow. It is suggested to act as scaffold protein connecting RhoA with the cortical ring components actin and myosin [73]. Anillin is also involved in the transition of contractile ring to midbody ring by both constituting the midbody structure and ensuring its plasma membrane linkage [74]. ALIX functions at the abscission step of cytokinesis and its interactions with Cep55 and ESCRT-III are essential for a successful cell division [27]. As all the mentioned proteins contribute to cytokinesis at different levels, we decided to dissect their interaction with CLIC4 to unveil its function in cell division.

To address whether CLIC4 cooperates with Ezrin, Anillin and ALIX during cytokinesis, we first examined their localization in dividing cells. Both CLIC4 and Ezrin accumulated at late furrow ingression site. Anillin and CLIC4 showed a similar co-localization pattern as Ezrin-CLIC4 pair. ALIX concentrated at the midbody along with CLIC4 at late telophase (**Figures 2.24, 2.25 and 2.26**).

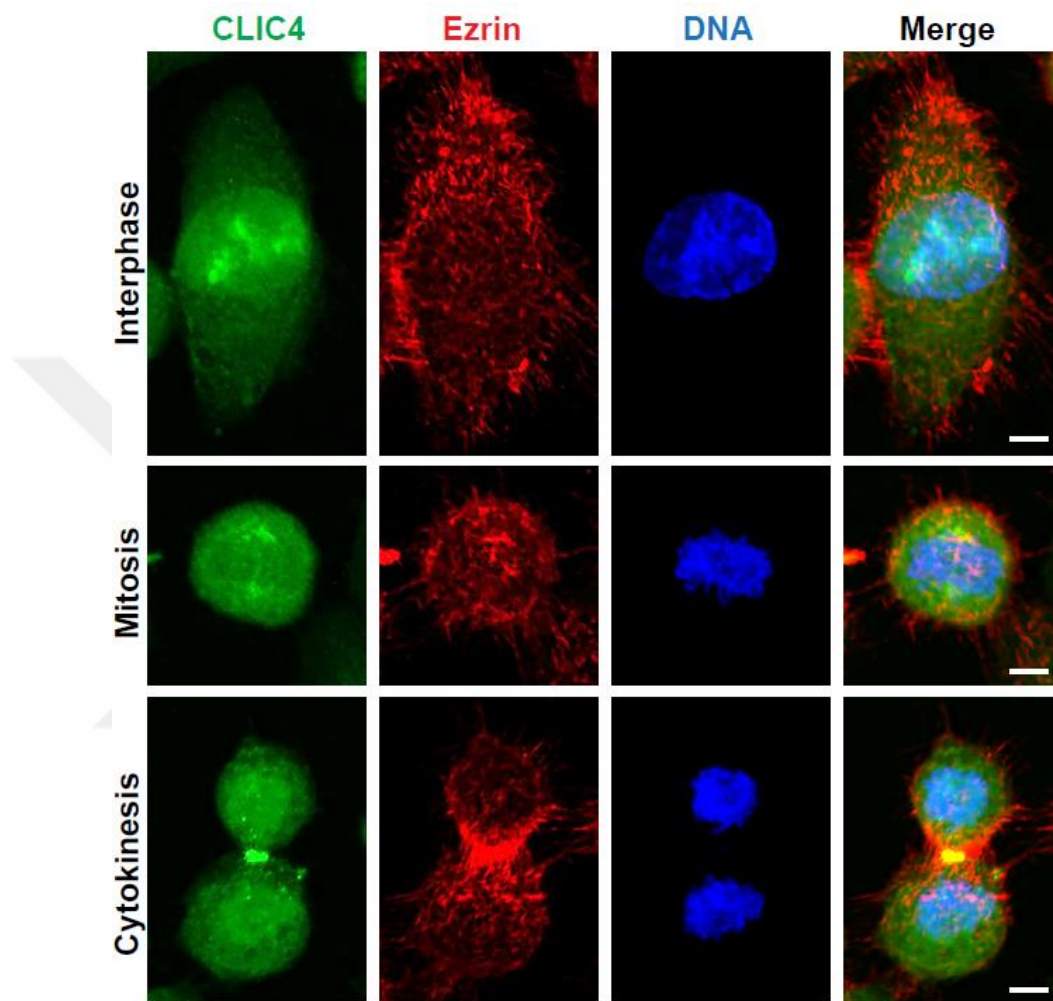


Figure 2.24: Localization of endogenous CLIC4 and Ezrin in interphase, mitosis and cytokinesis.

Scale bars: 5 μm .

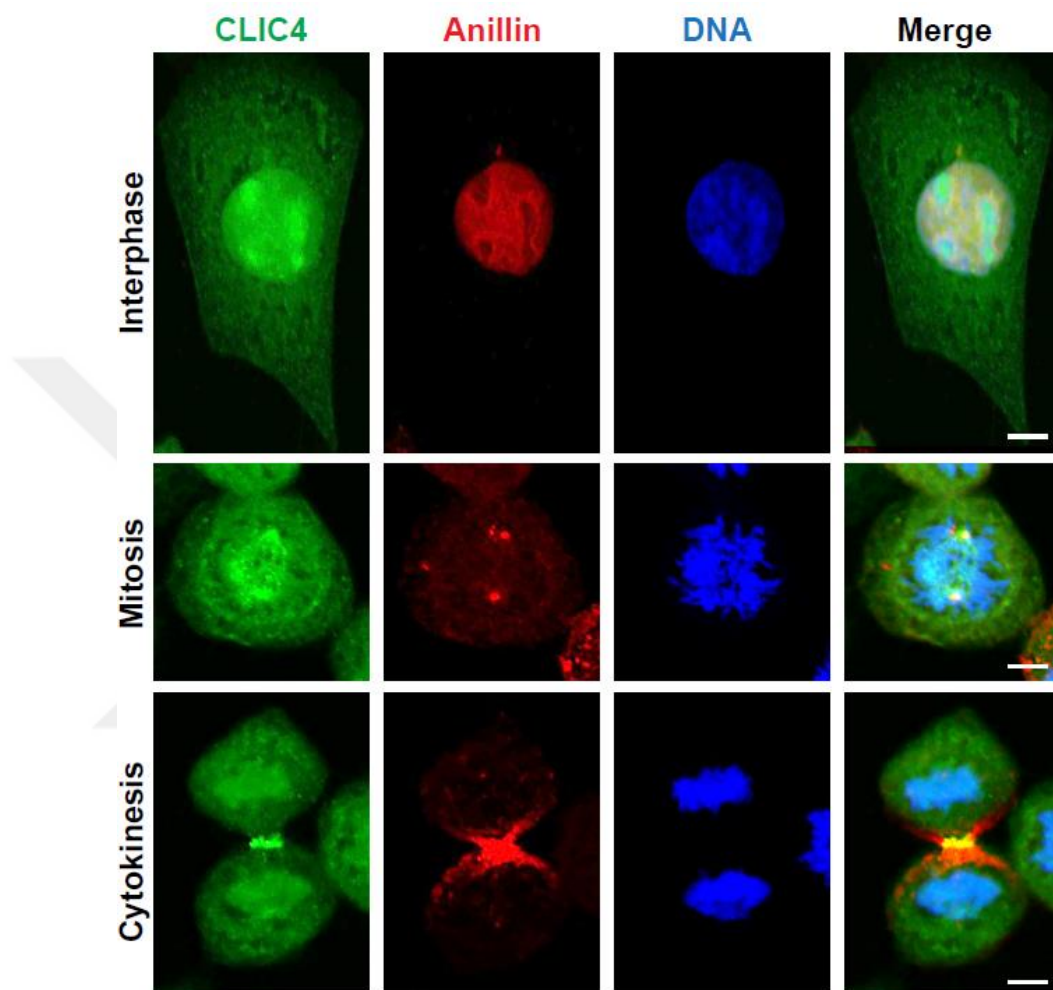


Figure 2.25: Localization of endogenous CLIC4 and Anillin in interphase, mitosis and cytokinesis.

Scale bars: 5 μm .

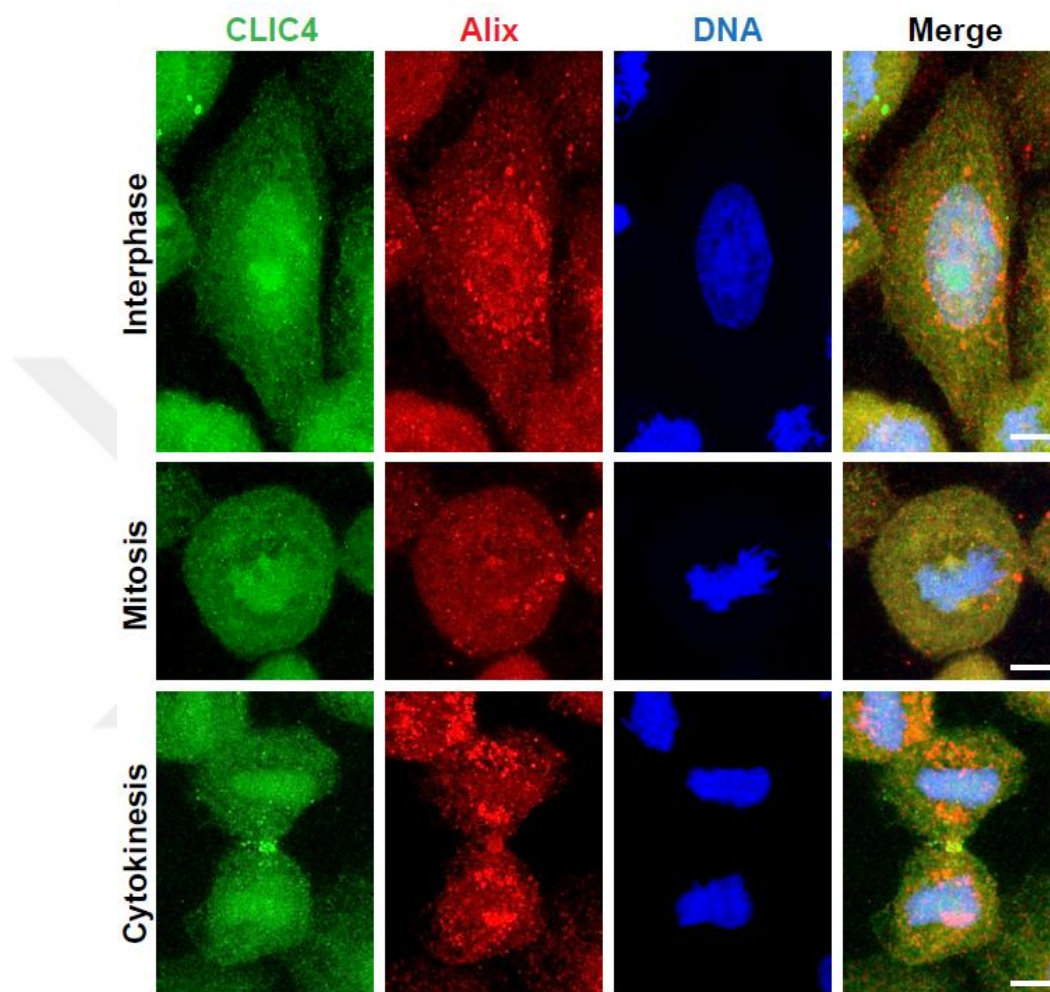


Figure 2.26: Localization of endogenous CLIC4 and ALIX in interphase, mitosis and cytokinesis.

Scale bars: 5 μm .

To further verify the physical interactions of Anillin, Ezrin and ALIX with CLIC4 during cytokinesis, we applied two different approaches using cytokinesis synchronized cells: (1) use of CLIC4-GFP fusion proteins to overexpress CLIC4 and then performing GFP-trap pulldown to analyze whether overexpressed CLIC4 interacts with Anillin, Ezrin

and ALIX; (2) use of proximity ligation assay (PLA) to analyze the interactions of endogenous CLIC4 with Anillin, Ezrin and ALIX in a time and place resolution. With the first approach, we revealed a mild interaction between CLIC4 and Ezrin, as well as CLIC4 and ALIX, but we could not detect CLIC4 and Anillin interaction (**Figure 2.27**).

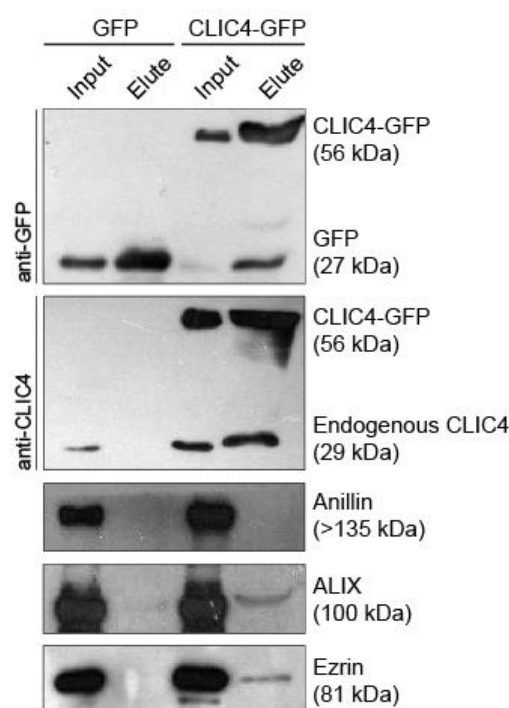


Figure 2.27: Western blotting analysis of GFP-Trap®_A pulldown of CLIC4-GFP interaction partners.

Stably GFP expressing cells were used as control.

This approach revealed that protein interactions of CLIC4 are highly transient and weak in strength as suggested in the literature [43]; therefore, the proximity ligation assay (PLA) [75] could be an alternative to immunoprecipitation experiments to identify and quantify transient and weak protein interactions at a spatiotemporal resolution in intact cells by

immunofluorescence imaging. For this, we used CLIC4-Ezrin, CLIC4-Anillin and CLIC4-ALIX antibody pairs and analyzed the protein-protein interactions at the spatial resolution during cytokinesis (**Figure 2.28**). As expected, either none or a few PLA signals were detectable in only CLIC4 antibody treated controls. We found strong interactions of CLIC4 with Ezrin, Anillin and ALIX in cytokinesis cells and the number of PLA signals were highest for the interactions with Ezrin and ALIX (**Figure 2.29**).

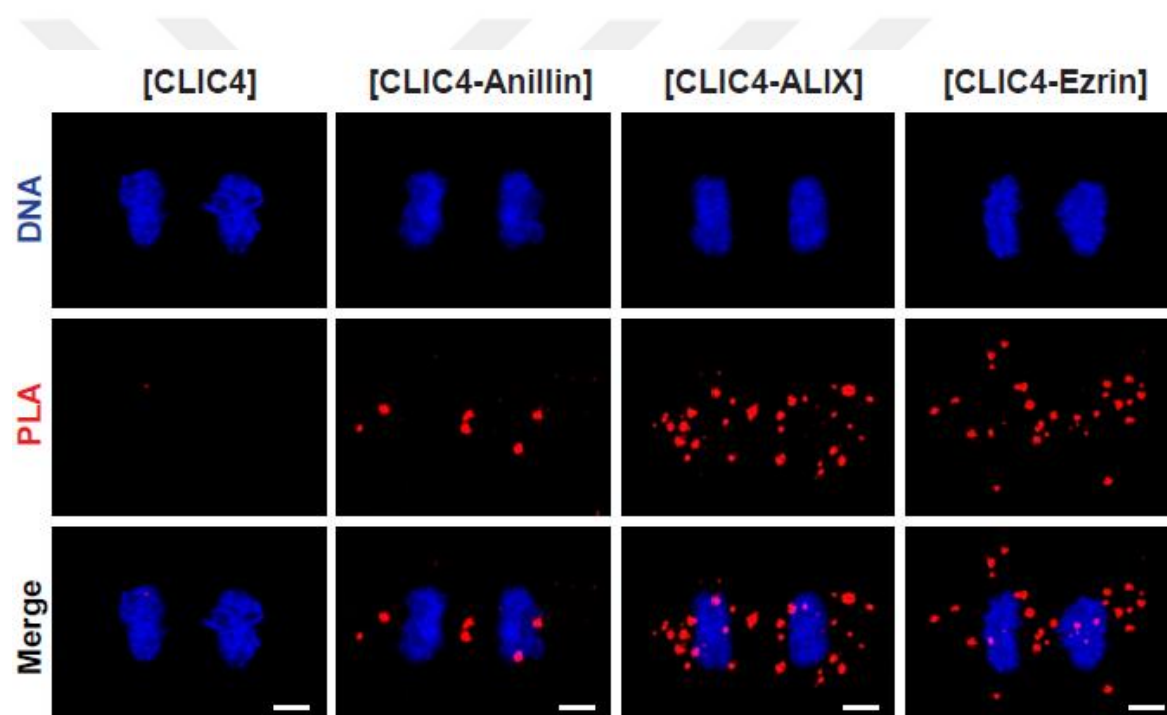


Figure 2.28: Spatial analysis of CLIC4-Anillin, CLIC4-ALIX and CLIC4-Ezrin interactions in cytokinesis by proximity ligation assay.

Scale bars: 5 μm .

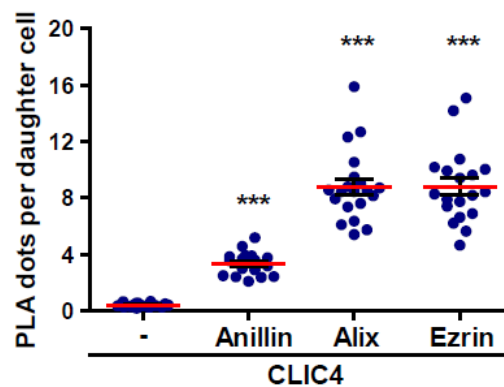


Figure 2.29: Quantification of PLA dots per daughter cell.

n=20 image frames having 456-757 nuclei in total were quantified for each condition. Unpaired two-tailed Student's t-test was used for significance analysis, *** $p < 0.0001$.

We performed live cell fluorescence imaging of HeLa S3 cells stably expressing CLIC4-GFP and Ezrin-RFP to analyze whether CLIC4 and Ezrin co-localize at different stages of cell division. They first colocalized at the mitotic cell surface and then were enriched at the equatorial cortex, where the cleavage furrow started to form. In telophase, both CLIC4 and Ezrin highly concentrated at the cleavage furrow (**Figure 2.30**).

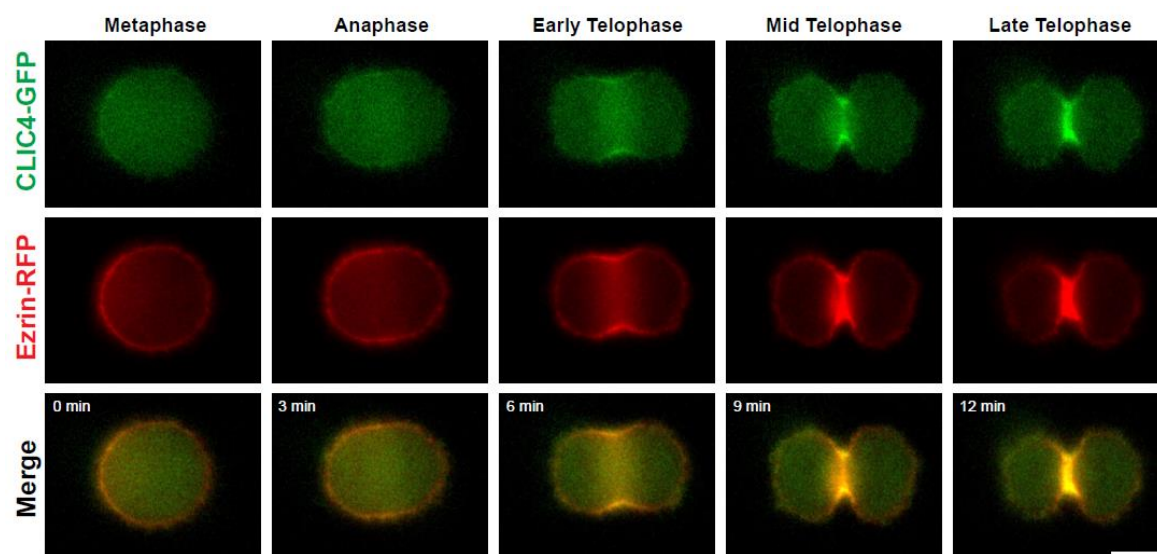


Figure 2.30: Live cell fluorescence imaging of CLIC4-GFP and Ezrin-RFP expressing cells.

Scale bar: 10 μ m.

To further assess whether the interaction between Ezrin and CLIC4 is essential for recruitment of CLIC4 to the cleavage furrow, we synchronized HeLa S3 cells to cytokinesis in the presence of Ezrin inhibitor NSC305787. NSC305787 inhibits the T567 phosphorylation of Ezrin caused by PKCI (protein kinase C iota type) and actin binding of endogenous Ezrin without altering cellular ezrin levels [65]. Endogenous CLIC4 staining in cytokinesis cells revealed that Ezrin inhibition results in a decrease in recruitment of CLIC4 to the cleavage furrow (**Figure 2.31**). Quantification of endogenous CLIC4 intensities at the cleavage furrow displayed a significant but mild decrease in the CLIC4 localization to the cleavage furrow following Ezrin inhibition (**Figure 2.32**).

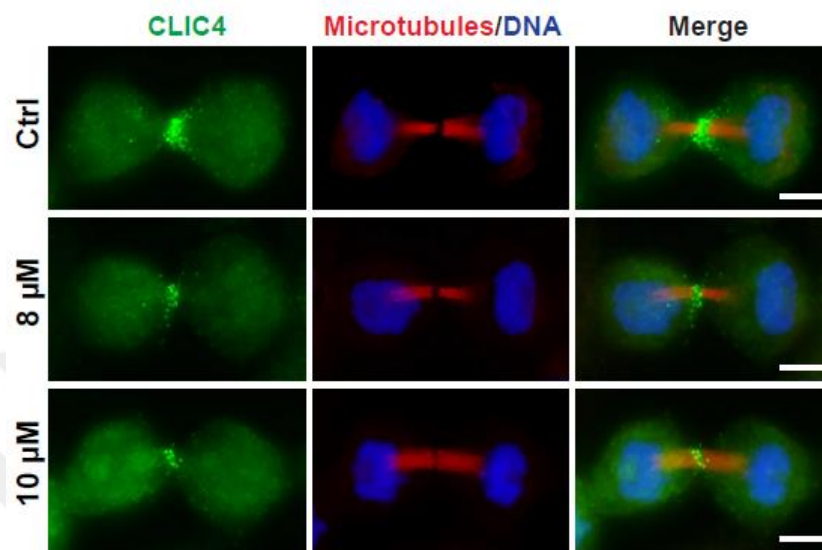


Figure 2.31: Localization of endogenous CLIC4 at the cleavage furrow after Ezrin inhibition.

Scale bars: 10 μ m.

