2015

RNA-Binding Protein CELF1 Controls MARCKS mRA Translation in Oral Cancer Cells

Mehul Z. Patel
Medical University of South Carolina

RNA-Binding Protein CELF1 Controls *MARCKS* mRNA Translation In Oral Cancer Cells

By

Mehul Z. Patel

Department of Oral Health Sciences and
Biochemistry and Molecular Biology

A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Masters in the College of Graduate Studies

Department of Oral Health Sciences and Biochemistry and Molecular Biology, 2015

Approved By:

Vasmsi Gangaraju (Chairman)

6/9/15

Viswanathan Palanisamy (Mentor)

Andrew G. Jakymiw

Dennis K. Watson

Lauren Ball

ABSTRACT

Post-transcriptional gene regulation plays an important role in controlling gene expression patterns in mammalian cells. CUG-binding and Embryonic Lethal vision-type RNA-binding protein 3 (ETR)-like Factor (CELF1), also known as CUGBP1, is a RNA-binding protein involved in post-transcriptional gene regulation through nuclear (alternative splicing) and cytoplasmic (mRNA turnover and translation) mRNA processing events. Primarily, CELF1 is known for its contribution to the development of myotonic dystrophy (DM1). But, published observations from our laboratory and others determined that CELF1 is overexpressed in head and neck squamous cell carcinoma (HNSCC) as well as multiple other cancers. Unpublished proteomic pulsed-stable isotope labeling by amino acids in cell culture (pSILAC) from our laboratory, has identified approximately putative 1350 CELF1 target proteins were controlled at the mRNA translation level in UM74B oral cancer cells. The pSILAC data and subsequent ribosome profiling validation experiments revealed that the protein expression of MARCKS (Myrisoylated alanine-rich kinase C substrate) is significantly reduced upon CELF1 knockdown in comparison to control oral cancer cells. MARCKS is known to play an important role in cell shape, cell motility, secretion, transmembrane transport, and regulation of the cell cycle in cancer cells. We have determined that MARKCS protein is overexpressed in HNSCC cell lines compared to primary normal oral keratinocytes. Moreover, CELF1 directly controls the expression of MARCKS, but its mRNA levels remain unchanged in oral cancer cells. CELF1 ribonucleoprotien immunoprecipitation (RNP-IP) experiments determined that *MARCKS* mRNA is directly associated with CELF1.

The cell migratory role of MARCKS was evaluated by a scratch wound healing assay and transwell migration assay. Depletion of CELF1 and MARCKS independently resulted in reduction of cell migration. Moreover, overexpression of CELF1 in normal non-malignant human oral keratinocyte cells resulted in overexpression of MARCKS and enhanced cell migration. Finally, shRNA-mediated reduction of CELF1 in association with exogenously expressed MARCKS in oral cancer cells, we were able to rescue the CELF1-mediated cell migration phenotype. In conclusion, our results demonstrate that CELF1 controls cell migration through regulating MARCKS protein translation in oral cancer cells.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF SCHEMATICS

LIST OF FIGURES

LIST OF ABBREVIATIONS

3′ UTR	3′ Untranslated Region
5′ UTR	5′ Untranslated Region
ARE	AU-Rich Element
Bru-3	Bruno-3
Brul	Bruno Like
CaM	Calmodulin Mediated
cAMP	Cyclic adenosine monophosphate
Cdk4	Cyclin-dependent kinase 4
CEBPβ	CCAAT/Enhancer-binding protein
CELF1-6	CUGBP and embryonic lethal abnormal vision like factor
cGMP	Cyclic guanosine monophosphate
CT	Computed tomography
CUP	An ovarian protein
DAG	Diacyl Glycerol
DEPC	Diethylpyrocarbonate
DM1	Myotonic Dystrophy Type 1
DMPK	Dystrophia myotonic protein kinase
DNΔCELF1	Dominant Negative CELF1
DNA	Deoxyribonucleic acid
EDEN-BP	Embryonic Deadenylation Element Binding Protein
eIFA4E	Eukaryotic Initiation Factor 4E
ERM	ezrin, radixin, and moesin proteins

Erk	Extracellular Signal-Related Kinase
ETR-1	ELAV- type RNA Binding Protein
F-Actin	Filamentous Actin
fMLP	N-formyl-L-methionyl-L-leucyl-phenylalanine
G-Actin	Globular Actin
GPCRs	G Protein Coupled Receptor Signaling
GRE	Guanine/Uridine-Rich Elements
HDACI	Histone deacetylase inhibitor
HNC	Head and neck cancer
hnRNPH	heterogeneous ribonucleoprotein
HNSCC	Head and neck squamous cell carcinoma
HOK	Human oral keratinocyte
HPV	Human Papilloma virus
IP_3	Inositol l 1, 4,5-triphosphate
IRES	Internal Ribosomal Entry Site
kip1	Cyclin dependent kinase inhibitor p27
LIP	Liver enriched inhibitor protein
MAPK	Mitogen Activated Protein Kinase
MARCKS	Myrisoylated alanine-rich kinase C substrate
MBNL1	Muscle Blind Like 1
MH2	Myristoylated amino-terminal domain
mRNP	Messenger ribonucleoproteins (mRNP)
miRNA	MicroRNA