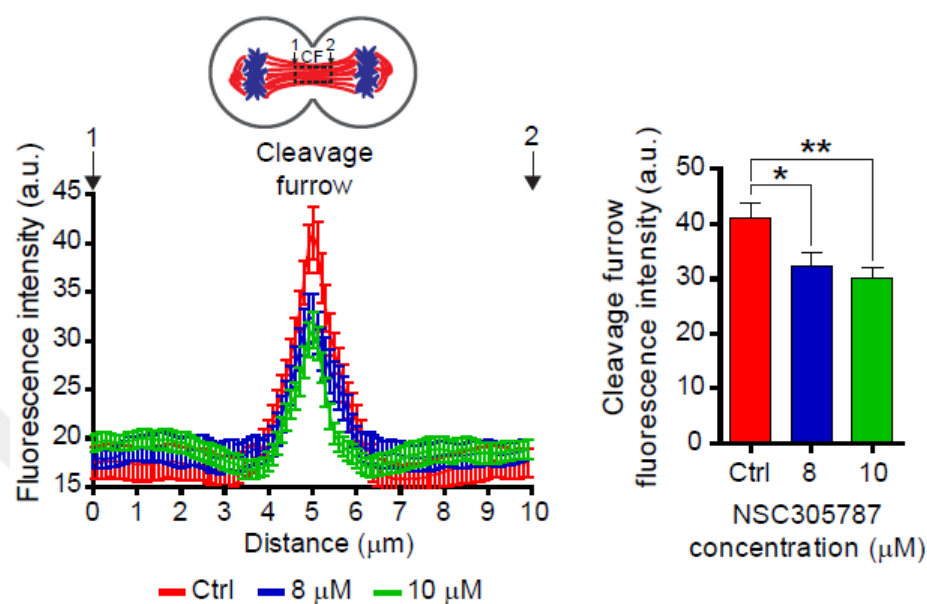


Figure 2.32: Quantification of endogenous CLIC4 signals at the cleavage furrow after Ezrin inhibition.

n=21-26 cells for each condition. Unpaired two-tailed Student's t-test was used for significance analysis, * $p < 0.05$; ** $p < 0.01$.

Collectively, these data suggest that activation of Ezrin by PKCI and interaction of active Ezrin with actin filaments favor the RhoA activation dependent plasma membrane localization of CLIC4.

2.3.6 Knockout of CLIC4 leads to multinucleation

To investigate the role of CLIC4 in cytokinesis, we generated CLIC4 knockout HeLaS3 cells using CRISPR/Cas9-based genome editing tool (**Figure 2.33**) and examined the extend of cell division failures in these cells.



Figure 2.33: Western blotting of CLIC4 knockout cell lines.

Anti-CLIC4 blotting was performed to observe endogenous CLIC4 levels and anti- α -Tubulin blotting was performed as loading control.

Moreover, stably CLIC4-GFP expressing cells in these two CLIC4 knockout background cell lines were generated (**Figure 2.34**). The knockout of CLIC4 led the cells to become multinucleated: while 3.0% of the control cells had multinucleation, this percentage significantly increased to 10.23% and 7.43% in CLIC4 knockout cell lines. CLIC4-GFP expression in CLIC4 knockout cells rescued this multinucleation phenotype resulting in a significant decrease to 4.88% and 4.08% in multinucleation percentage (**Figure 2.35**).

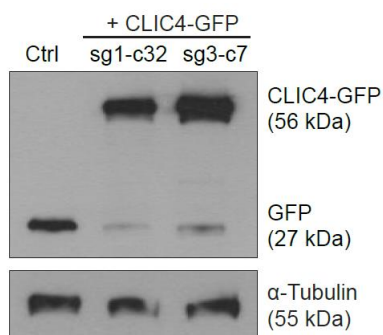


Figure 2.34: Western blotting of CLIC4-GFP expressing cell lines in CLIC4 knockout background.

Anti-GFP blot was performed to observe CLIC4-GFP expression levels. Anti- α -Tubulin blotting was performed as loading control.

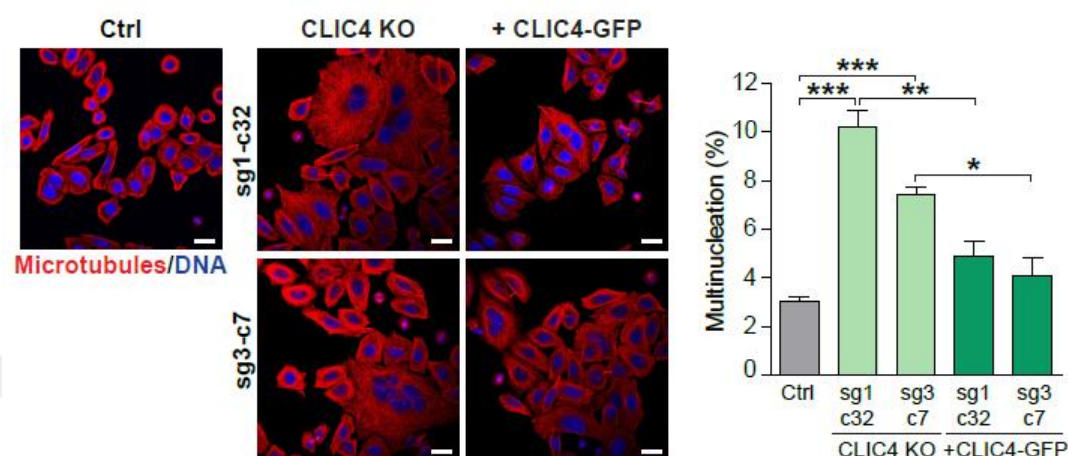


Figure 2.35: Quantification of multinucleation percentage in asynchronous populations of control, CLIC4 knockout and CLIC4-GFP expressing cells.

Data are presented as mean $\pm$ SEM ($n=3$); >300 cells were scored per experiment. Unpaired two-tailed Student's t-test was used for significance analysis, $*p<0.05$; $**p<0.01$; $***p<0.0001$. Scale bars: $20\ \mu\text{m}$.

2.3.7 Knockout of CLIC1 leads to multinucleation

Following multinucleation percentage analysis of CLIC4 knockout cells, we proposed that some redundant pathways can be present to compensate the role of CLIC4 in cell division. As CLIC1 and CLIC4 share 67.23% sequence identity showing a high degree of structural homology [34] (**Figure 2.36**) and both were enriched at the mitotic plasma membrane based on the previous proteomics data [1], CLIC1 caught our interest to address if it cooperates with CLIC4 during cell division.

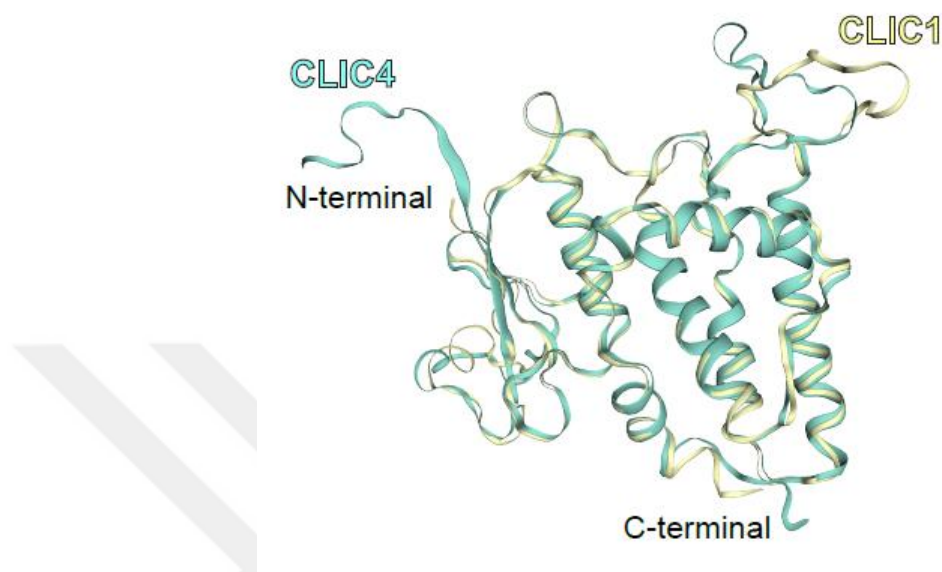


Figure 2.36: Three dimensional protein structure alignment of CLIC4 and CLIC1.

The three dimensional protein structures of CLIC4 (template: 2d2z.2.A) and CLIC1 (template: 3p90.1.A) obtained from the SWISS-MODEL Repository [41].

Live cell fluorescence imaging of CLIC1-GFP expressing cells showed that CLIC1 also localizes to the plasma membrane at mitosis and accumulates at the cleavage furrow during cytokinesis (**Figure 2.37**).

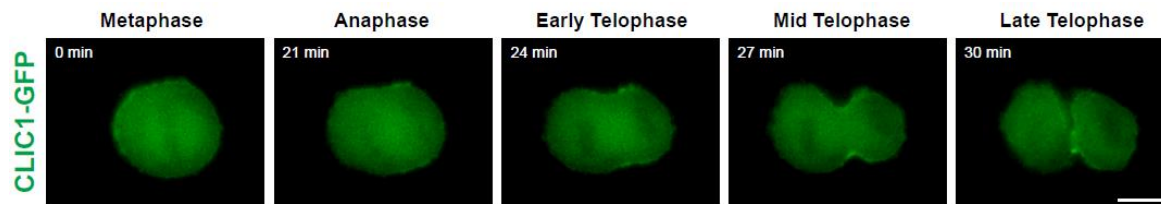


Figure 2.37: Live cell fluorescence imaging of CLIC1-GFP expressing cells.

Scale bar: 10 μm .

The immunostaining of endogenous CLIC1 and CLIC1-GFP in cytokinesis cells revealed a similar cleavage furrow localization as seen by live cell fluorescence imaging (**Figures 2.38** and **2.39**). Moreover, quantification of GFP intensities at the cleavage furrow further validated this localization pattern of CLIC1 during cytokinesis (**Figure 2.40**).

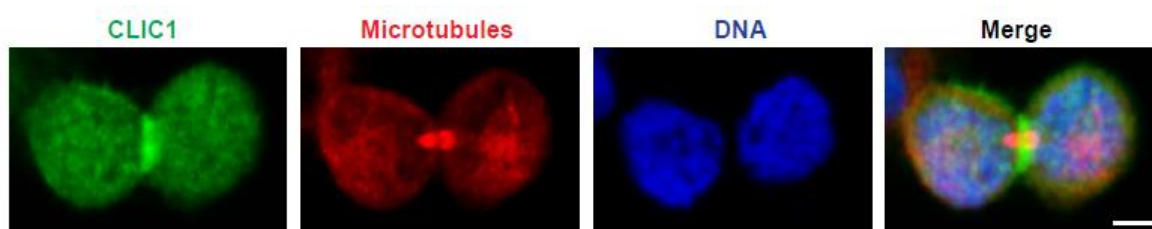


Figure 2.38: Endogenous CLIC1 localization in cytokinesis.

Scale bars: 5 μm .

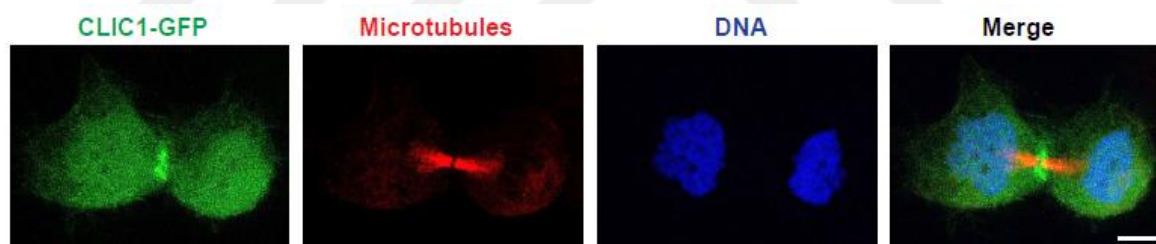


Figure 2.39: CLIC1-GFP localization in cytokinesis.

Scale bars: 5 μm .

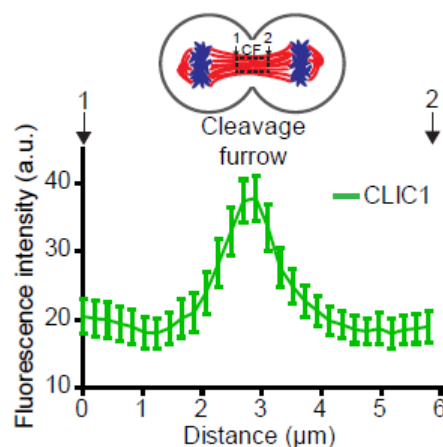


Figure 2.40: Quantification of CLIC1-GFP signals at the cleavage furrow.

n=14 cells were quantified.

As CLIC1 was also recruited to the mitotic plasma membrane and localized to the cleavage furrow during cytokinesis, we generated CLIC1 knockout cell lines using CRISPR/Cas9 system to probe its role in cell division (**Figure 2.41**).

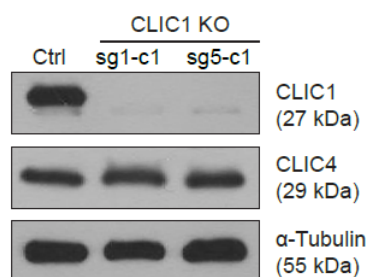


Figure 2.41: Western blotting of CLIC1 knockout cell lines.

Anti-CLIC1 and anti-CLIC4 blots were performed to observe endogenous CLIC1 and CLIC4 levels. Anti- α -Tubulin blotting was performed as loading control.

Ectopic CLIC1-GFP expression restored the CLIC1 expression in knockout cell lines (**Figure 2.42**). Analysis of multinucleation percentages of CLIC1 knockout and CLIC1-

GFP expressing cell lines revealed that in CLIC1 knockout cells, there is a significant increase in the multinucleation percentage (11.48% and 6.66%) in comparison to the control (3.95%) and this multinucleation phenotype was rescued upon ectopic CLIC1-GFP expression in these two different CLIC1 knockout cell lines (4.02% and 3.97%) (**Figure 2.43**).

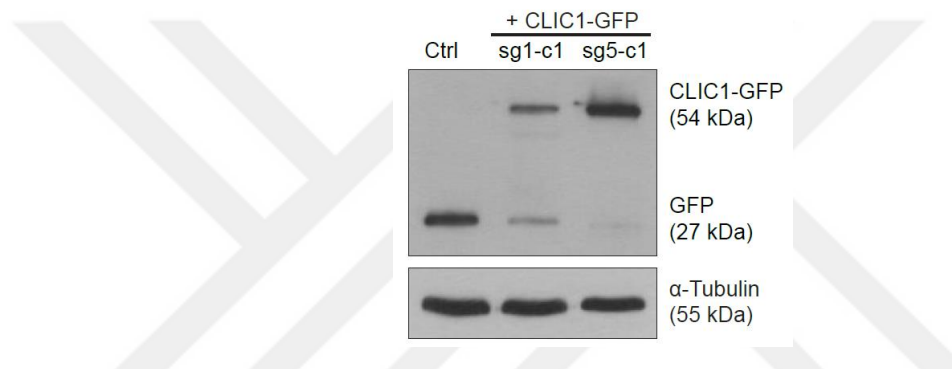


Figure 2.42: Western blotting of CLIC1-GFP expressing cell lines in CLIC1 knockout background.

Anti-GFP blot was performed to observe CLIC1-GFP expression levels. Anti- α -Tubulin blotting was performed as loading control.

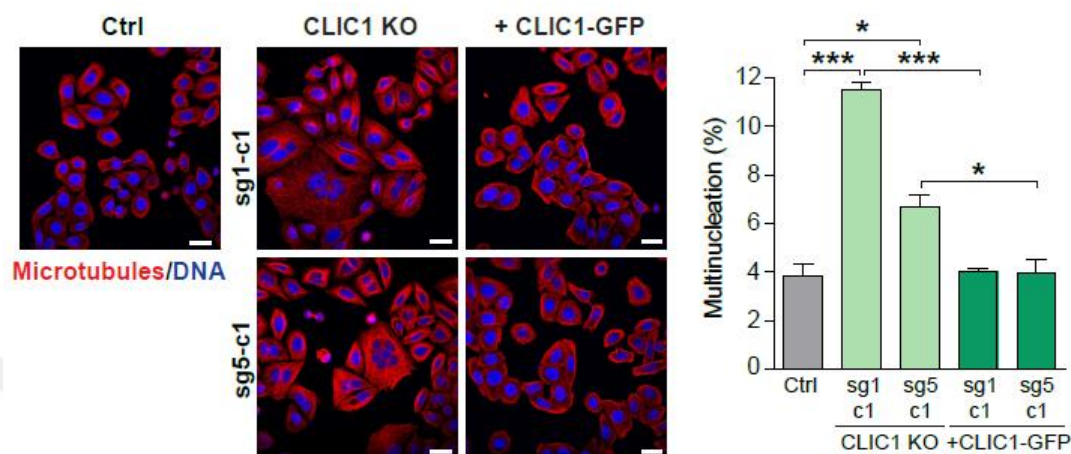


Figure 2.43: Quantification of multinucleation percentage in asynchronous populations of control, CLIC1 knockout and CLIC1-GFP rescue cells.

Data are presented as mean $\pm$ SEM (n=3); >300 cells were scored per experiment. Unpaired two-tailed Student's t-test was used for significance analysis, *p<0.05; ***p< 0.0001. Scale bars: 20 μ m.

2.3.8 Double knockout of CLIC1 and CLIC4 leads to late cytokinesis failure

To investigate whether CLIC1 and CLIC4 act together during cytokinesis i.e., whether they have additive effects on the progression of cytokinesis, we performed the double knockout of CLIC1 and CLIC4 in HeLa S3 cells (**Figure 2.44**).

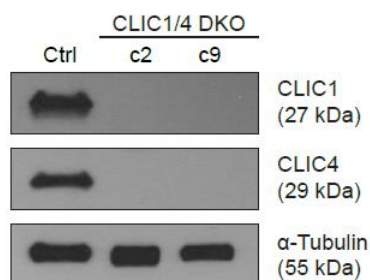


Figure 2.44: Western blotting of CLIC1/4 DKO cell lines.

Anti-CLIC1 and anti-CLIC4 blots were performed to observe endogenous CLIC1 and CLIC4 levels. Anti- α -Tubulin blotting was performed as loading control.

Double knockout cells exhibited a markedly increased cell size with the development of polyploid cells having multiple nuclei (**Figure 2.45**). Analysis of the multinucleation percentage of asynchronous CLIC1/4 DKO cells revealed that in the absence of both CLIC1 and CLIC4, multinucleation percentage of HeLa S3 cells is more than 16% (**Figure 2.46**) suggesting that CLIC1 and CLIC4 may have functional redundancy during cytokinesis.

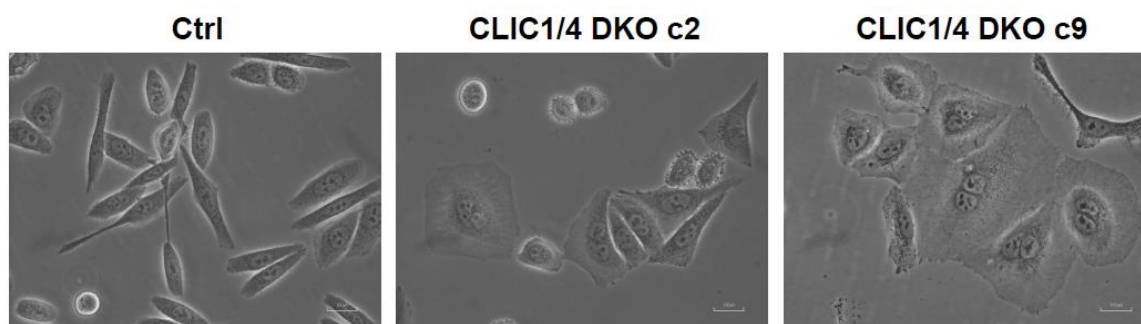


Figure 2.45: Bright-field images of control and CLIC1/4 DKO cell lines.

Scale bars: 100 μ m.

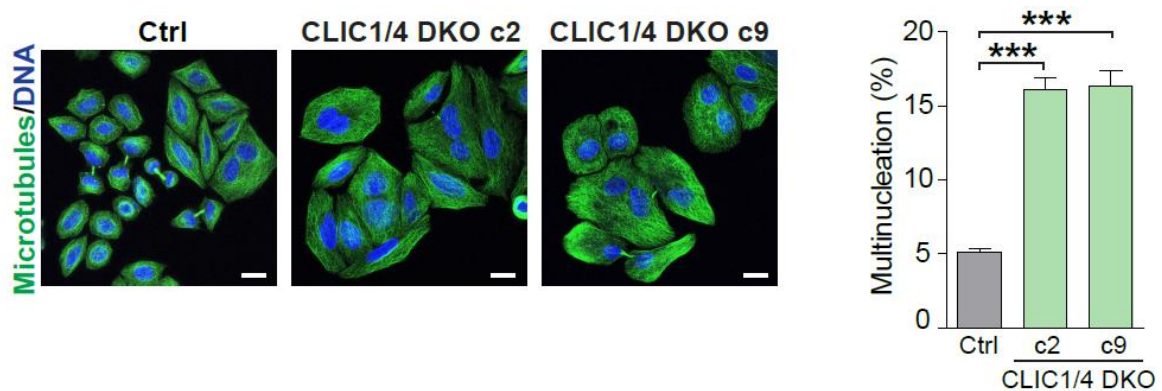


Figure 2.46: Quantification of multinucleation percentage in asynchronous populations of control and CLIC1/4 double knockout cells.

Data are presented as mean $\pm$ SEM (n=3); >200 cells were scored per experiment. Unpaired two-tailed Student's t-test was used for significance analysis, ***p< 0.0001. Scale bars: 20 μ m.

To characterize the defect leading to the multinucleated cell phenotype, we performed live cell imaging of control and CLIC1/4 DKO cells. Although CLIC1/4 DKO cells were able to round up normally, perform cleavage furrow ingression and midbody formation as normal cells (**Figure 2.47**), strikingly, they were more prone to form multinucleated cells due to the abscission defects in late cytokinesis. In abscission defective cells, incomplete abscission of the midbody leads to regression of the furrow and this increases number of multinucleated cells (**Figure 2.47**). The quantification of cell division phenotypes revealed that the percentage of abscission defective cell divisions were significantly higher in CLIC1/4 DKO cells when compared to control cells (**Figure 2.48**). These results suggest both CLIC1 and CLIC4 are required for the proper progression of late cytokinesis.

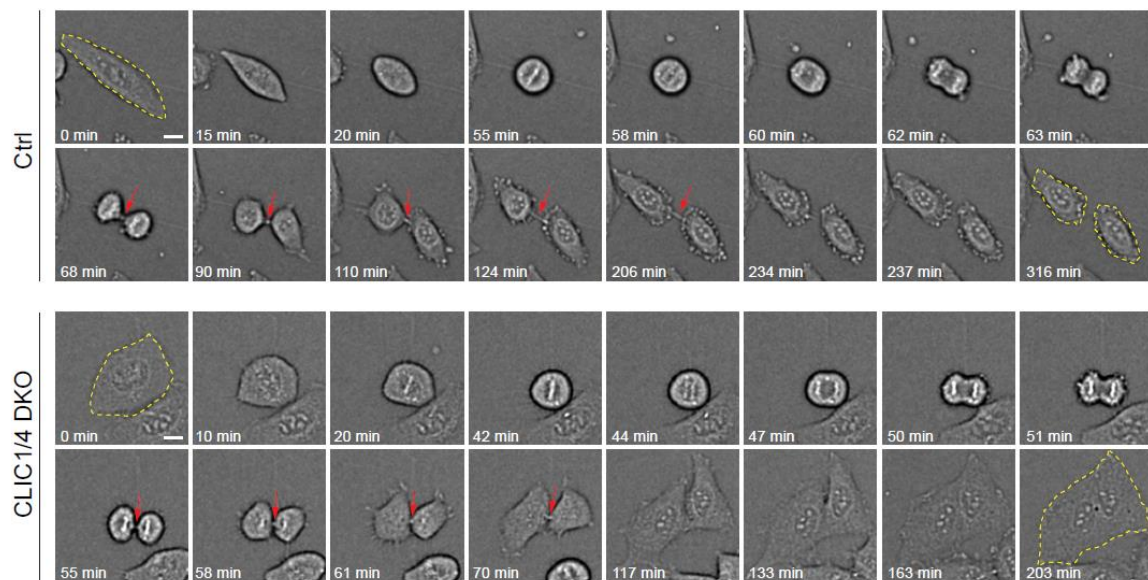


Figure 2.47: Normal cell division observed in control cells and abscission defective multinucleation observed in CLIC1/4 DKO cells were represented.