MRI	Magnetic resonance imaging
mRNA	Messenger RNA
NMD	Non-sense mediated decay
NMR	Magnetic Resonance Spectroscopy
NSD	Non-stop mediated decay
OHKC	non-tumorigenic normal oral keratinocytes
PARN	PolyA Ribonuclease
PDGF-BB	Platelet derived growth factor-BB
PET	Positron emission tomography
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PKS	Protein Kinase S
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-aceate
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PPK1	PKC and PKC-related Kinase 1
PSD	Phosphor-site domain
pSILAC	Pulsed stable isotope labeling of amino acids in cell culture
RIP-CHIP	RNA-binding protein Immunoprecipitation- Chromatin Immunoprecipitation

RBP	RNA binding protein
RNA	Ribonucleic acid
ROCK	Rho-associated kinase
RRM	RNA recognition motif
RTK	Receptor Tyrosine Kinase
SHMT	Hydroxymethyltransferase
siRNA	Small interfering RNA
SPR	Surface plasmon resonance
SELEX	Systemic evolution of ligands exponential enrichment
TIA1	T cell internal antigen 1
TNF-α	Tumor necrosis factor alpha
TPA	12-O-tetradecanylphorobol-13-acetate
TTCTG and TGTT	TC-Rich motifs
WASP	Wiskott-aldrich syndrome protein

Chapter 1

Introduction

Overview of HNSCC

Head and neck squamous cell carcinoma (HNSCC) represents a wide-variety of tumors that arise from base of the skull to the clavicles (1). HNSCC anatomic sites include: orbits, nasopharynx, oral cavity, oropharynx, hypopharynx, sinuses and larynx (1). HNSCC is considered to be the 6th most common cancer worldwide, with 400,000 or more new cases registered annually and of those cases, only 40-50% will survive within next 5 years (2). In the United States alone more than 42,000 cases and 8,300 deaths were predicted for this disease in 2014 (3). Factors such as tobacco carcinogens along with alcohol consumption have shown to be a major risk factor for HNSCC in Western countries (4). In the past few decades, up-rise of sexually transmitted infection, human papillomaviruses (HPV), among young people has emerged as a major risk factor (5, 6) for oral carcinogenesis. But recent advances in early diagnosis through three-dimensional imaging, such as computed tomography (CT scans) and magnetic resonance imaging (MRI) have allowed for better staging, and treatment planning for HNSCC patients (1).

Although, there have been many advances in HNSCC treatment, the overall survival rate of HNSCC patients only improved marginally over the past 30 years (2). Currently, nonselective treatments such as: surgery, radiation, and chemotherapy are being implemented for HNSCC patients (2). These nonselective treatments have high systemic toxicities and associated morbidity and mortality (2). Thus, understanding the molecular mechanisms and underlying cause of HNSCC will aid in the development of more selective and effective cancer treatment options for HNSCC patients.

Post-transcriptional control of gene expression

The central dogma of the cell states that DNA is transcribed into RNA, which is subsequently translated into protein (7). The complexity of the human proteome is derived from the control of post-transcriptional gene regulation. Disruption of post-transcriptional gene regulation often leads to cancer predisposition (8-10), viral infections (11-13) and neuromuscular disorders (14, 15). Post-transcriptional control of gene expression involves several steps, including mRNA processing, transport, stability and translation. The mRNA stability or turnover is either deadenylation-dependent or independent in mammalian cells (16) and these complex regulatory pathways control more than 50% of gene expression patterns. However, in general, the deadenylation-dependent mRNA decay process dominates the mRNA degradation process due to fact that most of the poly-A tail containing mRNAs undergo nuclear export and cytoplasmic mRNA translation and turnover (16).