Dividing cells were marked with yellow dashed lines before and after cell division. Red arrows indicate the presence of midbody. Scale bars: 10 μ m.

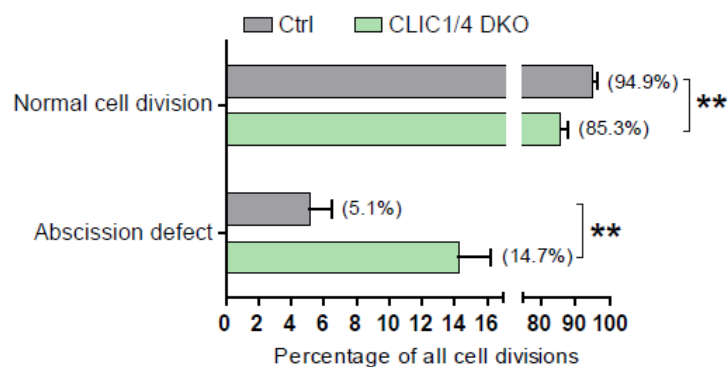


Figure 2.48: Quantification of normal and defective cell division percentages in asynchronous populations of control and CLIC1/4 DKO cells.

Data are presented as mean $\pm$ SEM; $n=6$ for control cells and ≥ 47 cell divisions were analyzed per experiment; $n=8$ for CLIC1/4 DKO cells and ≥ 43 cell divisions were analyzed per experiment. Unpaired two-tailed Student's *t*-test was used for significance analysis, $**p < 0.01$.

Following live cell analysis of CLIC1/4 DKO cells, we also examined the cell cycle dependent expression pattern of endogenous Ezrin and ERM phosphorylation levels by Western blotting. In order to analyze the ERM (ezrin/radixin/moesin) phosphorylations, we used anti-phospho-Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558) antibody. Western blotting analysis revealed that while Ezrin levels did not differ between control and double knockout cells, ERM phosphorylation was profoundly reduced in CLIC1/4 DKO cells (**Figure 2.49**).

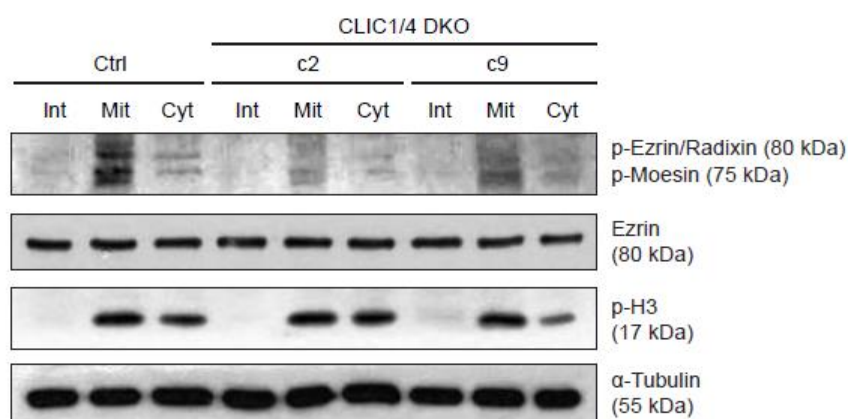


Figure 2.49: Western blotting analysis of Ezrin and phosphorylated ERM in double knockout cells.

Anti-phospho-H3 (p-H3) was used as mitosis marker and anti- α -Tubulin blotting was performed as loading control.

2.4 DISCUSSION

In this study, we investigated the cell cycle dependent localization dynamics of CLIC4 and its function during cytokinesis. During mitosis, CLIC4 enriches at the plasma membrane and mitotic spindle microtubules. While cells undergo cytokinesis, CLIC4 specifically localizes to the cleavage furrow and later concentrates at the midbody. We showed that during cytokinesis the accumulation of CLIC4 at the cleavage furrow depends on the presence of Cys35 and Phe37 residues which were previously shown to affect the plasma membrane translocation of cytosolic CLIC4 upon $G\alpha_{13}$ -mediated RhoA activation [45]. Moreover, the inhibition of RhoA activation during cytokinesis impairs the recruitment of CLIC4 to the cleavage furrow.

The identification of the proximity interactors of the wild type and C35A mutated CLIC4 proteins in cytokinesis cells gives new insights about the functions of CLIC4 during cytokinesis. Many nuclear, mitochondrial, cytoplasmic and cytoskeleton-related proteins were identified in the wild type and mutated CLIC4 interactomes. As expected, plasma membrane proteins are more enriched in the wild type CLIC4 interactome than in the mutated CLIC4 interactome since only wild type CLIC4 localizes to the plasma membrane and cleavage furrow. One of these plasma membrane proteins is Ezrin, a member of ERM (Ezrin, Radixin, Moesin) family, which functions as a linker protein between the plasma membrane-associated proteins and the actin cytoskeleton [76]. Ezrin has been shown previously to colocalize with CLIC4 in the apical microvilli and its active state directly binds to CLIC4 [47, 77]. During cytokinesis, Ezrin accumulates at the cleavage furrow and in the co-depletion of Anillin and Supervillin, its accumulation at the cleavage furrow

increases to aid ingression [78]. Same study also revealed that inhibition of RhoA activation does not abolish the localization of Ezrin at the cleavage furrow whereas the depletion of cholesterol from the plasma membrane abolishes this localization, suggesting that cleavage furrow localization of Ezrin is dependent on cholesterol [78]. Anillin also interacts with the wild type CLIC4 during cytokinesis. Anillin is an actin-binding protein and concentrates at the cleavage furrow in response to RhoA activation [73]. As a scaffold protein, Anillin links RhoA to the actin and myosin at the contractile ring and its depletion causes failures in contractile ring ingression and abscission [73, 79]. Another plasma membrane protein identified as wild type CLIC4 interactor is ALIX. ALIX interacts with proteins that function in abscission during late cytokinesis such as ESCRT-III pathway proteins and depletion of ALIX abolishes both early and late cytokinesis [27]. The colocalization, immunoprecipitation and PLA experiments clearly demonstrate the interaction of CLIC4 with Ezrin, Anillin and ALIX during cytokinesis.

The functional analysis of CLIC4 by CRISPR/Cas9-based knockout cells revealed that in the absence of CLIC4 expression, multinucleation percentage of cells increases slightly but significantly, which is likely due to the functional redundancy between the CLIC family members [80]. Given the high degree of structural homology between CLIC4 and CLIC1 [34], we analyzed the cell cycle dependent localization dynamics of CLIC1. Besides CLIC4, CLIC1 also localizes to the cleavage furrow and knockout of CLIC1 leads to the formation of multinucleated cells at similar levels observed with CLIC4 knockout cells. Our results suggest that CLIC4 and CLIC1 might be functionally redundant during cytokinesis. Double knockout of CLIC4 and CLIC1 in HeLa S3 cells results in a strong multinucleation phenotype and the formation of giant cells. Live imaging analysis of double knockout cells reveals the cytokinesis failure at the late cytokinesis stage; daughter cells fail to complete the abscission in late cytokinesis and reunite into a single cell by

regression of the cleavage furrow. Furthermore, the analysis of endogenous Ezrin and phosphorylated ERM levels in CLIC1/4 double knockout cells reveal that while Ezrin protein expression level is similar to wild type cells, phosphorylated ERM levels are profoundly decreased in CLIC1/4 double knockout cells. Moreover, the inhibition of Ezrin activation results in a decrease in the cleavage furrow accumulation of CLIC4 in wild type cells.

In summary, several lines of evidence coming from this study support the role of CLICs in cell division. Both CLIC1 and CLIC4 localize to plasma membrane in mitosis and then accumulates at the cleavage furrow. **Figure 2.50** represents our proposed model of how CLIC4 is involved in cytokinesis. During cytokinesis, CLIC4 localizes to the cleavage furrow in a RhoA activity dependent manner. Activation of Ezrin is required but not essential for the cleavage furrow localization of CLIC4. CLIC4 may mediate the plasma membrane and actin cytoskeleton coordination at the cleavage furrow through its interactions with scaffolding proteins such as Anillin and Ezrin. CLIC4 also interacts with ALIX, which is required for abscission in late cytokinesis. Knockout of CLIC4 and CLIC1 increases multinucleation due to the defects in the late cytokinesis revealing that CLIC4 mediates the proper completion of cytokinesis together with CLIC1. Further investigation of the molecular mechanisms underlying the regulation of CLIC4 and CLIC1 during cytokinesis will provide important clues about putative enzymatic activities and novel substrates of CLIC proteins.

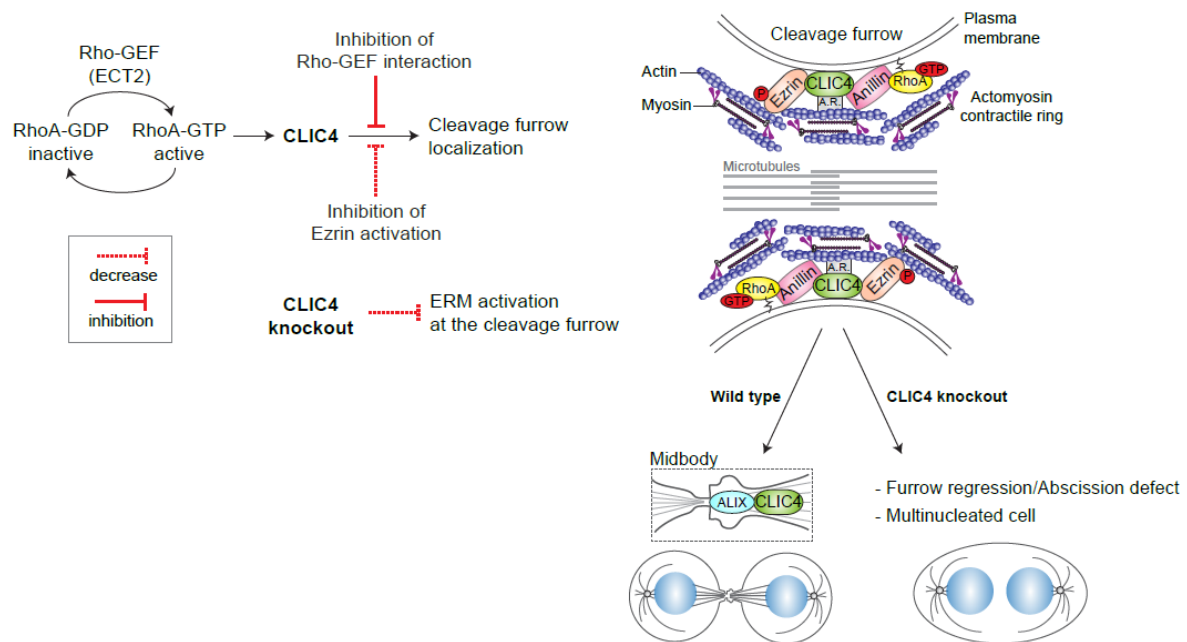


Figure 2.50: A proposed model of how CLIC4 is involved in cytokinesis.

We propose that CLIC4 provides a link between RhoA signaling and actin cytoskeleton-plasma membrane anchorage at the cleavage furrow and midbody via its interactions with Ezrin, Anillin, ALIX and other actin regulatory proteins (A.R.) which is required for the fidelity of cytokinesis.

Chapter 3

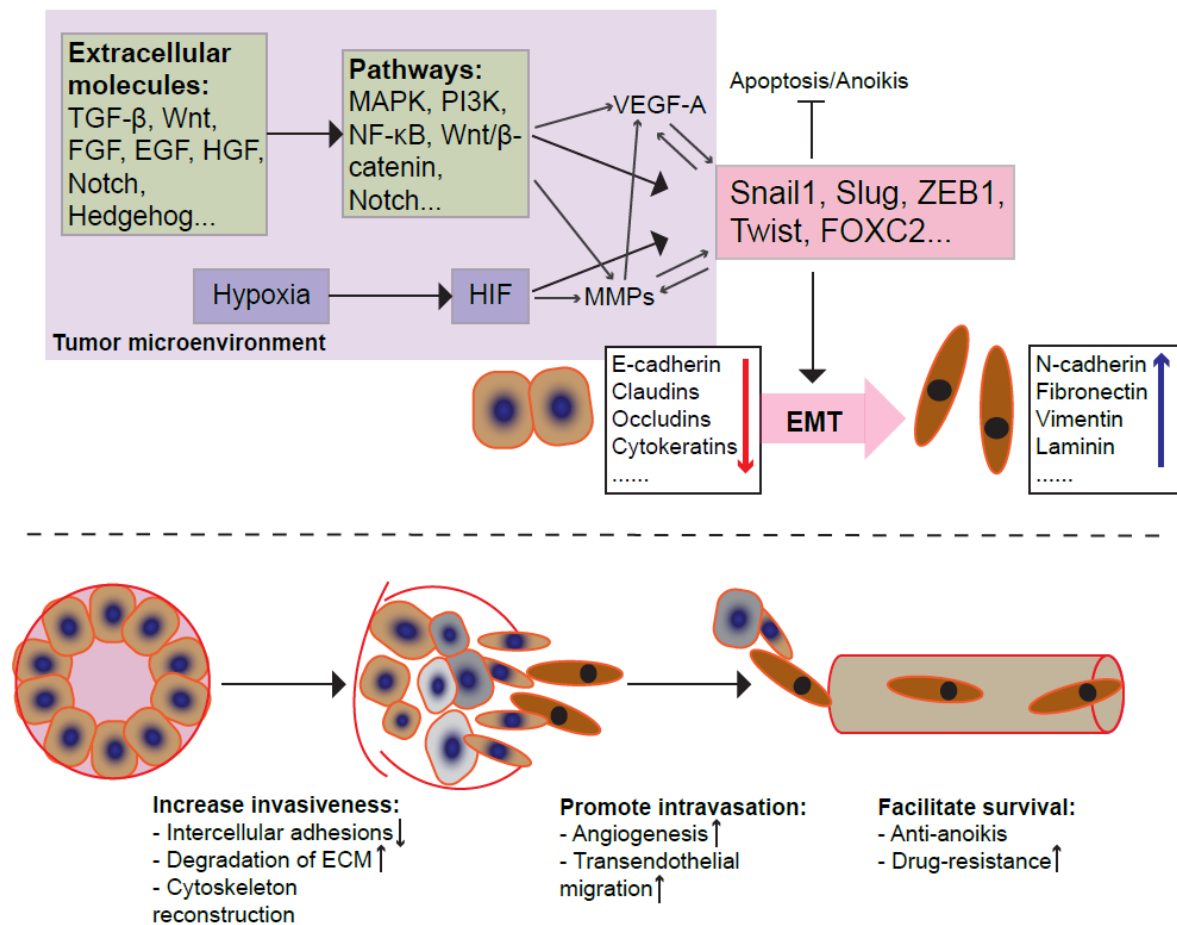
ANALYSIS OF BIOCHEMICAL CHANGES DURING EPITHELIAL-MESENCHYMAL TRANSITION

3.1 LITERATURE REVIEW

Carcinoma cells utilize epithelial-mesenchymal transition (EMT), a normally latent embryonic program, to facilitate the initiation and progression of metastasis. EMT can be induced via cell intrinsic and extrinsic factors upon the communication between epithelial cells and the nearby stromal cells, and plays a critical role in metastasis of cancer cells [2, 81-83]. In 1982, Greenburg and Hay first reported that epithelial cells can be converted into mesenchymal cells [84]. Subsequently, numerous studies described the molecular processes during EMT and suggested alternative mechanistic models that lead to EMT [85, 86]. EMT does not only trigger metastatic and invasive capabilities, but can also trigger cancer stem cell properties [84, 85, 87] and drug resistance [88-91].

Throughout EMT, epithelial cells lose cell-cell contacts and cell polarity [92]. Several signaling pathways that facilitate the activation of EMT have been discovered [85, 92, 93] and includes TGF- β , Wnt proteins, platelet-derived growth factors, Interleukin-6 and various receptor tyrosine kinases. All of these are involved in the activation of EMT inducing transcription factors (EMT-TF). These include Snail, Slug, Zeb1, Zeb2, and Twist [92]. Ectopic expression of Twist in epithelial cells leads to the loss of adherens junction

protein E-cadherin and expression of mesenchymal markers such as N-cadherin and Vimentin, suggesting the induction of EMT [2, 83, 94] (**Figure 3.1**).



Gene expression profiling of immortalized human mammary epithelial cells (HMLE) that underwent EMT by expressing Goosecoid, Snail, Twist, or TGF- β , or by knocking down the expression of E-cadherin identified a ‘EMT core signature’ which consists of 159 down-regulated genes and 87 up-regulated genes [2]. Although transcriptomic data represents gene expression changes during EMT process, it may not thoroughly predict the regulation at the protein level; i.e., the mRNA levels may not be perfectly correlated with the translated proteins due to the post-transcriptional modifications and miRNA-mediated regulation [96-98]. A comprehensive understanding of EMT requires combination of both transcriptomic and proteomic analyses. Therefore, we systematically compared biochemical changes during EMT of HMLE cells that was induced by overexpression of Twist (an EMT-TF), and we performed detailed comparison of previous transcriptome profile of the same EMT model system [2] with our proteome data.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture conditions

Human mammary epithelial cells (HMLE) (gift of R.A. Weinberg) were cultured in MEM Basal Medium (CC-3151, Lonza) supplemented with MEM SingleQuots (Bovine pituitary extract (BPE), hydrocortisone, human epidermal growth factor (hEGF) and insulin) (CC-4136, Lonza) at 37°C, 5% CO₂ in a humidified incubator. 1% penicillin/streptomycin (PS) (P4333, Sigma-Aldrich) was used in cell growth medium instead of gentamicin/amphotericin-B supplement (GA-1000) consisted in MEM SingleQuots. Freezing medium of HMLE cells was prepared using 50% fetal bovine serum (FBS) (TMS-013, Millipore), 40% HMLE growth medium and 10% dimethyl sulfoxide (DMSO) (276855, Sigma-Aldrich). For dissociation of cells from the adherent substrate, 0.05% Trypsin-EDTA solution (25300054, Thermo Fisher Scientific) was used. Dulbecco's Modified Eagle Medium (DMEM) (12-604F, Lonza) supplemented with 10% FBS and 1% PS was used to inhibit trypsinization process. Cells were spun down at 1,000 rpm for 4 min and washed once with phosphate buffered saline (PBS), pH 7.4. Then, cells were resuspended in HMLE growth medium. HEK293T cells were cultured in DMEM supplemented with 10% FBS and 1% PS at 37°C, 5% CO₂ in a humidified incubator. MCF7, MDA-MB-231 and MDA-MB-157 cells were cultured in DMEM supplemented with 10% FBS, 1% PS and 1% MEM non-essential amino acids solution (11140050, Gibco) at 37°C, 5% CO₂ in a humidified incubator.

3.2.2 Generation of HMLE-Vector and HMLE-Twist cell lines

For generation of epithelial HMLE-Vector cells and their mesenchymal derivatives, HMLE-Twist cells, we used pBABE-puro and pBABE-puro-mTwist retroviral mammalian

expression plasmids, respectively. pBABE-puro was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid # 1764 ; <http://n2t.net/addgene:1764> ; RRID:Addgene_1764) [99] and pBABE-puro-mTwist was a gift from Bob Weinberg (Addgene plasmid # 1783 ; <http://n2t.net/addgene:1783> ; RRID:Addgene_1783) [94].

pUMVC and pCMV-VSV-G packaging plasmids were used to produce retroviruses. pUMVC and pCMV-VSV-G were gifts from Bob Weinberg (Addgene plasmid # 8449 ; <http://n2t.net/addgene:8449> ; RRID:Addgene_8449 and Addgene plasmid # 8454 ; <http://n2t.net/addgene:8454> ; RRID:Addgene_8454) [56]. Moreover, pBABE-puro-GFP plasmid was a kind gift from Dr. Tamer T. Önder and used to evaluate transfection and transduction efficiencies using GFP expression.

HEK293T cell line was used for packaging of retroviral plasmids. Cells were seeded on 6 cm cell culture plates to be 25% confluent on the day of transfection. Transfection of cells was performed in the afternoon as follows: 1000 ng transfer plasmid, 1000 ng pUMVC and 100 ng pCMV-VSV-G were mixed in 90 μ l Opti-MEM (31985062, Thermo Fisher Scientific) for simultaneous transfection of cells. In a separate Eppendorf tube, 93 μ l Opti-MEM and 7 μ l FuGene transfection reagent (E2311, Promega) were mixed and incubated for 5 min at room temperature. Then, plasmid mix was added dropwise to transfection reagent solution and gently mixed. Final mix was incubated for 30 min at room temperature and then added directly to cells by dripping on top. Transfection mix was evenly distributed in cell growth medium by gently swirling the cell culture plate. Next day in the morning, transfection medium was replaced with 3 ml fresh cell growth medium. First viral supernatant was harvested at 48 hours post-transfection and stored at 4°C. Again, cells were given 3 ml fresh cell growth medium. Second viral supernatant was harvested at 72 hours post-transfection and pooled with the first one. Packaging cells were discarded

after bleach sterilization. The viral supernatant was passed through 0.45 μ m filter and aliquoted as 1 ml into Eppendorf tubes. Viruses were stored at -80°C for long-term storage.

For viral transduction, HMLE cells were seeded on 12 well cell culture plate to be 25% confluent on the day of transduction. Three wells were prepared for transduction by pBABE-puro (Vector), pBABE-puro-mTwist and pBABE-puro-GFP. In addition, one well was prepared as no transduction control to evaluate timing of puromycin selection. During viral transduction, protamine sulfate was used as adjuvant. Protamine sulfate (P4020, Sigma-Aldrich) was prepared as 8 mg/ml stock solution in water and used at 8 μ g/ml final concentration during transduction. Two successive transductions were performed to increase transduction efficiencies. First transduction of cells was performed on day 1 as follows: 200 μ l HMLE growth medium supplemented with 0.7 μ l protamine sulfate was given to cells in each well. Then, 500 μ l of corresponding viral supernatant for each well was added directly to cells by dripping on top. For control well, 500 μ l growth medium was given to cells instead of viral supernatant. Next day, GFP expression level was checked to evaluate transduction efficiency of HMLE cells. On day 3, first transduction medium was removed and second viral transduction was performed as on day 1. Depending on GFP expression level on day 4, cells were either kept in transduction medium one day more or given antibiotic selection medium. For selection of transduced HMLE cells, puromycin was used at 2 μ g/ml final concentration in growth medium for two days and all cells in control well died under puromycin selection. On the other hand, there were surviving cells in pBABE-puro (Vector), pBABE-puro-mTwist and pBABE-puro-GFP transduced cell populations. Since Twist overexpression promotes EMT [86], pBABE-puro-mTwist transduced cells were cultured for 16 days to obtain fully differentiated mesenchymal cells. Each cell population was expanded in culture and HMLE-Vector, HMLE-Twist and HMLE-GFP cell lines were obtained. Western blotting analysis of HMLE-Vector and

HMLE-Twist cell lysates were performed to evaluate the success of EMT process using anti-E-cadherin and anti-Vimentin antibodies, which are epithelial and mesenchymal protein markers, respectively. β -actin antibody was used as a loading control for Western blotting analysis. Following validation of expected EMT characteristics of each cell line by Western blotting, frozen stocks of each cell line were stored in liquid nitrogen.