Messenger RNA turnover rates determine the life span of an mRNA molecule. Messenger RNAs with fast turnover rates are considered short-lived mRNAs, conversely, long-lived mRNAs have slower turnover rates (17). Thus, in the context of changing environmental cues, these different mRNA turnover rates contribute to cellular gene expression. For example, vascular endothelial cells produce few or no growth factors, ILs (interleukins), or adhesion molecules, but with treatment of IL-1, gro-α mRNA is specifically stabilized (18). On the contrary, glucocorticoids, like estrogen have been shown to destabilize IL-1β (19), interferon (20) and collagen (21) mRNAs. Finally, regulation of mRNA half-life can occur through association of protein *trans*-acting factors and nucleotide *cis*-elements (22). The *cis*-elements such

as TG-rich motifs (TTCTG and TGTT) are located in the 3′ UTR and can promote mRNA decay and/or stability with the help of *trans*-acting factors such as RNA-binding proteins (RBPs) and noncoding RNAs (22, 23). High-throughput analysis messenger ribonucleoproteins (mRNPs) from mammalian cells showed that RBPs associated with unique subset of mRNAs to coordinately regulate their localization, translation and/or degradation (22, 24-27).

Deadenylation-dependent mRNA decay is the major pathway in mammalian cells. Most mammalian mRNAs are polyadenylated in the nucleus undergoes nuclear mRNA processing, transport into the cytoplasm, and subjected to translation and turnover. In the deadenylation-dependent mRNA decay pathway, one or a combination of deadenylases (Pan2-Pan3, Ccr4-Not and poly(A)-specific exoribonuclease (PARN)) removes the poly(A) tail located in the 3′ UTR of mRNAs (28, 29). Following deadenylation, exonucleases proceed with mRNA degradation in a 5′-3′ or 3′-5′ direction. Another pathway that controls mRNA decay, is the non-sense mediated decay pathway (NMD). NMD is a mRNA surveillance pathway that targets mRNAs with premature stop codons (16). Messenger RNA surveillance is the process of ensuring the destruction of mRNA that would otherwise produce nonfunctional protein due to either mutations in a gene, errors in pre-mRNA splicing that retain introns or nonproductive chromosomal rearrangements (30). An additional mRNA decay pathway that has been identified is called nonstop mRNA decay (NSD). This pathway has been proposed recently and illustrated that the translation of mRNAs that lacks the stop codon allows ribosomes to translate to the end of the 3′ UTR, leading to mRNA degradation (31).

GRE-Mediated mRNA Decay

During the investigation of T cell activation, a genome-wide database of mRNA half-lives has been established (32). The database contains many transcripts that possess both AU-rich elements (AREs) and GU-rich elements (GREs), and their half-lives under the activation of T cells. This observation led researchers to identify a canonical sequence in the form of UGUUUGUUUGU; which associates with the cytoplasmic protein CUGBP1 and Etr3 like Factor 1 (CELF1) (33). Further investigation uncovered a recruitment of PARN by CELF1 for decay of GRE containing mRNA (34). This mRNA decay mechanism is consistent with the observations made *in vitro* that show CELF1 association with mRNA correlates with enhanced deadenylation in both HeLa cells (35) and *Xenopus (36).*

CUG-binding and Embryonic Lethal abnormal vision-type RNA-binding protein 3 (ETR-3)-like Factor (CELF)

The CELF proteins are a family of RNA binding proteins that are known to play an important role in post-transcriptional gene regulation (37, 38). In humans and mice, there have been six members identified in the CELF family: CELF1-6, and CELF1 and CELF2 proteins are expressed ubiquitously and play important role in embryogenesis (39-43), CELF3-6 are expressed in adult tissues and mostly found in the nervous system (44, 45). CELF1 is well-studied protein in myotonic dystrophy, but its cancer biological role has not been described before. Hence, I plan to study in this thesis to decipher CELF1's cancer biological role in oral cancer cells.

CELF1 was first shown to be involved in alternative splicing of the mRNAs in the nucleus (46-48), and further studies revealed that CELF1 localized in the

cytoplasm (49), indicating that CELF1 could be involved in pre-mRNA splicing, turnover or translation. CELF1 was first identified as EDEN-BP (Embryonic Deadenylation Element-Binding Protein) in the *Xenopus Laevis* (36). EDEN-BP is a homolog of CELF1 and determined to cause a rapid decay of mRNAs. But, later it was determined that mRNAs that are being degraded by EDEN-BP occurred through binding to U/G rich sequences in 3′ UTR. The binding triggered deadenylation and translational repression of mRNA by recruitment of poly(A) ribonuclease (35).

In *Xenopus*, CELF1 was identified as a translational repressor by binding to a specific motif in the 3′ UTR of its targets mRNAs. These motifs were termed the "Embryonic Deadenylation Element" (EDEN) motifs. The binding triggers rapid deadenylation and therefore, the translation is inhibited and leads to degradation of the bound mRNA. Furthermore, human and *Xenopus* CELF1 have very high degree of sequence conservation and exactly 88% of the sequences are identical and the conserved. Due to similarities between these two proteins, they can compensate for one another and CELF1 was considered as a deadenylation factor (50). Based on the sequence similarities EDEN and CELF1 presumed to recognize similar subsets of mRNA targets (51). Overexpression of CELF1 causes muscular disease DM1 by imperfectly targeting its mRNAs leading to those encoding proteins misregultion in muscular functions (52-55).

Structure of CELF proteins

CELF proteins contain highly conserved RNA-Recognition Motifs (RRM), of which, two RRMs are located at the N-terminus and third RRM located at the C-

terminus, separated by the divergent linker domain. Furthermore, the RRMs have RNA binding activity and it is proposed that the divergent domain is an important site for functional regulation. This highly conserved family of proteins regulates the developmental processes and gene expression at post-transcriptional levels in *Gallus*, *Zebrafish*, *Drosophila* and *C. elegans* (36, 37, 56-59).

Protein domains of CELF1

The human CELF1 protein has the basic arrangement of three RRMs like other CELF family of proteins. The CELF1 is a 486 amino acid protein with a total mass of 52.1kDa. The RRMs are very important for CELF1 binding to its target mRNAs. There are multiple reports indicated that CELF1 targets mRNAs through AU-rich or GU-rich sequences (60, 61). Nuclear magnetic resonance spectroscopy (NMR) studies revealed that both the RRM1 and the RRM2 domains of CELF1 bind to 12-nucleotide targeted RNAs containing two UUGUU motifs. Moreover, binding of both RRM-1 and -2 domains have higher affinity compared to the binding of individual domains separately, suggesting a cooperative binding between these RRMs (62, 63). Further crystallographic studies showed that both RRM-1 and RRM-2 binds to GRE-RNAs, and RRM-1 is essential for crystal-packing interactions (64). Utilizing an NMR approach, RRM-3 has also been shown to bind specifically to UGU trinucleotide segment of bound $(UG)_3$ RNA. The recognition is through stacking and hydrogen bonding interactions within a pocket formed by the beta-sheet and the conserved N-terminal of RRM3 (60).

NMR studies along with yeast three-hybrid system, deletion and mutation analysis have been essential in understanding the interaction of various CELF1

domains with its target RNA. In an yeast three-hybrid system, deletion/mutation of RRM-1 or RRM-2 did not affect CELF1 binding to GU-rich RNAs, whereas RRM-3 has the ability to recognize GU-repeats more strongly than RRM-1 or RRM-2 (65). Additionally, combination of CELF1's divergent domain and RRM-3 known to poorly recognize the G/C-rich sequence of Cyclin D1 in the 5′ UTR (66).

Mechanism of CELF1 binding to Target mRNAs

CELF1's sequence recognition

Isolation and characterization of CELF1 and CELF2 demonstrated that they are novel heterogeneous nuclear ribonucleoproteins (hnRNPs). CELF1 has been shown to interact with two distinct types of RNA sequences: UG rich elements (67) and CUG repeats (68). An *in vitro* study by Timchenko et.al, showed that binding of CELF1 to the 3′ UTR of mytonic protein kinase mRNA occurred due to the presence of $(CUG)_8$ sequences (52, 54). Further SELEX (systemic evolution of ligands exponential enrichment) (69), SPR (surface plasmon resonance) (65) and yeast three-hybrid systems (67) revealed that CELF1 preferentially binds to GU-rich sequences (33, 70). These observations were confirmed in C2C12 cells utilizing RNA immunoprecipitation (RNA-IP) followed by microarray (RIP-Chip) analysis (71).

In *Xenopus,* EDEN-BP orthologs of CELF1 appears to also bind to GU-rich mRNA sequences, which contain the sequence $(UGUA)_{12}$ and function as a deadenylation signal in embryos after fertilization and deletion of EDEN-BP totally abolished EDEN mediated deadenylation activity (36, 50). In *Drosophila,* the CELF1 ortholog is Bru-3 and in *Xenopus,* the CELF1 ortholog is *EDEN-BP*; in both organisms CELF1 specifically bind to the sequence of $(UG)_{15}$ repeats (37). In

Zebrafish, a protein called Brul, which is 81% identical to EDEN-BP, preferentially binds to GU-rich mRNAs (48). Furthermore, in *C. elegans* the protein ETR-1 (ELAV-type RNA Binding Protein 1) is also a homolog of human CELF1. The reduction of ETR-1 caused failure of embryos to elongate and subsequent paralysis caused embryonic lethality (57). Interestingly, not only GU-rich sequences are required, but adjacent sequence elements are also important for assembly of CELF1 protein on RNA by allowing optimal secondary structure to facilitate the formation of RNA-binding protein complexes (72, 73). Findings in *Xenopus* and *C. elegans* models suggest that CELF1 homologs are critical for muscle development. Dysregulation of CELF1 is involved in the pathogenesis of the human neuromuscular disease, myotonic dystrophy type 1 (DM1) (74). CELF1 has also been shown to be a mRNA decay regulator in muscle tissue through GRE element recognition, therefore, dysregulated CELF1 mediated mRNA decay may be involved in pathogenesis of DM1 (74). In addition to conservation of biomedical mechanisms of CELF1-GRE regulation, these results also suggest that CELF1-GRE mediated regulation is preserved over evolution at the organismal level and regulate specific aspects of developmental programs.