3.2.3 Proteomic profiling of HMLE-Vector and HMLE-Twist cells

HMLE-Vector and HMLE-Twist cells were lysed in buffer containing 50 mM ammonium bicarbonate (pH 8.0) (A0751, Applichem), 8 M urea (33247, Sigma-Aldrich), 1 mM sodium orthovanadate (S6508, Sigma-Aldrich), cOmplete EDTA-free protease inhibitor cocktail (Roche) (1 tablet for 10 ml lysis buffer) and PhosSTOP phosphatase inhibitor cocktail (Roche) with ultrasonication for 2 min at 60 W using 20 cycles at 50% duty. Cells were disrupted by pulling the cell suspension through a thin needle. Cell debris was removed by centrifugation at 14,000 rpm for 45 min at 4°C. Protein concentration was measured using BCA protein assay kit (23227, Pierce). Proteins were reduced with 10 mM dithiothreitol (DTT) (43815, Sigma-Aldrich) for 45 min at 56°C and alkylated with addition of 20 mM iodoacetamide (IAA) (I6125, Sigma-Aldrich) for 30 min at room temperature in the dark. Cell lysate was diluted to a final urea concentration of 4 M with 50 mM ammonium bicarbonate and incubated for 4 h at 37°C using Lys-C (enzyme:protein ratio of 1:100). Finally, the solution was diluted further to a 1 M urea concentration with 50 mM ammonium bicarbonate and incubated at 37 C overnight after trypsin addition (enzyme: protein ratio of 1:100). The digestion was quenched by acidification using formic acid solution. Digests were desalted using Sep-Pak C18 cartridges, dried in Speed-Vacuum. Then, on column dimethyl labeling of peptides was performed as described previously [100]. The epithelial and mesenchymal samples were labeled with light and heavy dimethyl labels, respectively. For the second biological replicate, labels of the samples were

swapped. The labeled peptides were combined in equal ratios based on their average peptide intensities and fractionated into 40 fractions using a PolySULFOETHYL A 200×2.1 mm column (202SE05, PolyLC Inc.) and Agilent 1100 HPLC system (Agilent Technologies). The peptides were subjected to a reversed phase nanoLC-MS/MS (EASY-nLC, Thermo Fischer Scientific) connected to a Q Exactive quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific).

All raw data files were processed and quantified with Proteome Discoverer (version 1.4, Thermo Fischer Scientific). Peak lists containing HCD fragmentation were generated with Proteome Discoverer (PD) with a signal-to-noise threshold of 1.0. Produced data was searched against a Swissprot database version 2014_08 with taxonomy *Homo sapiens* (546,238 sequences) using the Mascot software (version 2.5.1, Matrix Science).

***** Proteomic profiling of HMLE-Vector and HMLE-Twist cells was performed by Özge Karayel and Ayşe Nur Polat. *****

3.2.4 Statistical analysis of the liquid chromatography mass spectrometry data

To determine regulated proteins in epithelial and mesenchymal states, epithelial and mesenchymal specific protein markers were used to define epithelial specific, mesenchymal specific and unchanged protein ratio distributions. By fitting normal distributions to Epithelial/Mesenchymal (E/M) ratios of these three groups, epithelial, mesenchymal and unchanged, the likelihood of each protein belonging to the respective groups was calculated by $p_{\text{relative}} = p_{\text{unchanged}} / (p_{\text{changed-epithelial}} \text{ or } p_{\text{changed-mesenchymal}})$. Proteins with $p_{\text{relative}} < 0.05$ were chosen as significantly regulated proteins during EMT process.

*** *Statistical analysis of the liquid chromatography mass spectrometry data was performed by **Erdem Şanal** and **Kathrin Trappe**.* ***

3.2.5 shRNA screen of the significantly regulated proteins during EMT

The analysis of the protein lists revealed many novel proteins significantly regulated during EMT process. For shRNA screen, 8 and 10 proteins that are significantly up-regulated were selected from the epithelial and mesenchymal specific protein lists, respectively. Two or three shRNA sequences against each target gene were designed using RNAi Codex database [101]. **Table 3.1** and **Table 3.2** display the epithelial and mesenchymal target proteins with their designed shRNA sequences, respectively. Red labeled sequences in each shRNA represent sense and antisense sequences separated by a loop sequence.

Table 3.1: Epithelial specific proteins and designed shRNA sequences targeting them.

NDRG1	sh1	TGCTGTTGACAGTGAGCGACGCCAGCACATTGTGAATGACTAGTGAAGCCACAGATGTA	GTCAATTCACAATGTGCTGGCGGTGCCTACTGCCTCGGA
NDRG2	sh2	TGCTGTTGACAGTGAGCGATGTGGGACTCCCAAGGAAACTAGTGAAGCCACAGATGTA	GTTCCCTTGGGAGTCCCACTGCCTACTGCCTCGGA
NDRG3	sh3	TGCTGTTGACAGTGAGCGACGTGGTGGAGTGCAACTCAATAGTGAAGCCACAGATGTA	TTTGAGTTGCACTCCACCACGGTGCCTACTGCCTCGGA
CDH1	sh1	TGCTGTTGACAGTGAGCGCGCCAACCTGGCTGGAGATTAATTAGTGAAGCCACAGATGTA	ATTAATCTCCAGCCAGTTGGCATGCCTACTGCCTCGGA
CDH1	sh2	TGCTGTTGACAGTGAGCGACCTGTTGGTGTCTTTATTATTAGTGAAGCCACAGATGTA	AATAATAAAGACCAACAAGGGTGCCTACTGCCTCGGA
CDH1	sh3	TGCTGTTGACAGTGAGCGACGAGAGAGTTTCCCTACGTATTAGTGAAGCCACAGATGTA	ATACGTAGGGAAACTCTCTCGGTGCCTACTGCCTCGGA
TLR2	sh1	TGCTGTTGACAGTGAGCGCCCAGGTAAGTGGAAACGTTATAGTGAAGCCACAGATGTA	TAACGTTTCCACTTTACCTGGAATGCCTACTGCCTCGGA
TLR2	sh2	TGCTGTTGACAGTGAGCGAGCCCTCTCTACAAACTTTAATTAGTGAAGCCACAGATGTA	ATTAAAGTTTGTAGAGAGGGCTGCCTACTGCCTCGGA
TLR2	sh3	TGCTGTTGACAGTGAGCGACGCAACTCAAAGAACTTTATATAGTGAAGCCACAGATGTA	TATAAAGTTCTTTGAGTTGCGGTGCCTACTGCCTCGGA
TFAP2A	sh1	TGCTGTTGACAGTGAGCGAGGTATTAACTCCAGATCAATAGTGAAGCCACAGATGTA	TTGATCTGGGATGTTAATACCTGCCTACTGCCTCGGA
TFAP2A	sh2	TGCTGTTGACAGTGAGCGACGTGTCCCTGTCCAAGTCCAAATAGTGAAGCCACAGATGTA	TTGGACTTGGACAGGGACACGGTGCCTACTGCCTCGGA
TFAP2A	sh3	TGCTGTTGACAGTGAGCGAGCCCTCAAGGCCATGGACAAATAGTGAAGCCACAGATGTA	TTTGCCATGGCCTTGAAGGGCTGCCTACTGCCTCGGA
LGALS7	sh1	TGCTGTTGACAGTGAGCGCTGCCAGCAGGTTCATGTAAATAGTGAAGCCACAGATGTA	TTTACATGGAACCTGCTGGCATGCCTACTGCCTCGGA
LGALS7	sh2	TGCTGTTGACAGTGAGCGCGCTCCAGGCTCTTTGAAATTAGTGAAGCCACAGATGTA	ATTTCCAAAGAGCCTGGGAGCTGCCTACTGCCTCGGA
LGALS7	sh3	TGCTGTTGACAGTGAGCGACCTCTCTCTCGCTAAACAACATAGTGAAGCCACAGATGTA	GTTGTTTAGGCGAGAGAGGGTGCCTACTGCCTCGGA
PI3	sh1	TGCTGTTGACAGTGAGCGACGTGTTCCATTCAATGGACAATAGTGAAGCCACAGATGTA	TTGTCCATTGAATGGAACACGGTGCCTACTGCCTCGGA
PI3	sh2	TGCTGTTGACAGTGAGCGCTCAGTTAAGGTCAAGATAAAATAGTGAAGCCACAGATGTA	TTTATCTTGACCTTTAACTGAATGCCTACTGCCTCGGA
PI3	sh3	TGCTGTTGACAGTGAGCGACCTAACCGCTGCTTGAAGATTAGTGAAGCCACAGATGTA	ATCTTTCAAGCAGCGTTAGGGTGCCTACTGCCTCGGA
CASP14	sh1	TGCTGTTGACAGTGAGCGACCTACCGACATGATCAGAAAGTAGTGAAGCCACAGATGTA	CTTCTGATCATGTGCGTAGGGTGCCTACTGCCTCGGA
CASP14	sh2	TGCTGTTGACAGTGAGCGCTCAGAAAGGCTCATGCTTTATTAGTGAAGCCACAGATGTA	ATAAAGCATGAGCCTTTCTGATTGCCTACTGCCTCGGA
ICAM1	sh1	TGCTGTTGACAGTGAGCGACCAACCAATGTGCTATTCAAAATAGTGAAGCCACAGATGTA	TTTGAATAGCACATTGGTTGGCTGCCTACTGCCTCGGA
ICAM1	sh2	TGCTGTTGACAGTGAGCGAGCCACGCATCTGATCTGTAGTTAGTGAAGCCACAGATGTA	ACTACAGATCAGATGCGTGGCTGCCTACTGCCTCGGA
ICAM1	sh3	TGCTGTTGACAGTGAGCGCAGACATGATTGATGGATGTTATAGTGAAGCCACAGATGTA	TAACATCCATCAATCATGTCTTGCCTACTGCCTCGGA

Table 3.2: Mesenchymal specific proteins and designed shRNA sequences targeting them.

LRP1	sh1	TGCTGTTGACAGTGAGCGAGGCCTGACTGTGTTTGAGAATTAGTGAAGCCACAGATGTAATTCTCAAAACAGTCAGGCCGTGCCTACTGCCTCGGA
LRP1	sh2	TGCTGTTGACAGTGAGCGACGACAATGACTGTGAGGATAATAGTGAAGCCACAGATGTAATATCCTCACAGTCATTGTCGCTGCCTACTGCCTCGGA
LRP1	sh3	TGCTGTTGACAGTGAGCGACCCGCGAGGACTACATTGAATTAGTGAAGCCACAGATGTAATTCAATGTAGTCCTCGCGGGCTGCCTACTGCCTCGGA
PDGFRB	sh1	TGCTGTTGACAGTGAGCGCGGACAGAAAGCTACATCTGCAATAGTGAAGCCACAGATGTAATGACAGATGTAGCTTCTGTCCTGCCTACTGCCTCGGA
PDGFRB	sh2	TGCTGTTGACAGTGAGCGCGGAGTTACTGAGGTGGTAAATTAGTGAAGCCACAGATGTAATTAACACCTCAGTAACCTCCATGCCTACTGCCTCGGA
CDH2	sh1	TGCTGTTGACAGTGAGCGCGGGCACATGCAGTAGATATTATAGTGAAGCCACAGATGTAATATCTACTGCATGTGCCCTGCCTACTGCCTCGGA
CDH2	sh2	TGCTGTTGACAGTGAGCGCGCAGATCTATTTACTTGATATTAGTGAAGCCACAGATGTAATATCAAGTAAATAGATCTGCATGCCTACTGCCTCGGA
CDH2	sh3	TGCTGTTGACAGTGAGCGAGGTAATCCTCCCAATCAAAATTAGTGAAGCCACAGATGTAATTGATTGGGAGGATTACCCGCCTACTGCCTCGGA
MME	sh1	TGCTGTTGACAGTGAGCGCGCTTGCTTGGAATGAATAATAGTGAAGCCACAGATGTAATATTCATTTCGAAGCAAGCTGCCTACTGCCTCGGA
MME	sh2	TGCTGTTGACAGTGAGCGCGGACAGCACCTTAATGGAATTAGTGAAGCCACAGATGTAATATCCATTAAAGGTGCTGTCATGCCTACTGCCTCGGA
MME	sh3	TGCTGTTGACAGTGAGCGCGCATTGTACAGGTCTTGATATAGTGAAGCCACAGATGTAATACAAGACCTGTACAATGCTGCCTACTGCCTCGGA
BIN1	sh1	TGCTGTTGACAGTGAGCGCGCAGTGCCTCCAGAAATTTCAATAGTGAAGCCACAGATGTAATGAAATCTGGACGCAGTCTGCCTACTGCCTCGGA
BIN1	sh2	TGCTGTTGACAGTGAGCGCCGACCTGCTGTGGATGGATATAGTGAAGCCACAGATGTAATATCCATCCACAGCAGGTGCTGCCTACTGCCTCGGA
BIN1	sh3	TGCTGTTGACAGTGAGCGCCGAGTCCCTCAAACCTGCCAATAGTGAAGCCACAGATGTAATGGCAGTTTGAAGGGACTCGTGCCTACTGCCTCGGA
CD81	sh1	TGCTGTTGACAGTGAGCGAGCCATCACCCGCTGTGTATATAGTGAAGCCACAGATGTAATATACACAGGCGGTGATGGCTGCCTACTGCCTCGGA
CD81	sh2	TGCTGTTGACAGTGAGCGCACACGTCGCCTTCAACTGTAAATAGTGAAGCCACAGATGTAATACAGTTGAAGGCGCAGTGTGCCTACTGCCTCGGA
CD81	sh3	TGCTGTTGACAGTGAGCGCCTGACTCCGTCATTTAATAATAGTGAAGCCACAGATGTAATTTAAATGACGGAGTCAAGGATGCCTACTGCCTCGGA
JUND	sh1	TGCTGTTGACAGTGAGCGATGTTGATTTGCCTATTATAGTGAAGCCACAGATGTAATAATAGGGCAGAATCGAACACTGCCTACTGCCTCGGA
JUND	sh2	TGCTGTTGACAGTGAGCGAACGCTGAGCCTGAGTGAGCAGTAGTGAAGCCACAGATGTAATGCTCACTCAGGCTCAGCGCTGCCTACTGCCTCGGA
JUND	sh3	TGCTGTTGACAGTGAGCGACGCCTCATCATCCAGTCCAATAGTGAAGCCACAGATGTAATGGAGTGGATGATGAGGCGCTGCCTACTGCCTCGGA
CADM3	sh1	TGCTGTTGACAGTGAGCGAAGCCCTTCGAGATAATCGAATTAGTGAAGCCACAGATGTAATTCGATTATCTCGAAGGGCTGCCTACTGCCTCGGA
CADM3	sh2	TGCTGTTGACAGTGAGCGCCAGGAAGATCCCAATGGTAAATAGTGAAGCCACAGATGTAATTAACATTGGGATCTTCTGTGCCTACTGCCTCGGA
ANPEP	sh1	TGCTGTTGACAGTGAGCGAGGCCATGAAGGCCGAGTTCAATAGTGAAGCCACAGATGTAATGAACCTCGGCCTTATGGCCGTGCCTACTGCCTCGGA
ANPEP	sh2	TGCTGTTGACAGTGAGCGACCTGTACCTCTTTACCTTTAGTGAAGCCACAGATGTAAGGTGAAAGAGGGTACAGGGCTGCCTACTGCCTCGGA
DNAJB4	sh1	TGCTGTTGACAGTGAGCGACGGAATGAGGAGAAGAATTATAGTGAAGCCACAGATGTAATATCTTCTCCTCATTCGGTGCCTACTGCCTCGGA
DNAJB4	sh2	TGCTGTTGACAGTGAGCGAACGGGTCAAATAAATAGGCAATAGTGAAGCCACAGATGTAATGCCTATTTATTTGACCCGTCTGCCTACTGCCTCGGA
DNAJB4	sh3	TGCTGTTGACAGTGAGCGCTGCAACTACTTGTTATGCATATAGTGAAGCCACAGATGTAATGCATAACAAGTAGTTGCAATGCCTACTGCCTCGGA

pSMP-Luc retroviral mammalian expression plasmid was used for the expression of shRNAs. pSMP-Luc was a gift from George Daley (Addgene plasmid # 36394 ; <http://n2t.net/addgene:36394> ; RRID:Addgene_36394) [102]. Each shRNA sequence was cloned into pSMP-Luc plasmid after the removal of Firefly luciferase targeting shRNA sequence

(5'-TGCTGTTGACAGTGAGCGCCCGCCTGAAGTCTCTGATTAAATAGTGAAGCCACAGATGTAATATCAGAGACTTCAGGGCGGGTGCCTACTGCCTCGGA-3'). First of all,

each designed shRNA sequence was flanked with forward shRNA primer containing XhoI cut site and reverse shRNA primer containing EcoRI cut site in PCR (5.0 ul of 10X ThermoPol Buffer; 1 ul of 100 mM MgSO₄; 2.5 ul of 100% DMSO; 10 ul of 5 M Betaine; 1 ul of 10 mM dNTPs; 5 ul of 1 uM shRNA oligo; 2.5 ul of 10 uM forward shRNA primer (5'-GATGGCTGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3'); 2.5 ul of 10 uM reverse shRNA primer (5'-GTCTAGAGGAATTCCGAGGCAGTAGGCA-3'); 0.5 ul of 2 U/ul Vent DNA Polymerase; 20 ul dH₂O; PCR conditions: Initial denaturation 94°C-5 min, Denaturation 94°C-30 sec, Annealing 54°C-30 sec, Extension 74°C-30 sec, 25 cycles, Final extension 75°C-2 min). Then, PCR products and pSMP-Luc plasmid were digested with restriction endonucleases which have recognition sites embedded in shRNA PCR primer pair (5 ul of 10X NEB4 Buffer; 0.5 ul of 100X BSA; 1 ul of EcoRI (R3101S, NEB); 1 ul of XhoI (R0146S, NEB); 1 ug shRNA oligo or 10ug pSMP-Luc plasmid; dH₂O up to 50 ul of total reaction volume; reaction conditions: Incubation 37°C-1 h, Heat inactivation 65°C-25 min). Digested pSMP plasmid was dephosphorylated with alkaline phosphatase enzyme (10 ul of 10X NEB4 Buffer; 50 ul plasmid digestion reaction; 4 ul of CIP (M0290S, NEB); 36 ul of dH₂O; reaction conditions: Incubation 37°C-1 h). Next, both digested shRNA oligos and CIP treated pSMP plasmid were run in 1.2% agarose gel electrophoresis and then extracted from the gel using NucleoSpin Gel and PCR Clean-up Kit (740609, Macherey-Nagel). Finally, digested and purified shRNA oligos and pSMP plasmids were used in the ligation reaction with vector:insert ratio of 1:3 using Quick Ligation Kit (M2200L, NEB) (50 ng of pSMP and 3-fold molar excess of shRNA oligos were mixed and dH₂O was added up to 10 ul total volume; 10 ul of 2X Quick Ligation Buffer; 1 ul of Quick T4 DNA Ligase; reaction conditions: Incubation 25°C-15 min and chill on ice).

Ligation products were used to transform chemical competent *E. coli* DH5 α bacteria according to the standard transformation protocol (5 μ l DNA + 100 μ l bacteria; 30 min incubation on ice; 1 min heat shock at 42°C; 2 min incubation on ice; addition of 900 μ l LB and incubation at 37°C with 700 rpm shaking for 1.5 h; centrifugation at 8,000 rpm for 10 min; discard 800 μ l of supernatant; resuspend the pellet; plate 100 μ l onto LB agar plate with selective antibiotic – pSMP plasmid has ampicillin resistance). Plates were incubated at 37°C overnight. Next day, single colonies from each plate were picked and inoculated into liquid LB cultures containing ampicillin. LB cultures were incubated at 37°C overnight with 200 rpm rotational shaking. DNA isolation from overnight bacteria cultures were performed using NucleoSpin Plasmid kit (740588, Macharey-Nagel) according to the manufacturer's instructions. Purified plasmids were sequenced with MSCV forward primer (5'-CCCTTGAACCTCCTCGTTCGACC-3') by Macrogen Company. After confirmation by sequencing, plasmids were used in the following experiments.

All packaging steps of retroviral shRNA plasmids were carried out as explained in **Section 3.2.2**. pSMP-Luc plasmid was packaged as control. For the virus-mediated shRNA-mediated knockdown of epithelial targets, epithelial cell line MCF-7 and for the that of mesenchymal targets, mesenchymal cell lines MDA-MB-231 and MDA-MB-157 were used. Viral transduction of cells was performed as explained in **Section 3.2.2**. For selection of transduced MCF7 cells, puromycin was used at 4 μ g/ml final concentration in growth medium for two days, whereas MDA-MB-231 cells were selected in growth medium containing 2 μ g/ml puromycin. Each cell population was expanded in culture and frozen stocks of each cell line were obtained. **Table 3.3** and **Table 3.4** show cell lines generated by stable expression of shRNAs in epithelial and mesenchymal cells, respectively.

Table 3.3: Epithelial cell lines generated by stable expression of shRNAs.

Cell line	shRNA	shRNA code
MCF7	LGALS7 sh1	1
MCF7	LGALS7 sh2	2
MCF7	LGALS7 sh3	3
MCF7	PI3 sh1	4
MCF7	PI3 sh2	5
MCF7	PI3 sh3	6
MCF7	CASP14 sh1	7
MCF7	CASP14 sh2	8
MCF7	ICAM1 sh1	9
MCF7	ICAM1 sh2	10
MCF7	ICAM1 sh3	11
MCF7	NDRG1 sh1	12
MCF7	NDRG1 sh2	13
MCF7	CDH1 sh1	14
MCF7	CDH1 sh2	15
MCF7	CDH1 sh3	16
MCF7	TLR2 sh2	17
MCF7	TLR2 sh3	18
MCF7	TFAP2A sh1	19
MCF7	TFAP2A sh2	20
MCF7	TFAP2A sh3	21
MCF7	Firefly Luciferase	FF

Table 3.4: Mesenchymal cell lines generated by stable expression of shRNAs.