Dimerization of CELF1

In *Xenopus, EDEN-BP* (homolog of CELF1) has been reported to undergo dimerization in a yeast two-hybrid assay, and the dimerization may have a role in RNA recognition (75, 76). However, no human CELF1 dimerization has been reported in the literature. The yeast two-hybrid observation does raise the question of whether dimerization is due to the formation of a true dimer or whether two CELF1

proteins dimerize as a result of binding to one RNA molecule, but this observation needs further investigation.

Phosphorylated CELF1 and RNA metabolism

CELF1 was considered as a phosphoprotein, and protein kinase C (PKC) can stabilize CELF1 by hyper-phosphorylating the protein in DM1 cells (49). Furthermore, it has been shown that hyper-phosphorylated CELF1 is unable to degrade TNF-α in DM1, suggesting that phosphorylation may interfere with CELF1 mRNA binding and decay (77). The support for a pathogenic role of CELF1 hyper-phosphorylation comes from a DM1 transgenic mouse model (78). Interestingly, mice treated with the specific inhibitor of the PKC resulted in cardiac abnormalities associated with the disease phenotype.

The CELF1 protein is also predicted to have phosphorylation sites for several phosphatases (79). Therefore, the phosphorylation of CELF1 has been suggested to play an important role in regulating its RNA binding preference, and interaction with other proteins (80-82). Phosphorylation has been shown to occur at Ser28 and Ser302; and demonstrated that phosphorylation affects the RNA binding of CELF1 (80). Akt kinase has been shown to phosphorylate CELF1 at the Ser28 residue and this specific residue has been shown to affect translation during differentiation of normal muscle myoblasts. Additionally, it has also been proposed that phosphorylation of CELF1 at Ser28 serves as a switch between binding to U/G- rich and C/G-rich sequences. For example, non-phosphorylated form of CELF1 binds to U/G- rich sequences but upon phosphorylation, CELF1 binds to C/G-rich sequences. Another example, CELF1 binds to the cyclin D1 mRNA upon the phosphomimetic

mutation S28D (66). Finally, phosphorylation of CELF1 at Ser302 alters its RNA-binding activity. For example, cyclin D3-Cdk4/6 complex phosphorylates CELF1 at Ser302, and effects the binding specificity of CELF1 to its targeted RNA (83). All together, these observations indicate that CELF1 functions in many aspects and in multiple disease states through phosphorylation.

CELF1 play a role in pre-mRNA splicing

Diversity within RNA transcripts and protein can be generated through pre-mRNA alternative splicing; and it has been determined that 90% of human genes produce alternatively spliced transcripts (84, 85). Alignment of the genomic regions adjacent to mammalian intron-exon splice sites, identified TG –rich motifs (TTCTG and TGTT) as conserved *cis*-elements that are associated with alternative splicing (86, 87). These identified C/UG-rich templates served as binding sites for CELF proteins, which can either activate or suppress the splicing of pre-mRNA targets depending upon the genomic environment (88).

In DM1, aberrant gain of CELF1 function and simultaneous loss of the splicing factor MBNL1 has been shown (89). Moreover, CELF1 and MBNL1 have opposite effects on exon/intron inclusion upon binding to specific sequences. The opposite effect is due to *mis*-splicing of various crucial genes (74). For example, minigene reporter system that contain alternative splice sites for CELF1 to identify pre-mRNA targets, these targets includes but not limited to cardiac troponin T (55), insulin receptor (90), and chloride channel 1 (46, 91). This system has shown that either loss or gain of these regulatory proteins occurs through pre-mRNA splicing events. Further studies have used cultured cells with transiently transfected

minigenes and results have identified a number of alternative gene regions that are regulated by CELF1 and other RBPs (46, 55, 60, 90-99).

An additional role of CELF1 mediated alternative splicing has emerged from early developmental studies that used transgenic CELF1 deficient mice which showed altered regulation of fetal to adult splicing pattern of several skeletal muscle transcripts (91, 100). In order to determine the global role of CELF1 in fetal-to-adult transition, mouse models have been used (89). In particular, splicing microarrays were utilized and demonstrated that half of the transcripts that underwent fetal-to-adult alternative splicing transitions occurred in the heart tissue due to overexpression of CELF1, which resulted in alternative splicing pattern of transcripts (89). Furthermore, in order to study CELF1-specific alternative splicing, a development of dominant negative and tissue specific transgenic mice was ideal for *in vivo* (92, 95, 100, 101). For example, under the control of a cardiac muscle-specific promoter expressing dominant negative CELF1 (DNΔCELF1) showed development of dilated cardiomyopathy and cardiac dysfunction over time (92).