Cell line	shRNA	shRNA code	Cell line	shRNA	shRNA code
MDA-MB-231	DNAJB4 sh1	22	MDA-MB-157	DNAJB4 sh1	22
MDA-MB-231	DNAJB4 sh2	23	MDA-MB-157	DNAJB4 sh2	23
MDA-MB-231	DNAJB4 sh3	24	MDA-MB-157	DNAJB4 sh3	24
MDA-MB-231	PDGFRB sh1	25	MDA-MB-157	PDGFRB sh1	25
MDA-MB-231	PDGFRB sh2	26	MDA-MB-157	PDGFRB sh2	26
MDA-MB-231	LRP1 sh1	27	MDA-MB-157	LRP1 sh1	27
MDA-MB-231	LRP1 sh3	28	MDA-MB-157	LRP1 sh3	28
MDA-MB-231	CDH2 sh1	29	MDA-MB-157	CDH2 sh1	29
MDA-MB-231	CDH2 sh2	30	MDA-MB-157	CDH2 sh2	30
MDA-MB-231	MME sh2	31	MDA-MB-157	MME sh2	31
MDA-MB-231	MME sh3	32	MDA-MB-157	MME sh3	32
MDA-MB-231	BIN1 sh1	33	MDA-MB-157	BIN1 sh1	33
MDA-MB-231	BIN1 sh2	34	MDA-MB-157	BIN1 sh2	34
MDA-MB-231	CADM3 sh1	35	MDA-MB-157	CADM3 sh1	35
MDA-MB-231	CADM3 sh2	36	MDA-MB-157	CADM3 sh2	36
MDA-MB-231	CD81 sh1	37	MDA-MB-157	CD81 sh1	37
MDA-MB-231	CD81 sh2	38	MDA-MB-157	CD81 sh2	38
MDA-MB-231	CD81 sh3	39	MDA-MB-157	CD81 sh3	39
MDA-MB-231	JUND sh1	40	MDA-MB-157	JUND sh1	40
MDA-MB-231	JUND sh2	41	MDA-MB-157	JUND sh2	41
MDA-MB-231	JUND sh3	42	MDA-MB-157	JUND sh3	42
MDA-MB-231	ANPEP sh2	43	MDA-MB-157	ANPEP sh2	43
MDA-MB-231	Firefly Luciferase	FF	MDA-MB-157	Firefly Luciferase	FF

3.2.6 Cell migration screen of shRNA treated epithelial and mesenchymal cells

The xCELLigence RTCA-DP system (ACEA Biosciences) was used for cell migration assay using FBS as chemo-attractant. Firstly, each bottom chamber well of CIM plate was filled with 160 μ l complete medium containing 10% FBS using an 8 channel pipette by preventing bubble formation. Then, top chamber was carefully placed onto the bottom chamber with the sensor surface facing down. 50 μ l complete medium containing 1% FBS was added to each well of the top chamber to cover the membrane surface. The CIM plate

was placed onto RTCA DP Analyzer inside the 37°C incubator and incubated for 1 h to allow the CIM membrane surface to reach an equilibrium with medium. Following 1 h incubation of the plate, the background measurement was performed. 100 μ l cell suspension was added to each well of the top chamber. The final number of cells per well should be 30,000 for MDA-MB-231 and 50,000 for MCF7. Duplicates were prepared for each experimental condition. The CIM plate was left in the hood at room temperature for 30 min after cell addition to allow the cells settle down on to the bottom surface of the top chamber. Finally, the CIM plate containing the cells was placed in the DP System inside the incubator, followed by the measurement with an interval of 15 minutes for 48 h.

*** *Cell migration screen of shRNA treated epithelial and mesenchymal cell lines was performed at Bilkent University, Ankara, Turkey with the help of Özge Saatci.* ***

3.2.7 Scratch wound healing assay

For scratch wound healing assay, 0.5×10^6 MDA-MB-231 cells per well were seeded in 6-well plates and grown to sub-confluence. Cell proliferation was blocked by a 8 h pretreatment of cells in serum-free medium. The cell monolayer was gently and slowly scratched with a 200 μ l pipette tip across the center of the well. After scratching, the well was gently washed twice with PBS to remove detached cells. Then, the well was replenished with fresh medium containing 1% FBS. The wound area was measured at the beginning and after 24 h following scratch formation using ImageJ software (Wayne Rasband, NIH). Three independent experiments were performed for each condition. Images were taken with 4x magnification using Nikon Eclipse inverted microscope (Nikon, Japan).

3.2.8 Cell viability assay

For cell viability assay, Cell Titer Glo Luminescent Cell Viability Assay kit (G7571, Promega) was used according to the manufacturer's protocol. 100 μ l MDA-MB-231 cell suspension containing 20,000 cells were added in 96-well as triplicates for each condition. Control wells containing medium without cells were prepared to obtain a value for background luminescence. Next day, 50 μ l of medium was removed from the wells and 50 μ l CellTiter-Glo Reagent was added to each well. Contents were mixed for 10 min on an orbital shaker in the dark to induce cell lysis and stabilize luminescent signal. 100 μ l supernatants from each well were transferred to opaque-walled 96-well plate (E121003H, Cellstar). Then, luminescence was recorded using 0.25–1 sec integration time.

3.2.9 Flow cytometry analysis

1×10^6 cells in 4 ml medium were placed into FACS tubes (2052, Falcon). Cells were spun down at 1,200 rpm for 5 min and supernatant was discarded. Cells were washed once with 3 ml of PBS containing 2% BSA and 0.02% NaN_3 . Then, cells were spun down at 1,200 rpm for 5 min and supernatant was discarded. Cell pellet was resuspended in approximately 100 μ l of remaining supernatant. PE-conjugated anti-CD81 antibody (JS-81, BD Biosciences) was a kind gift from Dr. Batu Erman. 2.5 μ l of PE-conjugated anti-CD81 antibody was diluted in 7.5 μ l PBS and added to cell suspension. Cell suspension was mixed by flicking and covered with foil to be kept at dark. The foiled tube was incubated on ice for 50 min. Cells were washed once with 3 ml of PBS containing 2% BSA and 0.02% NaN_3 . Then, cells were spun down at 1,200 rpm for 5 min and supernatant was carefully discarded. One more time, the centrifugation and washing of cells were performed as previous. Cell pellet was resuspended in 500 μ l of PBS containing 2% BSA and 0.02% NaN_3 . Finally, cell suspension was filtered into a new FACS tube and directly analyzed. In addition to the PE-conjugated anti-CD81 antibody stained samples, unstained

control samples were prepared for each cell line. All samples were analyzed using SONY LE-SH800 flow cytometer. All flow data were analyzed using FlowJo_V10 software.

3.2.10 EMT induction via expression of Twist-ER fusion protein in HMLE cells

HMLE-Twist-ER cell line was a kind gift from Dr. Tamer Önder. This cell line was generated by stable expression of retroviral mammalian expression plasmid pWZL-Blast-Twist-ER in HMLE cells. pWZL-Blast-Twist-ER was a gift from Bob Weinberg (Addgene plasmid # 18799 ; <http://n2t.net/addgene:18799> ; RRID:Addgene_18799) [103]. The cloned ER sequence in the plasmid has G525R point mutation preventing the interaction of ER with 17 β -estradiol but not with the synthetic ligand 4'-hydroxytamoxifen (4OHT). The Twist-ER fusion protein translocates to nucleus upon 4OHT treatment and stimulates transcription, which results in EMT in two weeks. For EMT induction, 4OHT treatment was performed as described previously [104]. Briefly, 4OHT (H7904, Sigma-Aldrich) was dissolved in ethanol to make 1 mM stock solution and stored at -20°C. For each treatment, 4OHT was freshly added into cell medium at a final concentration of 20 nM. 0.8x10⁶ cells were seeded on 6 cm cell culture plate and passaged every 2-3 days when they reached confluency. During each passage, cells were seeded at a ratio of 1 to 4 and the remaining cells were pelleted for Western blotting analysis. EMT induction by 4OHT treatment was performed for 16 days and at the end of treatment, 4OHT treated cells obtained a spindle-shaped mesenchymal morphology. Western blotting analysis of epithelial and mesenchymal marker proteins was performed to evaluate EMT induction efficiency.

3.2.11 Statistical analysis of cell line and patient datasets

For mRNA expression analysis of genes in different breast cell lines, the GSE40059 dataset [105] from NCBI GEO database was used. Epithelial and mesenchymal cell lines were assigned based on a previous study [106]. SK-BR-3 and MDA-MB-468 cell lines

were assigned as neither epithelial nor mesenchymal due to low expression of E-cadherin and the absence of mesenchymal markers, fibronectin and vimentin [106-108]. Mean mRNA expression values of two groups were statistically analyzed by two-tailed Student's t-test. TCGA mRNA data and EMT score values were downloaded from the cBioPortal for Cancer Genomics [109, 110] and TCGA database [111], respectively. Correlation analysis of mRNA z-scores with EMT scores was performed and r values were obtained for each gene. For metastasis-free survival analysis of patients, GSE2603 microarray data [112] for primary breast cancer patients (n=82) was utilized. Patients were separated into two groups at the median expression. Kaplan-Meier analyses were used to determine metastasis-free survival and statistical differences were determined by Log-rank (Mantel-Cox) test.

*** *Statistical analysis of cell line and patient datasets was performed with the help of Özge Saatci.* ***

3.2.12 Generation of shRNA treated MDA-MB-231-luc2-GFP cells

Luciferase and GFP dual labeled MDA-MB-231 cell line (MDA-MB-231-luc2-GFP) was a kind gift from Dr. Özgür Şahin. This cell line was used to perform shRNA mediated knockdown of target genes for animal experiments since luciferase expression allows bioluminescence imaging. Retroviral shRNA particles generated in **Section 3.2.5** were used to infect MDA-MB-231-luc2-GFP cells. Viral infection of cells was performed as explained in **Section 3.2.2**. For selection of transduced MDA-MB-231-luc2-GFP cells, puromycin was used at 2 ug/ml final concentration in growth medium for two days.

3.2.13 Animal experiments

All animal experiments were approved by the animal ethics committee of Bilkent University, Ankara, Turkey. 6–8 weeks old female athymic nu/nu mice were subcutaneously injected with 1×10^6 cells into mammary fat pad (MFP) without incision. Primary tumor growth was monitored by measuring the tumor volume twice a week with a caliper after tumors became palpable. Tumor volumes were calculated as $\text{length} \times \text{width}^2 / 2$. Spontaneous metastasis was evaluated with a luciferase assay. Due to heterogeneous distribution of metastasis in organs, three different parts were randomly collected from each organ to make a tissue pool and weighed for normalization. The lung tissues were ground in cold PBS by tissue homogenizer and treated with cell culture lysis 5X reagent (E153A, Promega). 200 μl lysis buffer was added to 50 mg weight. After 15 min incubation, 100 μl of luciferase substrate (Promega) was added to 50 μl of the lysed sample, and luminescence was measured with a luminometer. For tail vein metastasis assay, 1.5×10^6 cells were injected into tail-vein of each 6–8 weeks old female athymic nu/nu mice with 3–5 mice per group. Lung metastasis was monitored by bioluminescence imaging (BLI). Anesthetized mice were intraperitoneally injected with 150 mg/kg D-luciferin (Perkin Elmer). Bioluminescence images were acquired with Lumina III In Vivo Imaging System (Perkin Elmer). Analysis was performed with live imaging software by measuring total photon flux. For Bouin's fixation, harvested lungs were cleaned with PBS and placed into Bouin's solution on a shaker for overnight. After fixation, they were kept in 70% alcohol.

*** Animal experiments was performed by **Pelin Gülizar Ersan** at Bilkent University, Ankara, Turkey. ***

3.2.14 Western blotting

Approximately 3.2×10^6 cells were lysed in 1X PBS buffer containing 0.1% TritonX-100, cOmplete EDTA-free protease inhibitor cocktail (Roche) and PhosSTOP phosphatase inhibitor mixture (Roche). Inhibitor cocktail tablets were used as 1 tablet for 10 ml of lysis buffer. Cells were disrupted by pulling the cell suspension through a thin needle. Cell debris was removed by centrifugation at 14,000 rpm for 5 min at 4°C. Protein concentration was measured using BCA protein assay kit (23227, Pierce). Protein samples were prepared in 2X Laemmli sample buffer containing 4% (w/v) SDS, 20% glycerol, 120 mM Tris-Cl (pH 6.8), 0.02% (w/v) bromophenol blue and 100 mM dithiothreitol (DTT) as protein sample to 2X Laemmli sample buffer ratio of 1:1 (v/v). Samples were heated at 95°C for 5 min, cooled prior to loading onto SDS-PAGE gel. 10% and 12% SDS-PAGE gels were used for the separation of proteins. After electrophoresis of samples at 120 V for 100 min, proteins were transferred to nitrocellulose membranes (BA85, Whatman Protran) by the Trans-Blot Turbo transfer system (Bio-Rad). After 45 min blocking with 4% milk in 0.1% Tween20 containing 1X TBS, the membrane was incubated with primary antibodies: 1:1000 anti-E-cadherin (24E10) (3195, Cell Signaling), 1:1000 anti-Vimentin (D21H3) (5741, Cell Signaling), 1:1000 anti-Snail (C15D3) (3879, Cell Signaling), 1:1000 anti- β -actin (AC15) (ab6276, Abcam), 1:100 anti-DNAJB4 (R-07) (sc-100711, Santa Cruz) and 1:100 anti-CD81 (B-11) (sc166029, Santa Cruz) overnight at 4°C or 3 hours at room temperature. The membrane was rinsed with 0.1% Tween20 containing 1X TBS Buffer for 10 min three times and incubated with secondary antibodies: 1:2000 anti-mouse IgG, HRP-linked antibody (7076S, Cell Signaling) or 1:2000 anti-rabbit IgG, HRP-linked antibody (7074S, Cell Signaling) at room temperature for 1.5 hours. Finally, membranes were rinsed with 0.1% Tween20 containing 1X TBS Buffer for 10 min three times and proteins were visualized with the ECL Western Blotting Substrate system (32106, Pierce).

3.3 RESULTS

3.3.1 Proteomic profiling identifies differentially regulated proteins during EMT

Twist ectopic expression has been shown to induce epithelial cells to undergo EMT, thereby promoting mesenchymal cell characteristics [94]. To identify differentially expressed proteins during EMT in epithelial HMLE cells and in their mesenchymal derivatives HMLE-Twist cells, we took the advantage of quantitative proteomics approach. First of all, we generated HMLE-Vector and HMLE-Twist cells and validated their epithelial and mesenchymal characteristics by showing the protein level down-regulation of epithelial marker, E-cadherin, and protein level up-regulation of mesenchymal marker, Vimentin during the transition process by Western blotting. At the end of 16 days of Twist overexpression, HMLE-Twist cells became fully mesenchymal cells (**Figure 3.2**).

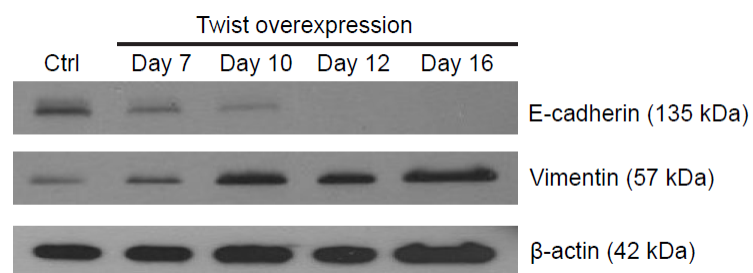


Figure 3.2: The generation of HMLE-Twist cells.

HMLE-Twist cells were generated at the end of 16 days of Twist overexpression upon pBABE-puro-mTwist transduction of cells. Ctrl: HMLE-Vector cells generated by transduction of pBABE-puro. β -actin was used as loading control.

The experimental workflow of proteomic analysis is presented in **Figure 3.3**. HMLE cells exhibiting characteristics of epithelial cells (HMLE-Vector) were systematically

compared with HMLE cells that stably express Twist (HMLE-Twist) using dimethyl labelling (**Figure 3.3**).

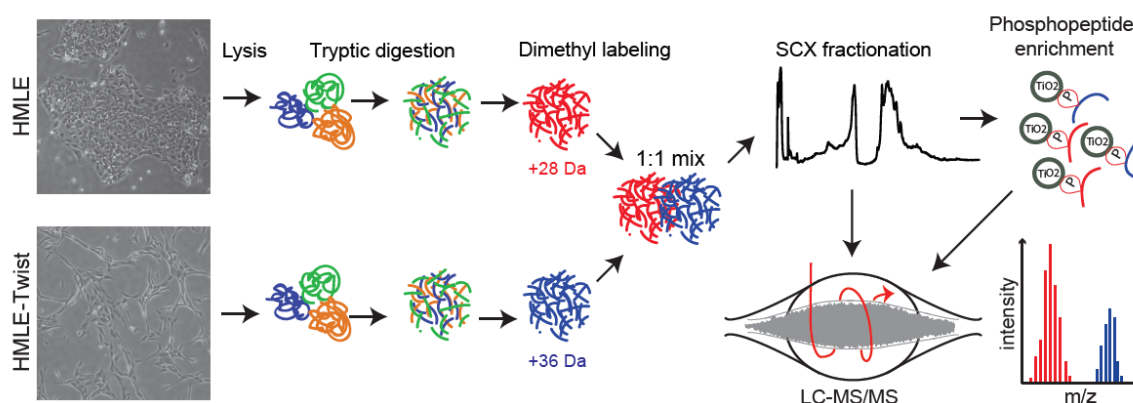


Figure 3.3: Experimental workflow of the liquid chromatography mass spectrometry analysis.

To perform a systematic quantitative profiling of EMT, the equal amount of cell lysates from HMLE and HMLE-Twist cells were digested using Lys-C and Trypsin. The resulting peptides were differentially labelled with light and heavy isotopes using stable isotope dimethyl labelling [100]. The labels were swapped to eliminate any labelling bias in biological repeats. Two technical replicates were performed for each biological replicate. After combining differentially labelled peptides in equal amounts, the pooled peptides were separated by column-based SCX chromatography into 40 fractions, and each fraction was analyzed by LC-MS/MS in two replicates. All raw MS data were processed using the Proteome Discoverer software to identify and quantify peptides in two conditions.

Based on our statistical analysis (see **Section 3.2.4**), out of more than 6,000 uniquely identified proteins, 168 and 146 proteins are significantly up-regulated in epithelial and mesenchymal cells, respectively. Almost all known epithelial and mesenchymal markers

were identified indicating high accuracy of our dataset. To show what we can gain from the proteomic data beyond the transcriptomic data, we compared our proteomic profiles with a previously published transcriptome data in the same cell lines [2]. Despite the significant correlation ($r=0.53$, $p\text{-value} < 0.005$) between proteome and transcriptome data, around 65% of the significantly regulated proteins are not altered at the transcriptome level during EMT (**Figure 3.4**). This result highlights the necessity of proteome level analyses in addition to the transcriptomic profiling to identify the complex molecular mechanisms behind EMT process.

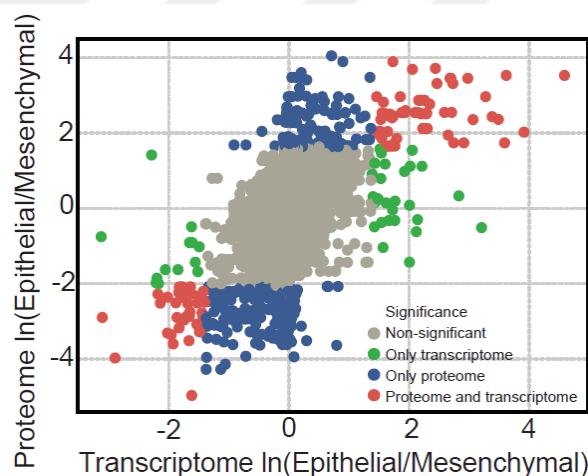


Figure 3.4: Comparison between proteome and transcriptome during EMT.

Epithelial/Mesenchymal ratios of genes were compared at the mRNA [2] and protein levels (grey: no change, green: only mRNA level change, blue: only protein level change, red: both mRNA and protein levels change).

3.3.2 Novel epithelial and mesenchymal specific proteins affect the migration of breast cancer cells

After obtaining the list of proteins significantly regulated during EMT, we further focused on a sub-class of novel epithelial and mesenchymal specific proteins for an initial screen. We selected eight proteins (CASP14, PI3, TLR2, ICAM1, LGALS7, CDH1, TFAP2A, and NDRG1) whose protein level up-regulation is specific to epithelial state and ten proteins (MME, CDH2, JUND, DNAJB4, BIN1, PDGFRB, CADM3, CD81, ANPEP, and LRP1) whose protein level up-regulation is specific to mesenchymal state. To analyze the effect of these proteins on cells, we first performed an shRNA screen by retrovirus-mediated shRNA knockdown of selected epithelial specific proteins in MCF7 cells and selected mesenchymal specific proteins in MDA-MB-231 and MDA-MB-157 invasive breast cancer cells as explained in **Section 3.2.5**. Then, we performed real time migration analysis of shRNA treated MCF7 and MDA-MB-231 cell lines using xCELLigence RTCA-DP system (ACEA Biosciences) as explained in **Section 3.2.6**. For the RTCA analysis, the depletion of E-cadherin (CDH1) in MCF7 cells and the depletion of N-cadherin (CDH2) in MDA-MB-231 cells were used as positive controls (**Figure 3.5** and **Figure 3.6**). As expected, the depletion of E-cadherin resulted in an increase in the migration of MCF7 cells whereas the depletion of N-cadherin in MDA-MB-231 cells decreased cell migration. When epithelial specific proteins CASP14, PI3, TLR2, and TFAP2A were depleted, we observed that the migration of MCF7 cells increased as in the case of E-cadherin depletion (**Figure 3.7**). On the other hand, the depletion of mesenchymal specific proteins BIN1, PDGFRB, CADM3, ANPEP, DNAJB4, and CD81 decreased the migration of MDA-MB-231 cells like the depletion of N-cadherin (**Figure 3.8**).

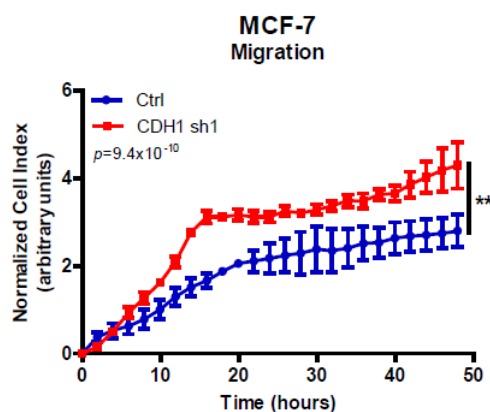


Figure 3.5: The depletion of E-cadherin increases migration ability of MCF7 cells.

50,000 cells were seeded per well and duplicates were prepared for each experimental condition. Ctrl: MCF7 cells generated by transduction of control shRNA, pSMP-Luc. The measurements were taken with an interval of 15 min.

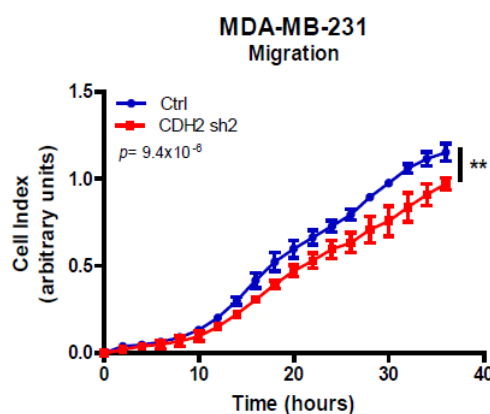


Figure 3.6: The depletion of N-cadherin decreases migration ability of MDA-MB-231 cells.

30,000 cells were seeded per well and duplicates were prepared for each experimental condition. Ctrl: MDA-MB-231 cells generated by transduction of control shRNA, pSMP-Luc. The measurements were taken with an interval of 15 min.

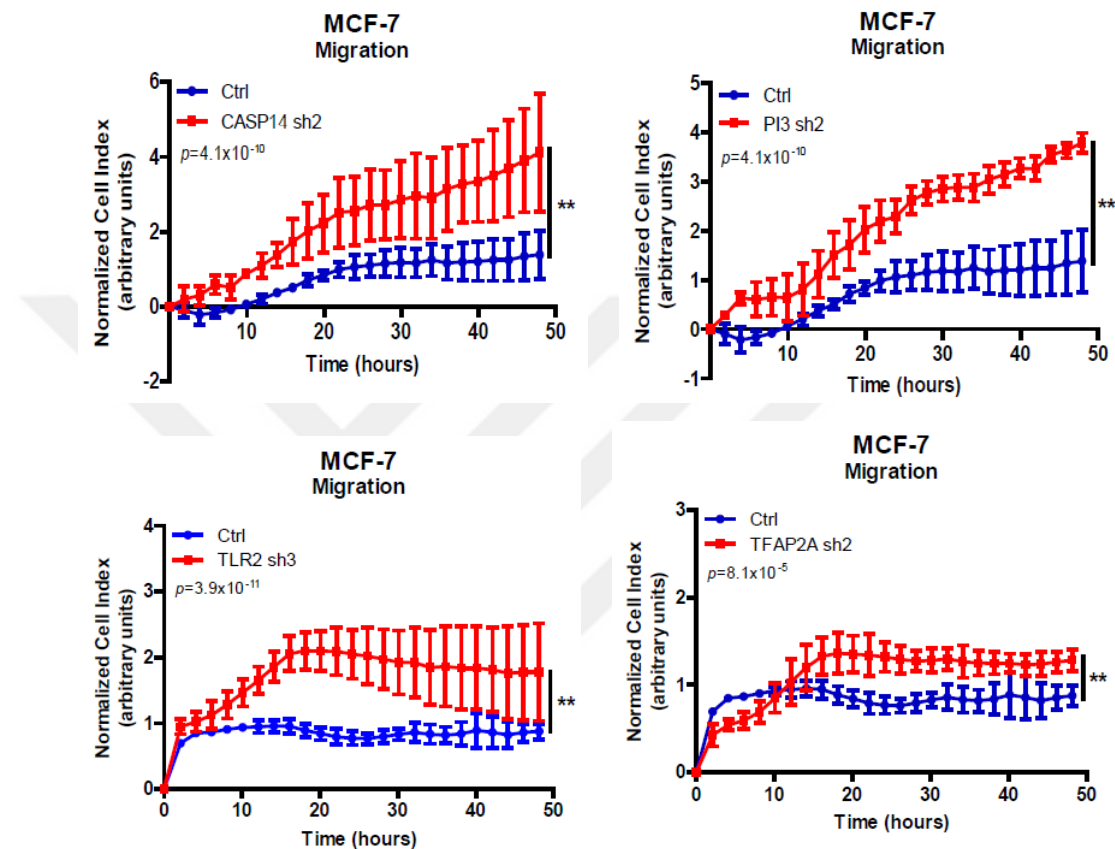


Figure 3.7: The depletion of epithelial specific proteins, CASP14, PI3, TLR2, and TFAP2A, increases migration of MCF7 cells.

50,000 cells were seeded per well and duplicates were prepared for each experimental condition. Ctrl: MCF7 cells generated by transduction of control shRNA, pSMP-Luc. The measurements were taken with an interval of 15 min.

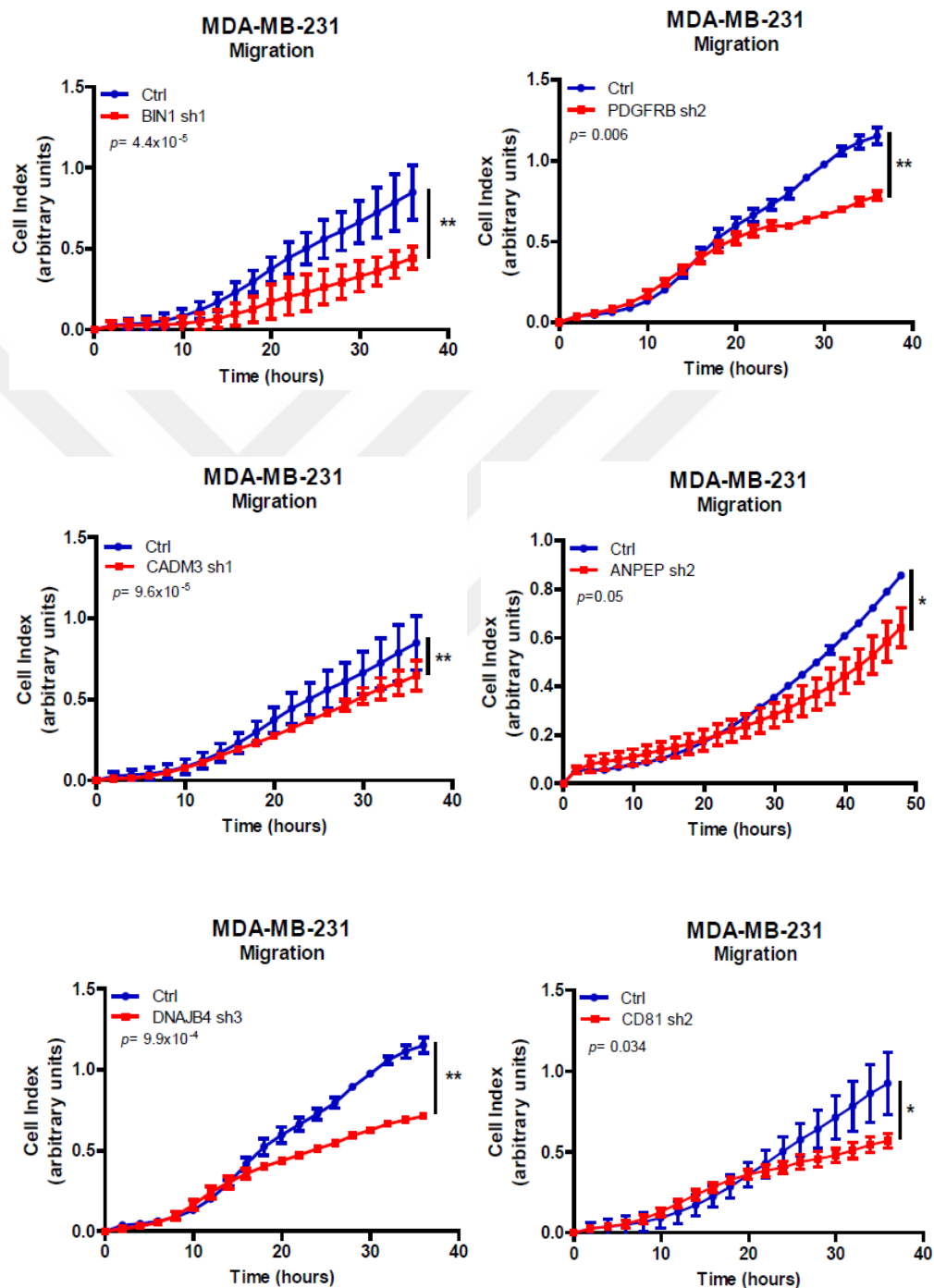


Figure 3.8: The depletion of mesenchymal specific proteins, BIN1, PDGFRB, CADM3, ANPEP, DNAJB4 and CD81, decreases migration of MDA-MB-231 cells.

30,000 cells were seeded per well and duplicates were prepared for each experimental condition. Ctrl: MDA-MB-231 cells generated by transduction of control shRNA, pSMP-Luc. The measurements were taken with an interval of 15 min.

In addition to cell migration analysis, we analyzed the mRNA expression levels of selected proteins in epithelial and mesenchymal breast cancer cell lines using the study by Luo *et al.* [105]. This sub-class of proteins was also analyzed in the transcriptome data obtained from Taube *et al.* [2]. Selected proteins were either regulated at only proteome level or at both transcriptome and proteome levels. All results including cell migration analysis were summarized in **Table 3.5**. For each selected protein, we showed median ln ratio (epithelial/mesenchymal or mesenchymal/epithelial), number of quantified peptides in a replicate, percentage of maximum coverage and its up-regulation direction (epithelial or mesenchymal). Transcription up-regulation direction of selected proteins were indicated using previous EMT microarray data [2]. We further analyzed GSE40059 dataset [105] in Gene Expression Omnibus (GEO) database to represent mRNA expression profiles of selected EMT associated proteins in breast cancer and normal cell lines which were grouped as epithelial and mesenchymal cell lines [106-108]. In the last column, we showed what happens to migration ability of cells when the selected proteins were depleted by shRNA-mediated knockdown. Based on these results, we focused on two proteins, DNAJB4 and CD81, to further address their effects on EMT both *in vitro* and *in vivo*.

Table 3.5: The selected sub-class of novel epithelial and mesenchymal specific proteins.

Gene	Protein name	Median ln ratio	Number of peptides	Percentage of coverage	Protein expression direction	EMT transcription direction*	Breast cancer cell lines transcription direction**	Effect of depletion on cell migration
CASP14	Caspase-14	3.77	3	15	Epithelial	No change	No change	Increase
PI3	Elafin	3.60	1	8	Epithelial	Epithelial	No change	Increase
TLR2	Toll-like receptor 2	3.14	2	3	Epithelial	No change	No change	Increase
ICAM1	Intercellular adhesion molecule 1	2.76	2	5	Epithelial	No change	No change	No change
LGALS7	Galectin-7	2.51	3	28	Epithelial	Epithelial	No change	No change
CDH1	E-cadherin	2.36	5	7	Epithelial	Epithelial	Epithelial	Increase
TFAP2A	Transcription factor AP-2-alpha	2.21	1	4	Epithelial	No change	No change	Increase
NDRG1	Protein NDRG1	2.14	9	35	Epithelial	No change	No change	No change
MME	Nephrilysin	-3.32	17	27	Mesenchymal	No change	No change	No change
CDH2	N-cadherin	-2.92	1	2	Mesenchymal	Mesenchymal	Mesenchymal	Decrease
JUND	Transcription factor jun-D	-2.73	2	8	Mesenchymal	No change	No change	No change
DNAJB4	DnaJ homolog subfamily B member 4	-2.55	13	43	Mesenchymal	Mesenchymal	Mesenchymal	Decrease
BIN1	Myc box-dependent-interacting protein 1	-2.54	9	22	Mesenchymal	Mesenchymal	Mesenchymal	Decrease
PDGFRB	Platelet-derived growth factor receptor beta	-2.44	1	2	Mesenchymal	Mesenchymal	No change	Decrease
CADM3	Cell adhesion molecule 3	-2.36	4	18	Mesenchymal	Mesenchymal	No change	Decrease
CD81	CD81 antigen	-2.22	3	25	Mesenchymal	No change	No change	Decrease
ANPEP	Aminopeptidase N	-2.02	30	30	Mesenchymal	Mesenchymal	No change	Decrease
LRP1	Prolow-density lipoprotein receptor-related protein 1	-1.88	21	7	Mesenchymal	No change	No change	No change

* based on Taube *et al.*, 2010** based on Luo *et al.*, 2013

3.3.3 The effect of DNAJB4 knockdown on cell migration of mesenchymal breast cancer cells

In agreement with our quantitative proteomics data, Western blotting analysis showed dramatically increased DNAJB4 protein expression in HMLE-Twist cells compared to HMLE cells (**Figure 3.9**). DNAJB4 is regulated at both transcriptome and proteome level during EMT and shows increased transcription in mesenchymal breast cancer cell lines (**Table 3.5** and **Figure 3.10**).

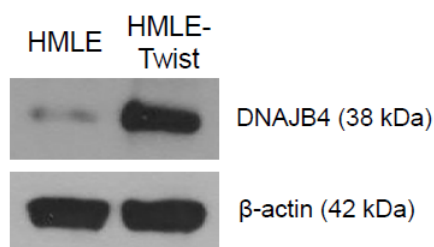


Figure 3.9: Western blotting analysis showing the increased protein expression of DNAJB4 in HMLE-Twist cells.

HMLE: HMLE-Vector cells generated by transduction of pBABE-puro. β-actin was used as loading control.

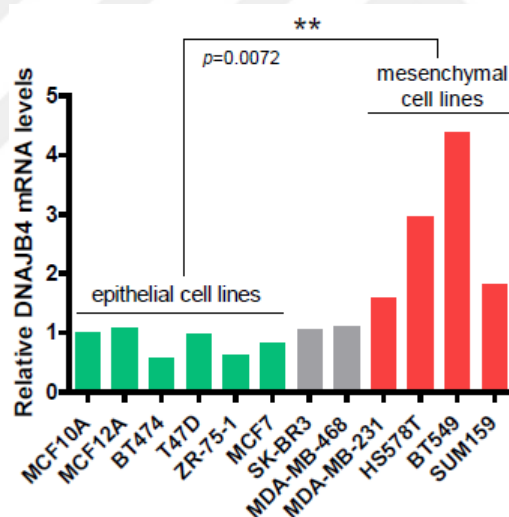


Figure 3.10: mRNA expression of DNAJB4 in GSE40059 dataset based on the classification of different breast cell lines as epithelial and mesenchymal.

To examine the roles of DNAJB4 expression on mesenchymal cells, we performed shRNA-mediated knockdown of DNAJB4 in MDA-MB-231 invasive breast cancer cells and analyzed the DNAJB4 expression by Western blotting (**Figure 3.11**). We revealed that shRNA mediated depletion of DNAJB4 in MDA-MB-231 cells was successful and the

suppression of DNAJB4 does not affect cell viability (**Figure 3.12**), but results in a significant inhibition of migration in mesenchymal cells as observed by real-time migration assay (**Figure 3.8**). We further validated the results of real time cell migration assay by a scratch wound healing assay (**Figure 3.13**). The reduction percentage of the scratch wound area by DNAJB4 depleted MDA-MB-231 cells was significantly lower when compared to control cells.

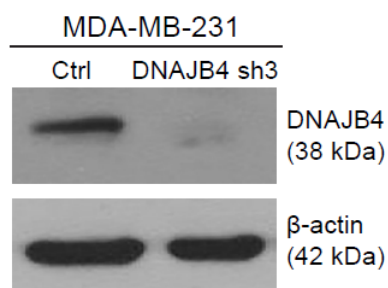


Figure 3.11: Western blotting analysis of shRNA mediated knockdown of DNAJB4 protein expression in MDA-MB-231 cells.

Ctrl: MDA-MB-231 cells generated by transduction of control shRNA, pSMP-Luc. β -actin was used as loading control.

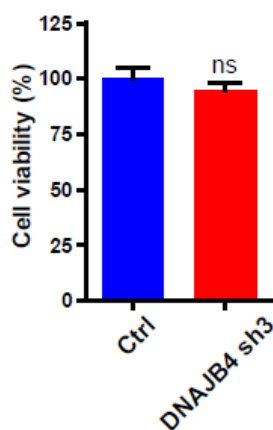


Figure 3.12: Cell viability analysis of MDA-MB-231 cells stably expressing the control and DNAJB4 shRNAs.

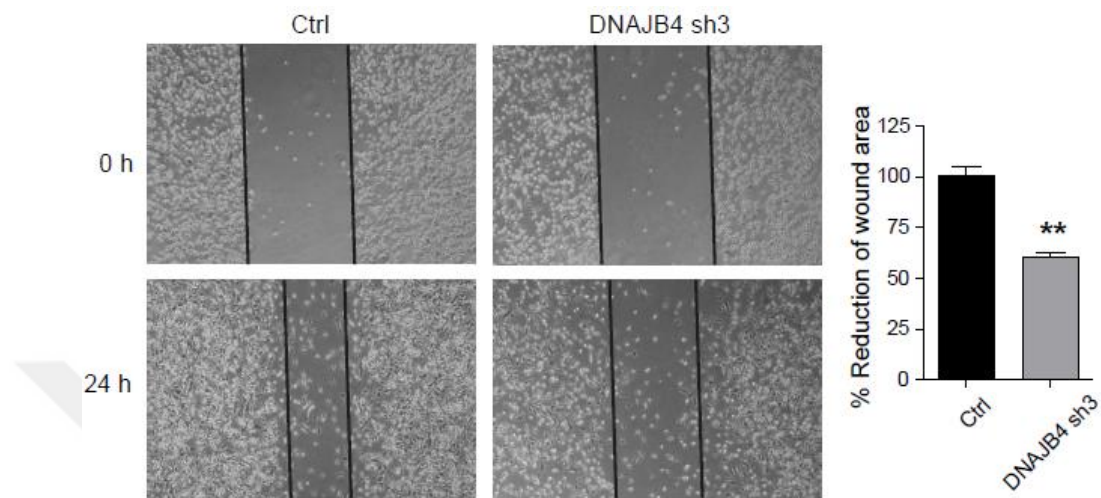


Figure 3.13: Scratch wound healing assay of DNAJB4 depleted MDA-MB-231 cells and quantification of percent reduction of the wound area.

Previously, the pan-cancer EMT signature including breast, lung, colon, ovarian, and bladder cancers were investigated using patient tumors [113]. By using this publicly available dataset, we performed the correlation analysis of DNAJB4 mRNA expression and EMT scores of breast cancer patients. TCGA mRNA data and EMT score values were downloaded from the cBioPortal for Cancer Genomics [109, 110] and TCGA database [111], respectively. Accordingly, we revealed that DNAJB4 mRNA expression levels positively correlate with the EMT scores of breast cancer patients (**Figure 3.14**). Similarly, the metastasis-free survival analysis of patients revealed that high level of DNAJB4 mRNA expression significantly associates with worse metastasis-free survival of breast cancer patients (**Figure 3.15**).

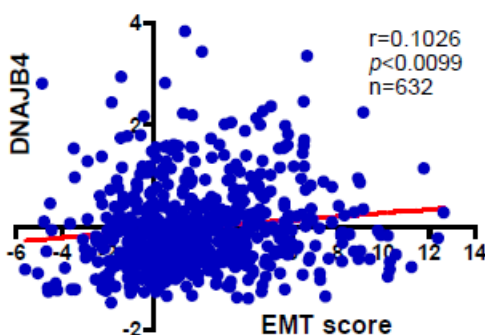


Figure 3.14: Correlation analysis of the mRNA expression of DNAJB4 with the EMT score in breast cancer patients from TCGA dataset.

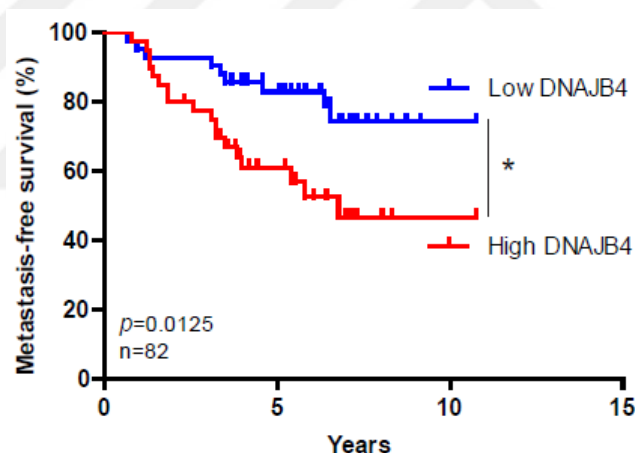


Figure 3.15: Metastasis-free survival of breast cancer patients in GSE2603 dataset based on mRNA expression of DNAJB4.

In addition to DNAJB4, the mRNA expression level of DNAJB5, which was also identified as a member of up-regulated proteins in a mesenchymal specific manner, is higher in mesenchymal breast cancer cell lines (**Figure 3.16**) and positively correlates with EMT scores of breast cancer patients (**Figure 3.17**). These results suggest that both

DNAJB4 and DNAJB5 contribute to the mesenchymal characteristics of cells undergoing EMT.

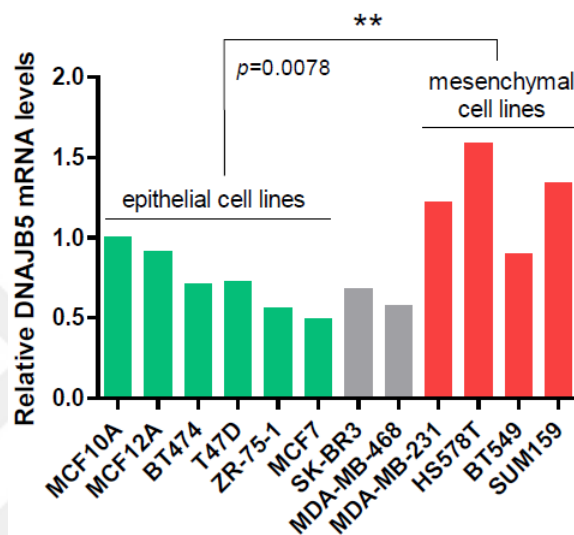


Figure 3.16: mRNA expression of DNAJB5 in GSE40059 dataset based on the classification of different breast cell lines as epithelial and mesenchymal.

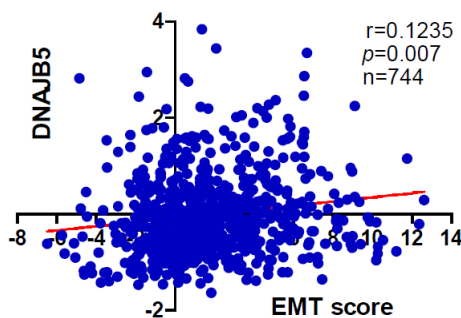


Figure 3.17: Correlation analysis of the mRNA expression of DNAJB5 with the EMT score in breast cancer patients from TCGA dataset.

3.3.4 The effect of CD81 knockdown on cell migration of mesenchymal breast cancer cells

Another prominent hit in our migration assay was a member of tetraspanins family, CD81 (**Table 3.5**). Although most of the members of tetraspanins were present in the list of epithelial specific proteins, CD81 is among the proteins which are up-regulated in a mesenchymal specific manner. Our Western blotting analysis confirmed that CD81 protein expression dramatically increases in HMLE-Twist cells as expected from our quantitative proteomics data (**Figure 3.18**). As a potential cell surface marker, we tested whether CD81 expression increases on the mesenchymal cell surface using unpermeabilized HMLE and HMLE-Twist cells in flow cytometry. Our analyses revealed that surface CD81 expression increased 79% in HMLE-Twist cells (**Figure 3.19**).

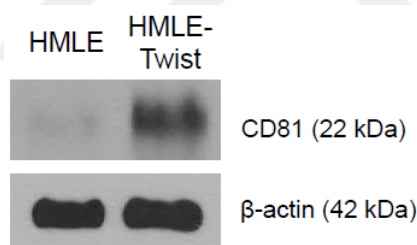


Figure 3.18: Western blot analysis showing the increased protein expression of CD81 in HMLE-Twist cells.

HMLE: HMLE-Vector cells generated by transduction of pBABE-puro. β -actin was used as loading control.

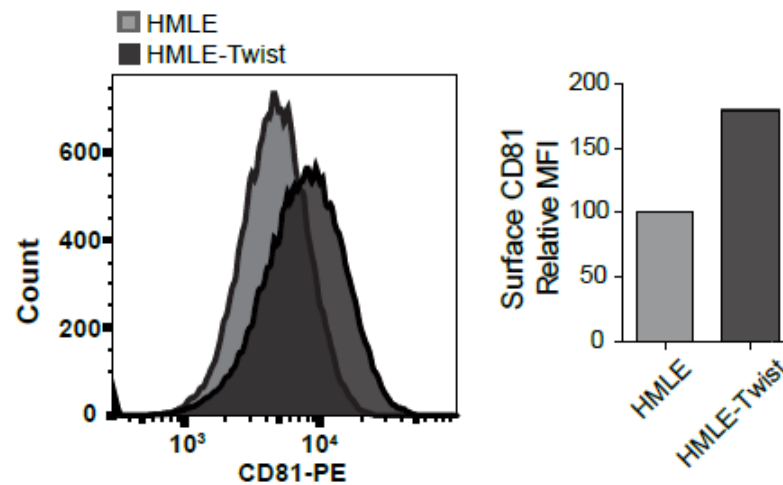


Figure 3.19: Cell surface expression analysis of CD81 on epithelial HMLE and mesenchymal HMLE-Twist cells by flow cytometry.