CELF1 as an mRNA Translation Regulator

Protein translation is an important part of post-transcriptional control of gene expression that is regulated and adapted to various environmental and developmental changes. CELF1 at some extend activates (102) and inhibits (83, 103) the translation of various mRNAs that are involved in multiple developmental stages. Some homologs of CELF1 have also been shown to regulate translation. For example, in the *Drosophila* oocyte, when Bruno, a homolog of CELF1, binds to Bruno response elements (BREs) within the 3′ UTR of oskar mRNA, protein

translation was inhibited. Additionally, Bru-3 (another CELF1 ortholog) bound to the 3′ UTR GU-rich sequence of gurken, cyclin A and oskar mRNA also lead to inhibition of translation (56). The proposed translation inhibition mechanism of Bru-3 is mediated through formation of Bru-3/eIFA4E/5′-cap complex at various stages of embryo development (104).

In mammalian cells, CELF1 has also been identified as a translational regulator of selective mRNAs. For example, CELF1 has been demonstrated to promote the translation of alternative isoforms of the transcription factor CCAAT/enhancer-binding protein (CEBPβ) (68, 105, 106). This regulation was first described in a rat model of partial hepatectomy. In this model, partial hepatecomy caused CELF1 phosphorylation, which led to formation of a CELF1 and eIF2 (eukaryotic initiation factor 2) complex. This complex initiated translation of the liver enriched inhibitor protein (LIP), an isoform of CCAAT/enhancer-binding protein (82). The confirmation of this experiment was demonstrated in liver cells. Additionally, it was shown that CELF1 goes through hyper-phosphorylation during normal aging via the GSK3beta-cyclin D3-cdk4 kinase pathway (107). In an age-associated model, just like the partial hepatectomy model, the cdk4-mediated hyper-phosphorylation of CELF1 also induced the CELF1-eIF2 complex formation (80). In addition, in a rat aging model, phosphorylation of CELF1 caused its interaction with a GC-rich sequence in the 5′ UTR of p21 mRNA inhibiting its translation and senescence in fibroblasts (108). In myocytes, stabilized p21 mRNA was observed in stress granules. However, only in late senescence did p21 localize in stress granules which interfere with its translation (109). Furthermore, under normal muscle cell

differentiation, CELF1 increased the translation of p21 (110), and Mef2a29 (111) via direct interaction with $(GC)_n$ repeats in the 5′ UTR. Additionally, binding of CELF1 to the 3′ UTR of the serine hydroxymethyltransferase (SHMT) mRNA (112, 113) and cyclin dependent kinase inhibitor p27 (kip1) (114), found to regulate translation activation of an internal ribosome entry site (IRES). These studies suggested CELF1's role in IRES mediated initiation of mRNA translation. Fox J. et.al, have shown that IRES translation occurs through the formation of CELF1/hnRNPH complex, which promotes circularization of RNA transcripts by interaction of 5′/3′ ends (112).

Taken together, these data suggest that CELF1 mediates translational regulation through G- and C-rich motifs and other various motifs in the 5′ UTR, whereas CELF1 mediated splicing and degradation affects occur through G- and U-rich motifs in introns and the 3′ UTR. However, a global analysis of CELF1-mediated translational regulation remains to be determined. Thus, our interest remains to investigate CELF1 mediated translation in oral cancer cells.

Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS)

In 1982, MARCKS was first identified as an 80kDa protein (115) and that is expressed in neural and central nervous system (CNS) tissues (116, 117), skeletal muscle (118), connective tissue (119) and leukocytes (120-123). MARCKS has been characterized and shown to be present in mammalians and non-mammalian species. The MARCKS protein only varies slightly among various species due to the conserved domains (116, 124-126). The MARCKS gene itself contains three-conserved domains such as, mristoylated amino-terminal domain, MARCKS

homology domain 2 (MH2) and phospho-site domain (PSD). The PSD site serves as the phosphorylation site for PKC (127, 128) and the MH2 domain contains an intron-splice site, but no known function has been demonstrated (127).