HMLE: HMLE-Vector cells generated by transduction of pBABE-puro.

CD81 is regulated only at proteome level, and its transcription level does not significantly change during EMT and in breast cancer cell lines with epithelial or mesenchymal phenotype (**Table 3.5** and **Figure 3.20**). We performed shRNA-mediated knockdown of CD81 in MDA-MB-231 invasive breast cancer cells. As a result, CD81 was effectively depleted (**Figure 3.21**) and viability of cells was not affected (**Figure 3.22**). In agreement with the real-time migration assay (**Figure 3.8**), the *in vitro* scratch wound healing assay also revealed the impairment of migration ability of MDA-MB-231 invasive breast cancer cells upon CD81 knockdown (**Figure 3.23**). These results suggest that CD81 contributes to the mesenchymal characteristics of cells undergoing EMT and has a potential to become a novel cell surface marker of mesenchymal cells.

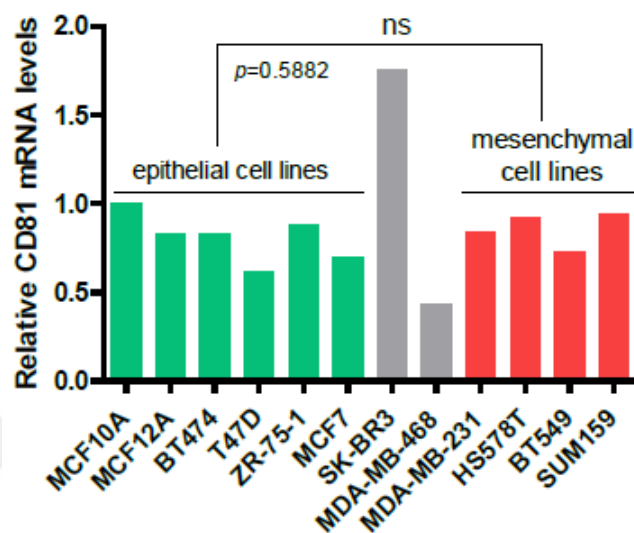


Figure 3.20: mRNA expression of CD81 in GSE40059 dataset based on the classification of different breast cell lines as epithelial and mesenchymal.

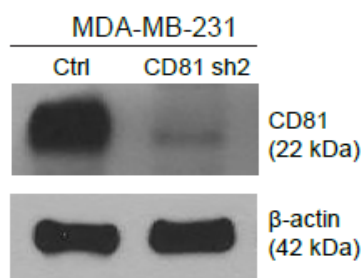


Figure 3.21: Western blotting analysis of shRNA mediated knockdown of CD81 protein expression in MDA-MB-231 cells.

Ctrl: MDA-MB-231 cells generated by transduction of control shRNA, pSMP-Luc. β-actin was used as loading control.

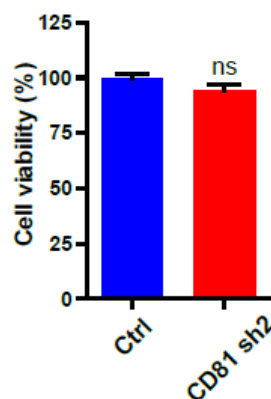


Figure 3.22: Cell viability analysis of MDA-MB-231 cells stably expressing the control and CD81 shRNAs.

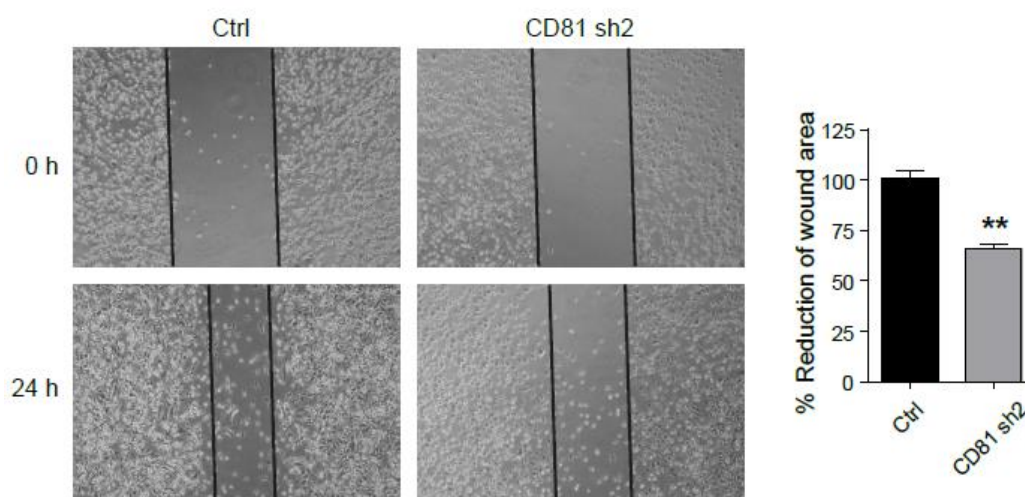


Figure 3.23: Scratch wound healing assay of CD81 depleted MDA-MB-231 cells and quantification of percent reduction of the wound area.

3.3.5 The suppression of DNAJB4 and CD81 results in decreased primary tumor growth and metastasis

To analyze temporal expression of DNAJB4 and CD81 during EMT, we used EMT inducible HMLE-Twist-ER cells as described in **Section 3.2.10**. Briefly, the cells were treated with 20 nM 4OHT for 16 days to induce EMT and collected at different days to analyze their protein expression pattern by Western blotting. We found that both DNAJB4 and CD81 expression increased gradually in a time dependent manner in inducible HMLE-Twist-ER cells as EMT progressed by 4OHT treatment (**Figure 3.24**). The down-regulation of epithelial marker, E-cadherin, and the up-regulation of mesenchymal marker, Vimentin during the transition process revealed the successful induction of EMT by 4OHT treatment.

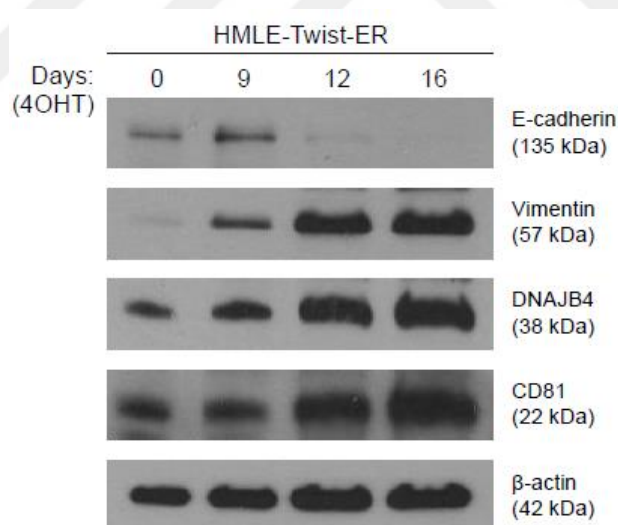


Figure 3.24: Induction of EMT with tamoxifen-inducible Twist (Twist-ER) in HMLE cells.

Cells were induced to undergo EMT in a time dependent manner and analyzed for DNAJB4 and CD81 protein expressions. β -actin was used as loading control.

We then examined the roles of DNAJB4 and CD81 in primary tumor growth and metastasis *in vivo*. We performed retrovirus-mediated shRNA knockdown of DNAJB4 and CD81 in MDA-MB-231-luc2-GFP cells as described in **Section 3.2.12**. To observe the effects of DNAJB4 and CD81 knockdown on the primary tumor growth, we developed primary tumor xenografts with control and knockdown cells in nude mice. Knockdown of CD81 and DNAJB4 significantly impaired the tumor growth (**Figures 3.25, 3.26 and 3.27**).

*** Animal experiments and ex vivo luciferase assays were performed by **Pelin Gülizar Ersan** at Bilkent University, Ankara, Turkey. ***

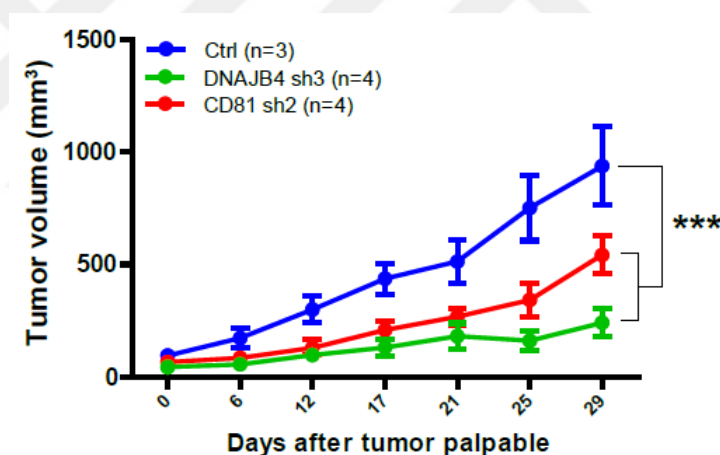


Figure 3.25: Primary tumor progression in xenografts generated by mammary fat pad injection of MDA-MB-231-luc2-GFP control (n=3), CD81 shRNA (n=4) and DNAJB4 shRNA (n=4) treated cells into nude mice.

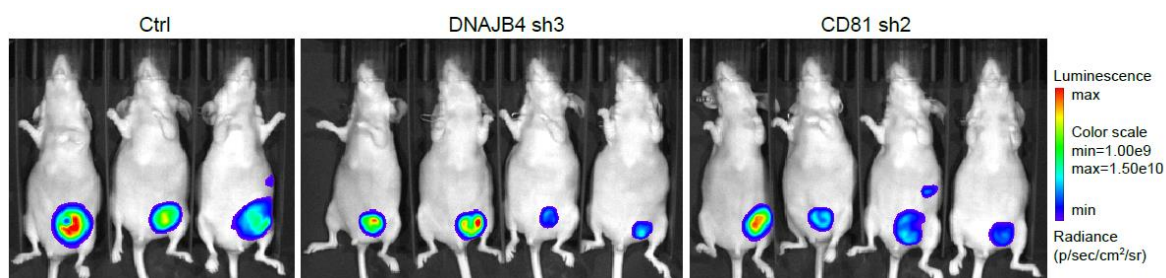


Figure 3.26: Bioluminescence images of nude mice from Figure 3.25.

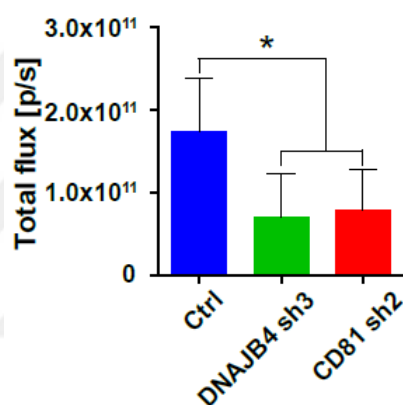


Figure 3.27: Quantification of the luminescence signals from mice in Figure 3.25, given as total flux.

To test the effect of two candidates on the spontaneous metastasis, tumor growth has been monitored until tumors in each group reached to 1000 mm^3 (**Figure 3.28**). One month after surgical removal of primary tumors, an *ex vivo* luciferase assay was performed. Knockdown of CD81 and DNAJB4 significantly reduced both lung and liver metastases (**Figure 3.29**).

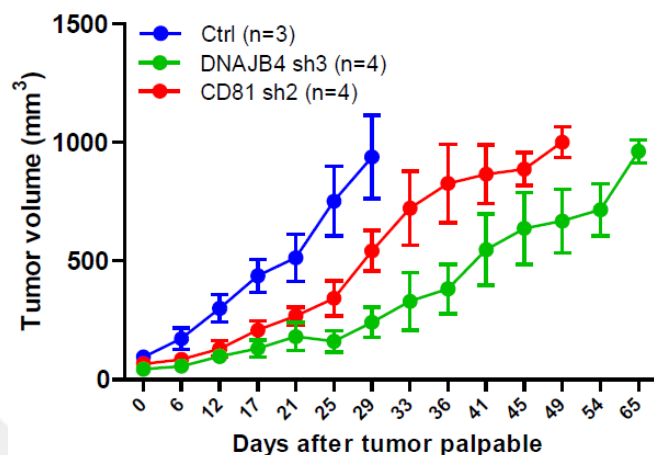


Figure 3.28: The growth rate of primary tumors for MDA-MB-231-luc2-GFP control (n=3), CD81 shRNA (n=4) and DNAJB4 shRNA (n=4) treated cells before they were surgically removed to observe spontaneous metastasis in different organs.

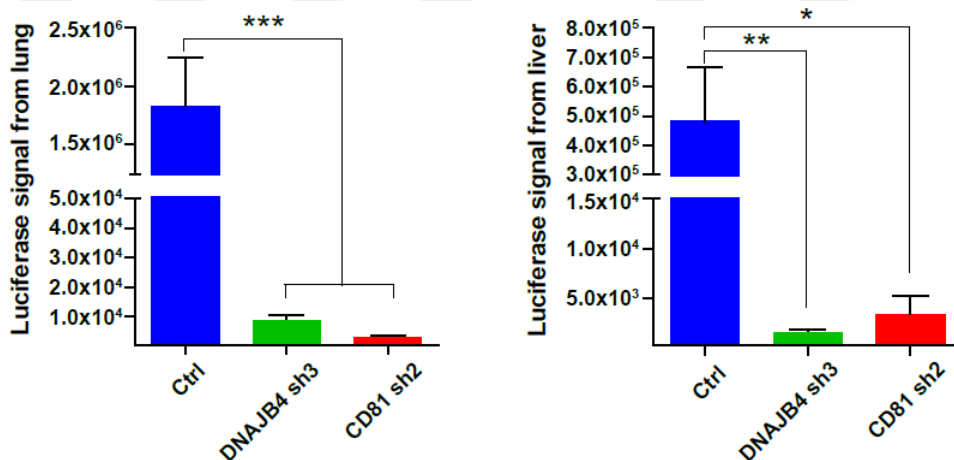


Figure 3.29: Luciferase signal coming from lungs and livers of mice in Figure 3.26 as quantified by luciferase assay.

(MDA-MB-231-luc2-GFP control, n=3; CD81 shRNA, n=4; DNAJB4 shRNA, n=3).

Next, we tested whether DNAJB4 and CD81 play a role in extravasation/colonization of MDA-MB-231 invasive breast cancer cells in lungs by tail-vein metastasis assay. After 6 weeks from intravenous injection of DNAJB4 and CD81 depleted cells into nude mice, we compared their lung metastasis with control cells (**Figure 3.30**). Mice groups injected with either CD81 or DNAJB4 knockdown cells showed a low level of lung metastasis compared to control (**Figure 3.31**).

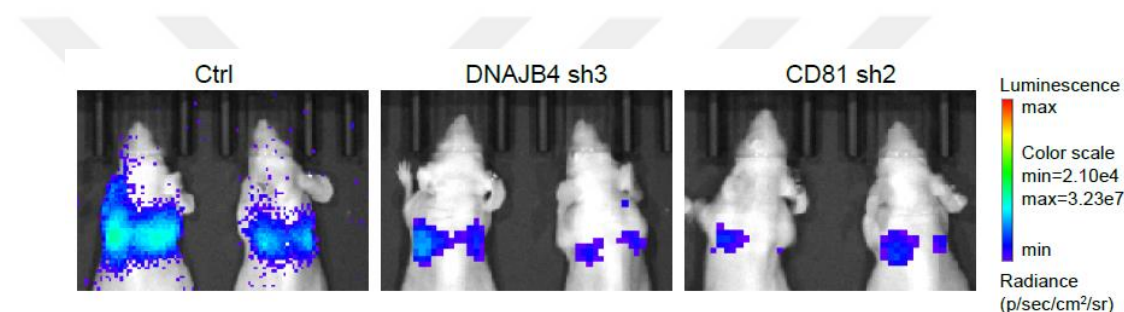


Figure 3.30: Bioluminescence images of nude mice intravenously injected with MDA-MB-231-luc2-GFP control, DNAJB4 shRNA and CD81 shRNA treated cells.

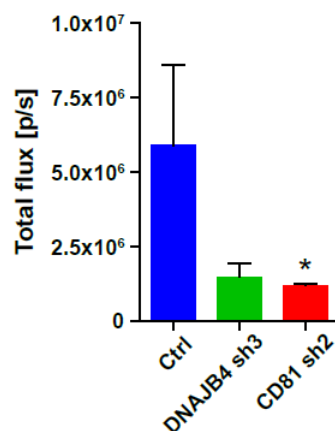


Figure 3.31: Quantification of the luminescence signals from mice in Figure 3.30, given as total flux.

Similarly, we observed less nodules in lungs of the knockdown group mice compared to control mice after fixation with Bouin's solution (**Figure 3.32**). Metastatic nodules on lung surface appear white after overnight fixation in Bouin's solution. Mice injected with Ctrl cells have more nodules on the lung surface compared to DNAJB4 and CD81 depleted cells injected mice. Hematoxylin and eosin (H&E) staining confirmed the differences in lung metastases in control, DNAJB4 knockdown and CD81 knockdown groups (**Figure 3.33**). These results demonstrate that both DNAJB4 and CD81 have roles in primary tumor growth and metastasis of breast cancer cells *in vivo*.

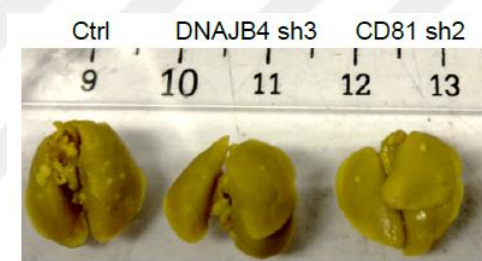


Figure 3.32: Representative images of lungs collected from the groups given in Figure 3.30.

Mice were sacrificed at sixth week and lungs were fixed in Bouin's Solution.

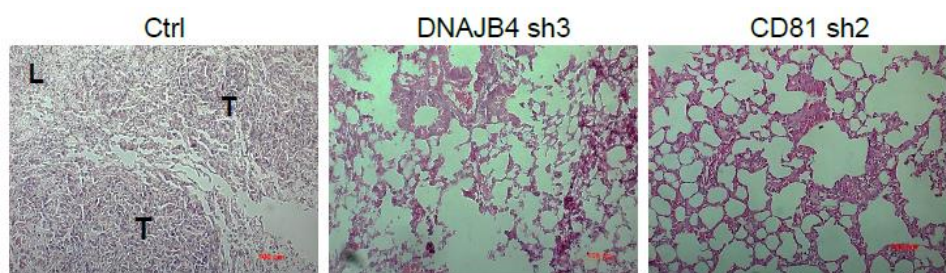


Figure 3.33: Representative images of lungs stained with Hematoxylin-Eosin.

T: for tumor, L: for lung.

3.4 DISCUSSION

The transition from epithelial to mesenchymal state involves a major reorganization of multiple cellular components triggered by EMT transcription factors. This study used quantitative proteomics to identify biochemical changes upon activation of EMT transcription factors. Previously, Taube *et al.* obtained the transcriptome profiles of HMLE cells in epithelial and mesenchymal states as ‘EMT core signature’ [2]. By using HMLE-Twist cells, we profiled the biochemical changes at the protein level. Strikingly, around 65% of significantly regulated proteins did not exhibit any detectable regulation at the mRNA level. These findings suggest that besides the gene expression level regulation, EMT is regulated at multiple post-transcriptional levels including protein sub-cellular localization and posttranslational modification.

The reconstruction of epithelial and mesenchymal specific protein-protein interaction networks based on our proteomics data revealed the key signaling pathways of cells undergoing EMT. One of the interesting signaling pathways enriched for the mesenchymal state was unfolded protein response (UPR). DNAJB4 and DNAJB5, members of the human heat shock proteins 40 family, were identified in this signaling network. Although previous studies have shown DNAJB4 as tumor-suppressor protein [114-116], our proteomic data and patient dataset analyses describe DNAJB4 as a metastasis inducer in breast cancer cells. When we compare the mRNA expression levels of DNAJB4 in epithelial and mesenchymal breast cancer cells [105], we found that DNAJB4 mRNA expression levels are significantly increased in mesenchymal cells. Our EMT induction experiment shows that DNAJB4 protein level gradually increases as EMT progresses. Importantly,

knockdown of DNAJB4 in MDA-MB-231 cells leads to a decrease in migration, impairs lung metastasis of invasive breast cancer cells, and decreases primary tumor growth rate. In parallel with this, metastasis-free survival analysis of primary breast cancer patients reveals that higher levels of DNAJB4 significantly associates with a worse metastasis-free survival. Recent research demonstrated that cells in their mesenchymal state have increased synthesis and secretion of large quantities of extracellular matrix (ECM) proteins which then triggers ER stress and activation of unfolded protein response, and UPR is required for survival, invasion and metastasis of the cells [117-120]. However, how these two mechanisms, EMT and UPR, act in parallel has not been completely resolved. It is plausible that DNAJB4 and DNAJB5, as chaperone proteins may increase due to the activation of unfolded protein response during EMT. Deciphering the molecular mechanism of DNAJB4 overexpression during EMT would bring new perspectives into the relationship between EMT and unfolded protein response.

One motivation for this study was to find biomarkers for EMT. Our network analysis identified multiple cell surface proteins as epithelial and mesenchymal specific. CD81 was one of the mesenchymal specific cell surface markers. As a tetraspanin family member, CD81 has two extracellular domains and two intracellular tails. It was first identified in 1990 [121] and studied in the immune system [122]. Recent studies have shown that CD81 interacts with 14-3-3 protein and thereby may relay extracellular cues to intracellular signaling pathways [123] and its activation stimulates the ERK/MAPK pathway and tumor cell proliferation [124]. CD81 is also required for the lamellipodia formation in dendritic cells and its absence disrupts the cell front actin protrusions and Rac signaling causing decrease in cell migration [125]. Interestingly, the CD81 mRNA level does not significantly alter during EMT [2] and between epithelial and mesenchymal breast cell lines. However, we identified CD81 as one of the significantly up-regulated proteins during

the EMT process. CD81 protein levels increased gradually as cells undergo EMT. In parallel, CD81 level increased 79% on the surface of HMLE-Twist cells. Functional assays supported its potential of being a marker for EMT process. Upon CD81 knockdown, we observed a decrease in the migration of mesenchymal breast cancer cells as reported in Zhang *et al.* [126]. Moreover, *in vivo* experiments revealed that CD81 knockdown in MDA-MB-231 cells decreased primary tumor growth rate and impaired lung metastasis. Its strong mesenchymal characteristics suggest that CD81 may have a value as mesenchymal cell marker.

In summary, our analysis uses a quantitative proteomic approach to provide large scale biochemical changes as HMLE cells undergo EMT and reveals key signaling pathways during this process. Considering the key involvement of EMT in both metastasis and drug resistance, detailed characterization of novel EMT regulators may open new avenues for targeting metastatic tumors and overcoming drug resistance.