In resting cells, MARCKS is anchored to cellular membranes by its myristoylation motif and electrostatic interaction between the amino acids of the PSD and the acidic phospholipid bilayer, which is enriched in phosphatidylserine and PIP_2 (129, 130). MARCKS has been shown to be myristoylated post-transcriptionally, and an addition of myristoyl moiety (C_{14} saturated fatty acid) to the amino-terminal glycine residue. The myristoyl moiety allows MARCKS to insert itself hydrophobically into the phospholipid bilayer of the plasma membrane (118, 127, 130-135). A combination of myristoylation and electrostatic interaction helps MARCKS anchor itself to cellular membranes and does so in an independent manner (132, 133, 136, 137). Furthermore, upon MARCKS phosphorylation, negative charges get incorporated into the PSD, resulting in the weakening of the electrostatic interactions between MARCKS and the phospholipid bilayer. This weakening causes MARCKS to completely dissociate from cellular membranes resulting in cytosolic localization (118, 132, 133, 135). This mechanism has been termed the “myristoyl-electrostatic switch” mechanism (136) and has been presumed to play a role in cell migration.

Function of phosphorylated MARCKS

Initially, MARCKS was described as a substrate of PKC, which can phosphorylate MARCKS, either directly or indirectly. It has been shown in a rat model that that PKS and the PKC-related kinase 1 (PPK1) can phosphorylate Ser152, Ser156, and Ser163 of MARKCS within PSD. Additionally, G protein

coupled receptor signaling (GPCRs) and receptor tyrosine kinase (RTK) can activate PKC; in turn PLC cleaves PIP2 into inositol 1,4,5-triphosphate (IP_3) and diacyl glycerol (DAG). DAG is stationed in the plasma membrane and IP_3 opens calcium channels in the endoplasmic reticulum (138). Opening of calcium channels increases intracellular calcium concentrations. Interestingly, calcium is known to activate many isoforms of PKC. For example, conventional PKCs (α, βI, βII, γ) isoforms require DAG and Ca^{2+}; novel PKCs (δ, ε, θ, η) isoforms only require DAG; and atypical PKCs (ζ, ι, λ), do not require DAG or Ca^{2+} (138). Phosphorylation of MARCKS has been shown to occur by both novel PKCs (ε, δ, θ) and classical PKCs (α, βII) (118, 139-141). Furthermore, MARCKS phosphorylation through PKC causes MARCKS to localize from cellular membranes to cytosol (142). Using a rat model, residue Ser152 of MARCKS shown to be phosphorylated through PKC activation and PKC has been shown to control the regulation of MARCKS ability to bind phosphatidylserine on the plasma membrane (143).

In contrast, it has been shown that upon inhibition of the Rho-associated kinase (ROCK) signaling pathway and treatment of LPA leads to human MARCKS phosphorylation at Ser159 (Ser152 in mice), thus depicting an example of MARCKS indirect phosphorylation by PKC, as ROCK activates PKC (144-148). In parotid cells, activation of protein kinase A (PKA), a serine/threonine kinase, by adenylyl cyclase and cyclic AMP (cAMP) resulted in activation of PKCδ and released amylase (149). This indicated that MARCKS could potentially be phosphorylated and that the cAMP dependent/PKA pathway could indirectly regulate MARCKS function.

Other than PKC activation, MARCKS also be phosphorylated by Protein Kinase G (PKG) through activation of cyclic GMP (cGMP) (150) in NIH-3T3 fibroblasts. However, total reduction of MARCKS has also been observed in these cells, perhaps indicating that the PKG pathway is involved in its degradation (151). Additionally, in mouse, the p42 mitogen activated protein kinase (MAPK) has been shown to phosphorylate MARCKS on Ser113, a residue is present outside of the PSD (152).

MARCKS phosphorylation also leads to its localization from the plasma membrane to cytosol (122, 128, 136, 153) or lysosome (154). Cytosolic phosphorylated MARCKS is dephosphorylated by protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A) or calcineurin (calcium-bound protein) (155-159). Upon de-phosphorylation, MARCKS has been shown to bind to calmodulin, get cross-linked by filamentous actin (F-actin), or re-associate with cellular membranes, hence, validating MARCKS proposed role in cell migration.

MARCKS role in reorganization of the actin cytoskeleton:

Many studies have determined that MARCKS is capable of binding to actin but the underlying mechanism is unclear. A general accepted hypothesis of MARCKS is that the PSD has two actin-binding motifs on the N-terminus and C-terminus that are responsible for F-actin cross-linking. In addition, phosphorylation of MARCKS leads to conformational change within the PSD, which blocks one of the two acting binding domains. However, dephosphorylation of MARCKS also results in a conformational change that reveals both actin-binding sites for F-actin cross-linking (160-162).

MARCKS has the ability to cross-link F-actin is promoted by both myristoylation and cleavage of MARCKS (163), as well as MARCKS phosphorylation at Ser25 (164). Furthermore, the PSD of MARCKS is able to bundle F-actin filaments and does so by binding to negatively charged residues of F-actin filaments, thus eliminating the electrostatic repulsion between F-actin filaments (165). Additionally, the MARCKS PSD is able to polymerize globular actin (G-actin) to F-actin. This mechanism is dependent upon the pentalysine sequence on the N-terminus of the PSD. Upon being phosphorylated by PKC or calmodulin binding, the ability of MARCKS to polymerize G-actin is inhibited (166).