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VITA

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Appendix A: Proximal interaction partners of CLIC4 in cytokinesis

Table A.1: Wild type CLIC4 specific proximal protein interactions

Gene Name	Σ coverage	Σ # Proteins	Σ # Unique Peptides	Σ # Peptides	Σ # PSMs
CLIC4 WT	66.34	7	22	28	59
HIST1H2BK	65.08	5	1	12	56
MORF4L2	61.19	5	1	3	4
VAT1	50	4	1	2	2
FLYWCH2	43.57	2	1	2	3
H3F3A	43.38	10	4	7	16
HN1L	40	5	1	4	4
NME2	38.16	10	1	3	4
HIST1H1D	37.56	6	11	12	23
PRDX5	35.88	4	2	4	6
SLC25A3	33.98	6	2	7	11
RAB7A	31.34	3	1	2	2
RPS21	29.63	3	1	1	1
CSNK1A1	26.11	5	1	5	9
TUBB4A	24.55	17	1	11	31
DIS3	23.49	4	1	13	15
PDAP1	23.2	1	1	3	4
SHQ1	22.7	2	1	8	11
RNMTL1	22.38	2	1	4	5
EIF4H	22.37	2	1	2	2
SLC13A3	22.25	1	1	3	9
PSMD2	20.96	3	1	8	8
PUS1	20.14	9	1	4	5
APEX1	19.81	5	1	5	6
EZR	19.8	5	1	10	13
CPSF7	18.72	4	1	2	6
ANLN	17.97	4	2	13	17
MRPL16	16.73	2	1	3	5
NAMPT	16.7	3	1	4	4
KIF11	15.34	1	2	9	12
MCM5	13.62	3	1	4	5
EIF4B	13.09	9	2	6	7
RAVER1	11.77	4	1	5	9
CPSF6	11.07	4	2	4	6
PDCD6IP	11.06	2	1	5	6
OGDH	11.05	5	1	6	7
EIF2B3	9.96	1	1	2	2
GMPS	9.76	2	1	4	4
MCMBP	9.22	2	1	3	3
RPN1	9.06	2	2	5	7
PAN2	8.41	3	1	7	8
REPIN1	8.29	5	1	3	4
PCDHA9	8.11	2	1	4	4
FLNB	8.08	10	1	10	11
FSIP2	7.62	2	1	28	106
GPRIN3	7.47	1	1	4	26
GP1BB	7.06	1	1	2	2
KMT2A	6.23	3	1	16	16
PRRC2C	5.87	9	1	12	16
PPAN	5.65	3	1	2	2
HCFC1	5.06	1	1	6	9
KMT2C	4.56	2	1	13	15
NOB1	3.64	1	1	1	1
SLC7A5	3.55	1	1	1	2
THRAP3	2.72	1	1	3	3

Table A.2: CLIC4 C35A specific proximal protein interactions

Gene Name	Σ coverage	Σ # Proteins	Σ # Unique Peptides	Σ # Peptides	Σ # PSMs
CLIC4 C35A	60.73	7	21	24	46
MAT2B	62.65	6	1	4	5
BOLA1	54.74	1	1	3	4
ACAA2	44.08	2	7	9	15
MRPL27	38.1	4	1	3	4
HIST1H3A	36.76	10	4	6	9
CTTN	26.5	5	2	8	11
RBM39	26.41	5	1	4	4
HNRNP LL	24.77	8	1	6	7
C19orf10	24.28	3	1	2	3
PFKP	24.27	4	1	2	3
GSR	23.18	1	1	7	17
DDX46	22.89	1	1	14	17
CRKL	22.44	1	1	4	6
CORO1C	22.01	5	1	4	5
PHF5A	21.82	1	1	2	4
SARS	21.79	2	1	5	5
HNRNP L	21.22	4	2	7	20
RPS13	21.19	2	1	2	2
CDC42	20.42	2	1	3	4
TXNL1	20.07	2	2	3	5
CRK	16.12	3	1	3	4
PKLR	14.98	2	1	4	26
MRE11A	14.97	5	1	7	8
RPSA	14.24	3	1	2	3
IDH1	13.77	2	1	3	4
PLK1	13.1	2	1	4	4
AHCTF1	12.75	3	1	13	17
ACADVL	12.39	4	1	5	5
MCM6	12.3	1	1	6	9
MPG	12.08	5	1	2	3
PALLD	11.71	7	1	9	11
PGAM1	11.42	2	1	2	3
PRDX6	10.27	1	1	1	1
PPP6C	4.26	1	1	1	1

Table A.3: Common proximal protein interactions of wild type and mutant CLIC4

Gene Name	Σ Coverage	Σ # Proteins	Σ # Unique Peptides	Σ # Peptides	Σ # PSMs
HMGN3	36.92	3	1	3	3
SUPT20H	16.27	7	1	4	4
UQCRC1	17.08	1	1	4	6
UBA1	19.57	3	4	14	23
KRT10	21.75	1	3	7	9
HMGN2	22.22	1	2	2	3
TAGLN2	48.24	5	2	6	9
CCT8	23.18	4	2	7	9
EIF4A3	21.9	18	1	5	10
EIF4G1	9.32	15	2	7	8
FLNA	11.86	5	1	15	28
TRMT1	14.05	6	1	3	4
ZC3HAV1	30.93	4	3	9	20
ADD2	31.63	1	1	2	5
EIF2A	19.47	6	2	7	10
TOP1	17.39	1	1	8	10
ELAVL1	16.26	2	2	4	5
USP14	14.57	4	1	4	5
RPL27	53.68	4	4	7	12
CCT7	19.15	4	1	6	8
UBAP2L	10.03	11	1	5	9
PRPF19	27.18	3	4	8	10
RPS27	22.73	5	1	2	3
MTCH2	22.44	2	3	6	12
ZNF775	14.53	1	1	5	23
AHNAK	27.42	1	19	66	113
EFHD2	22.92	1	2	6	7
HUWE1	10.56	3	1	19	26
RSL1D1	36.49	4	1	6	6
TIMM50	17.11	6	1	4	5
GLUD2	29.21	4	2	9	14

Appendix B: Generation of CLIC4 knockout HeLa S3 cell lines

One non-targeting single guide RNA (sgRNA) as control and three different sgRNAs targeting early exons of CLIC4 together with Cas9 enzyme were individually expressed in HeLa S3 cells by transient transfection of the lentiCRISPR plasmid [54]. All the generated cell lines were analyzed for CLIC4 expression by Western blotting (**Figure A.1**). sgRNA-1 (sg1) and sgRNA-3 (sg3) targeted HeLa S3 cell lines were selected for single cell clone isolation experiments due to their better CLIC4 depleted phenotype when compared to sgRNA-2 (sg2) targeted cell line. Single cell clones were expanded to obtain cell lines and analyzed by Western blotting (**Figure A.2**). To eliminate off-target effects, one single cell clone from sg1 targeted cells (sg1-c32) and one single cell clone from sg3 targeted cells (sg3-c7) were randomly selected for following experiments.

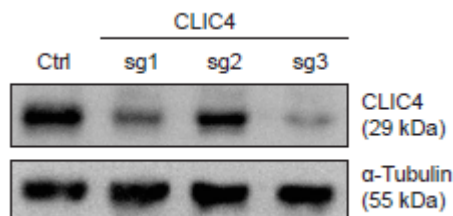


Figure A.1: Anti-CLIC4 blotting of HeLa S3 cell lines treated with different sgRNAs targeting *CLIC4* gene (sg1, sg2 and sg3).

Ctrl cell line was treated by non-targeting sgRNA. sg1, sg2 and sg3 target exon1, exon2 and exon3 of *CLIC4*, respectively. Anti- α -Tubulin blotting was performed as loading control.

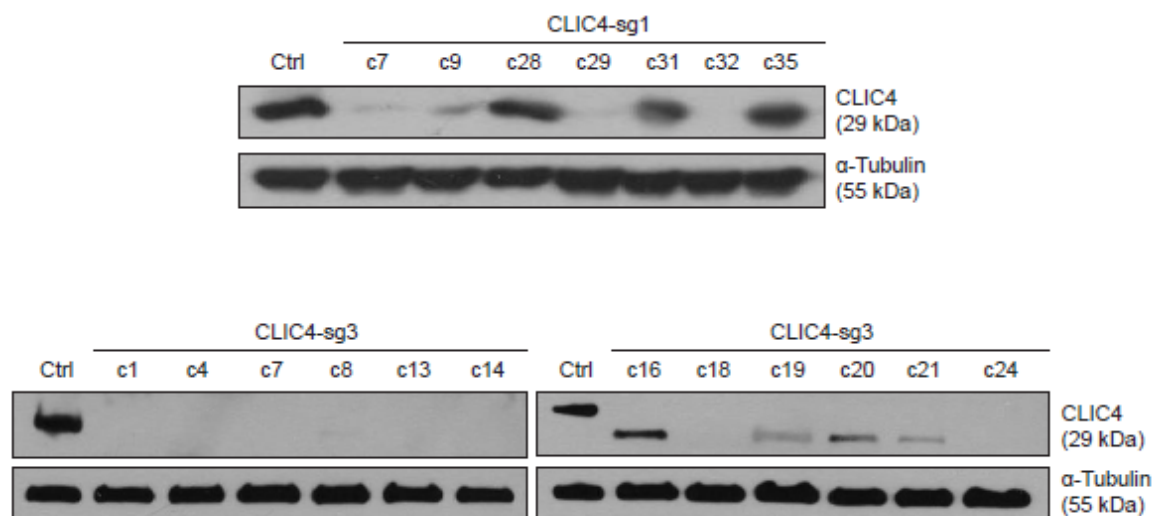


Figure A.2: Anti-CLIC4 blotting of single cell clones selected from CLIC4-sg1 (top panel) and CLIC4-sg3 (bottom panel) treated HeLa S3 cell lines.

Ctrl cell line was treated by non-targeting sgRNA. Anti- α -Tubulin blotting was performed as loading control.

Appendix C: Generation of CLIC1 knockout cell lines

Four different sgRNAs targeting early exons of CLIC1 together with Cas9 enzyme were individually expressed in HeLa S3 cells by lentiviral transduction of the lentiCRISPR plasmid [54]. All sgRNA targeted cell lines were analyzed for CLIC1 expression by Western blotting (**Figure A.3**). sgRNA-1 (sg1) and sgRNA-5 (sg5) targeted HeLa S3 cell lines were used to select single cell clones and CLIC1 expression of all single cell clones were analyzed by Western blotting (**Figure A.4**). To eliminate off-target effects, two CLIC1 knockout cell lines, sg1-c1 and sg5-c1, were selected for following experiments.

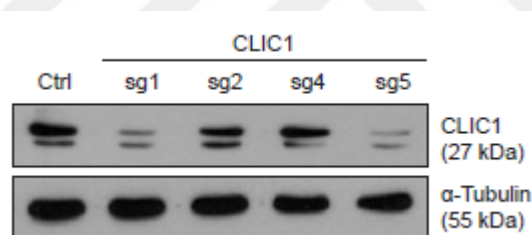


Figure A.3: Anti-CLIC1 blotting of HeLa S3 cell lines treated with different sgRNAs targeting *CLIC1* gene (sg1, sg2, sg4 and sg5).

Ctrl cell line was treated by non-targeting sgRNA. sg1 and sg2 target exon1 while sg4 and sg5 target exon2 of *CLIC1*. Anti- α -Tubulin blotting was performed as loading control.

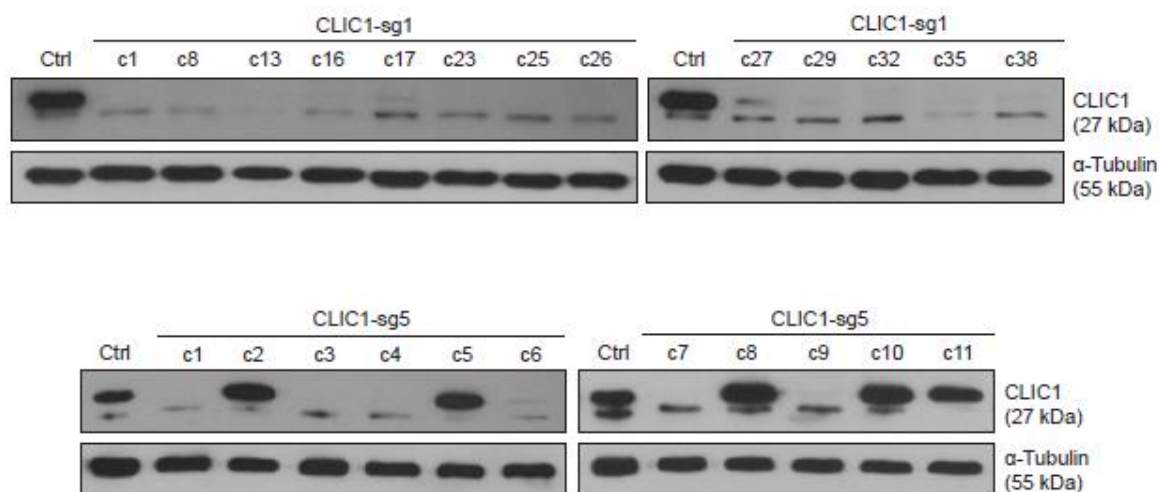


Figure A.4: Anti-CLIC1 blotting of single cell clones selected from CLIC1-sg1 (top panel) and CLIC1-sg5 (bottom panel) treated HeLa S3 cell lines.

Ctrl cell line was treated by non-targeting sgRNA. Anti- α -Tubulin blotting was performed as loading control.

Appendix D: Mutational analysis of *CLIC4* and *CLIC1* after CRISPR/Cas9 genome editing

Mutational analysis of human *CLIC4* gene, located on chromosome 1, in sg3-c7 cell line revealed that one allele has single nucleotide insertion whereas other allele has ten nucleotides deletion (**Figure A.5**).

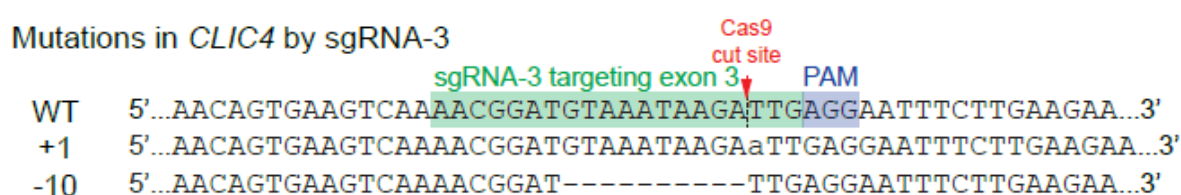


Figure A.5: Mutations generated in the *CLIC4* exon3 by CRISPR/Cas9 based genome editing.

WT: wild type allele, +1: one nucleotide insertion, -10: ten nucleotides deletion.

Mutational analysis of human *CLIC1* gene, located on chromosome 6, in sg1-c1 cell line revealed that one allele has single nucleotide deletion whereas other allele has five nucleotides deletion (**Figure A.6**).

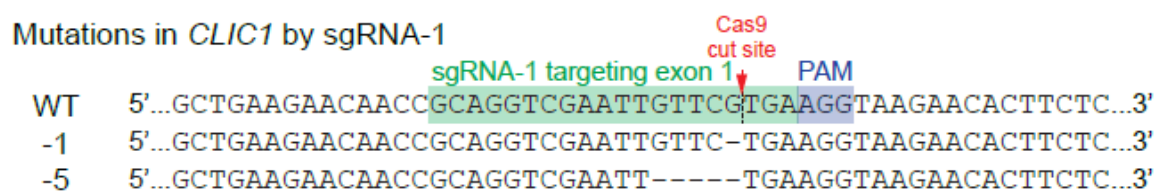


Figure A.6: Mutations generated in the *CLIC1* exon1 by CRISPR/Cas9 based genome editing.

WT: wild type allele, -1: one nucleotide deletion, -5: five nucleotides deletion.

Appendix E: Generation of CLIC1 and CLIC4 double knockout cell lines

CLIC1 and CLIC4 double knockout (CLIC1/4 DKO) cell line was generated by the infection of CLIC4 KO sg3-c7 cell line with the CLIC1-sg1 lentiviruses (**Figure A.7**). This cell line was used to select single cell clones and single cell clones were analyzed for CLIC1 expression by Western blotting (**Figure A.8**). Two CLIC1/4 DKO cell lines, c2 and c9, were selected for following experiments.

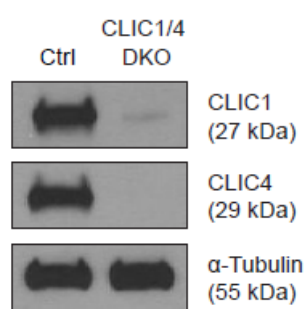


Figure A.7: Anti-CLIC1 and anti-CLIC4 blotting of CLIC1/4 DKO cell line.

Ctrl cell line was double treated by non-targeting sgRNA (first one: transient transfection, second one: viral transduction). Anti- α -Tubulin blotting was performed as loading control.

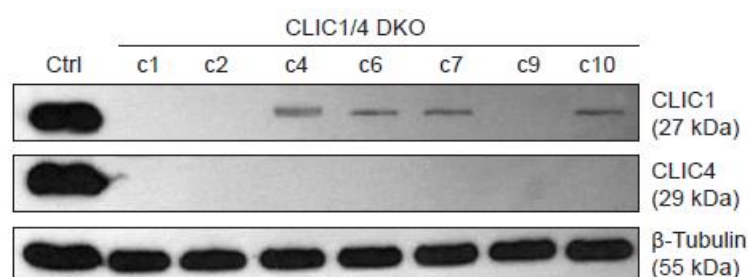


Figure A.8: Anti-CLIC1 and anti-CLIC4 blotting of single cell clones selected from CLIC1/4 DKO cell line.

Ctrl cell line was treated twice by non-targeting sgRNA. Anti- β -Tubulin blotting was performed as loading control.



Appendix F: shRNA-mediated knockdown of PCDH1 in HeLa S3 cells

I designed two shRNA sequences against human Protocadherin-1 (PCDH1) using RNAi Codex database [101] and performed their cloning into pSMP plasmid. Red labeled sequences in each shRNA represent sense and antisense sequences separated by a loop sequence:

PCDH1-sh1 5'-
TGCTGTTGACAGTGAGCGCGGTGACTTTGTTATCCAGAATTAGTGAAGCCACA
GATGTAATTCTGGATAACAAAGTCACCATGCCTACTGCCTCGGA-3' and PCDH1-
sh2, 5'-

TGCTGTTGACAGTGAGCGACCTTATGGACTCTTCCAGATTAGTGAAGCCACAG
ATGTAAATCTGGAAGAGTCCATAAGGGTGCCTACTGCCTCGGA-3'. Cloning of
shRNA oligos into pSMP-Luc retroviral mammalian expression plasmid and retroviral
packaging was performed as explained in **Section 3.2.5**. Viral transduction of HeLa S3
cells was performed as described in **Section 3.2.2** and transduced cells were selected using
2 ug/ml puromycin in growth medium for two days. Protein lysates of shRNA treated HeLa
S3 cells were prepared as in **Section 3.2.14**. Western blotting analysis was performed using
1:200 anti-PCDH1 (ab170812, Abcam), 1:1000 anti- α -Tubulin (DM1A) (3873, Cell
Signaling), 1:2000 HRP-linked anti-rabbit IgG (7074, Cell Signaling) and 1:2000 HRP-
linked anti-mouse IgG (7076, Cell Signaling) antibodies. The ECL (Amersham) was used
for detection and blots were visualized using X-ray film. Western blotting analysis reveals
expression levels of PCDH1 protein in shRNA treated HeLa S3 cells. PCDH1 sh2 induced
a decrease in the expression of PCDH1 protein when compared to control cells (**Figure
A.9**).

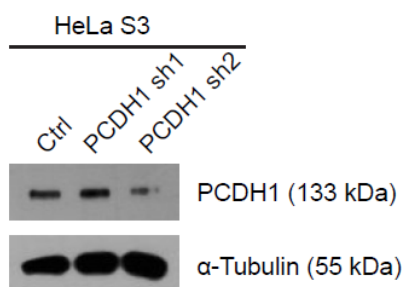


Figure A.9: Western blotting analysis of shRNA mediated knockdown of PCDH1 protein expression in HeLa S3 cells.

Ctrl: HeLa S3 cells generated by transduction of control shRNA, pSMP-Luc. α -Tubulin was used as loading control.

Appendix G: Real time cell migration assay of PCDH7 knockout HeLa S3 cells

I used the xCELLigence RTCA-DP system (ACEA Biosciences) for cell migration assay of PCDH7 knockout HeLa S3 cells. For cell migration analysis, FBS was used as chemo-attractant and experimental setup was prepared as explained in **Section 3.2.6**. 30,000 cells were seeded per well of the CIM plate and duplicates were prepared for each experimental condition. Measurements were done with an interval of 15 minutes for 48 hours. The migration of HeLa S3 cells was significantly decreased upon PCDH7 knockout (**Figure A.10**). The statistical analysis of the results were done by the unpaired two-tailed Student's t-test.

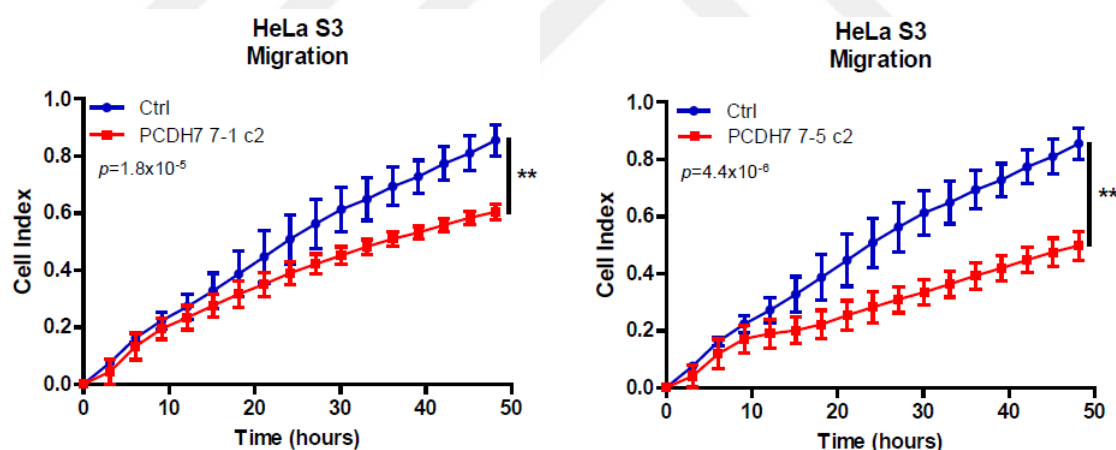


Figure A.10: The knockout of PCDH7 decreases migration ability of HeLa S3 cells.

30,000 cells were seeded per well and duplicates were prepared for each experimental condition. The measurements were taken with an interval of 15 min.

Appendix H: Generation of HRP-TM expressing stable MCF7 and MDA-MB-231 cell lines

The lentiviral mammalian expression plasmids, pLenti CMV Hygro DEST empty backbone and HRP-TM subcloned pLenti CMV Hygro DEST, were packaged as explained in **Section 2.2.7**. For viral transduction, MCF7 and MDA-MB-231 cells were seeded on 12 well cell culture plates to be 25% confluent on the day of transduction. During viral transduction, protamine sulfate was used as adjuvant. Protamine sulfate (P4020, Sigma-Aldrich) was prepared as 8 mg/ml stock solution in water and used at 8 ug/ml final concentration during transduction. Two successive transductions were performed to increase transduction efficiency. First transduction of cells was performed on day 1 as follows: 200 ul growth medium supplemented with 0.7 ul protamine sulfate was given to cells in each well. Then, 500 ul of empty backbone viral supernatant and HRP-TM viral supernatant were added directly to the corresponding wells by dripping on top of cells. For control wells, 500 ul growth medium was given to cells instead of viral supernatant. On day 3, first transduction medium was removed and second viral transduction was performed as on day 1. Next day, transduction medium was removed from each well and cells were selected for two weeks using 150 ug/ml hygromycin for MCF7 cells and 400 ug/ml hygromycin for MDA-MB-231 cells.