A generally accepted hypothesis of MARCKS is that it helps in the movement of F-actin around the cell and helps other cytoskeletal proteins interact with F-actin as well. It has been established that myoblast alpha-5 integrin mediated focal adhesion needs functional MARCKS (140), and that MARCKS and F-actin are colocalized in blebs, lamellae of fibroblasts and membrane ruffles (167). Additional studies have shown colocalization of MARCKS with cytoskeletal proteins such as α_3-integrins and tetraspanins, proteins that form membrane complexes with integrins and participate in integrin-mediated cellular migration (168, 169).

Furthermore, an additional mechanistic hypothesis of MARCKS regulation of actin cytoskeleton has been demonstrated by sequestration of PIP_2. It has been demonstrated that the continuous presence of PIP_2 in the plasma membrane regulates actin dynamics. Wiskott-Aldrich syndrome protein (WASP) and ERM (ezrin, radixin, and moesin) proteins are actin-binding proteins that are activated by PIP_2. Increased concentrations and decreased concentrations of PIP_2 are the

signals for anchoring and releasing of the actin cytoskeleton from membranes (166, 170, 171). Additionally, the MARCKS PSD binds to the plasma membrane phospholipids phoshatidylserine and PIP_2 through electrostatic interactions (129, 134, 172, 173). The unphosphorylated PSD of MARCKS binds to PIP_2 and inhibits PLC mediated hydrolysis of PIP_2 (174). On the plasma membrane, MARCKS clusters PIP_2 molecules and PKC and Calmodulin mediated (CaM) displacement of MARCKS from the plasma membrane releases the sequestrations of PIP_2, resulting in the hydrolysis of PIP_2 by PLC (166, 174, 175). Taken together, these results demonstrate that sequestration of PIP_2 is one of the mechanisms whereby MARCKS regulates actin dynamics.

MARCKS participates in cell migration:

MARCKS role in cell migration should not be a surprise given that it regulates the actin cytoskeleton. MARCKS is required in the migration of fibroblasts (167), myoblasts (156), human embryonic kidney cells (176), human hepatic stellate cells (177) and vascular smooth muscle cells (178). The first step during cell migration is the cells ability to adhere to the extracellular matrix; and the role in MARCKS in regulation of cell adhesion and migration has been established (121, 140, 167, 169, 179). Mutated MARCKS, where the myristoyl moiety is replaced by a palmitoyl moiety, interferes with the “MARCKS myristoyl-electrostatic switch mechanism”. This results in retraction of fibroblasts spreading on a fibronectin substrate. The palmitoylated MARCKS construct also interfered with early stages of cell spreading; this observation was confirmed by a round morphology with multiple membrane blebs. Additionally, fibroblasts expressing palmitoylated MARCKS exhibited

decreased numbers of membrane ruffles and lamellae at the leading edge of the cell. Thus, results from this study suggested that the defect was due to the inability of the cell to adhere to a fibronectin substrate (167).

A role for MARCKS in cell migration has been elucidated in myoblast cells. Myoblasts express either wild type or non-myristoylated MARCKS and were capable of spreading on fibronectin, but myoblasts expressing MARCKS PSD null mutants were unable to spread on fibronectin. This observation suggested that the combination of phosphorylation and bi-lateral translocation of MARCKS was required for cell migration.

An additional mechanism of MARCKS dependent cell migration occurs through stimulation of platelet derived growth factor (PDGF-BB). After stimulation with PDGF-BB, MARCKS shown to translocates from membrane to cytosol in Swiss 3T3 fibroblasts (180). Further, upon PDGF-BB stimulation in hepatic stellate cells PKCε phosphorylated MARCKS. Additionally, MARCKS has been shown to associate with the PDGF-BB receptor but not focal adhesion kinase either in non-stimulated and stimulated hepatic stellate cells. However, the non-stimulated hepatic stellate cells showed greater association of MARCKS with PDGF-BB receptor. Overexpression of MARCKS in hepatic stellate cells resulted in decreased PDGF-BB mediated chemotaxis, whereas silencing of MARCKS using siRNA increased PDGF-BB mediated chemotaxis (160). These data support the concept that bi-lateral translocation of MARCKS is needed for the regulation of cell migration. Additionally, data also suggests that in migratory cells MARCKS is involved in stabilizing the actin

cytoskeletal structure. However, MARCKS needs to be phosphorylated within PSD in order to promote cell migration and reorganize the actin cytoskeleton.

Contrary to the above mentioned observations, overexpression of MARCKS in cholangiocarcinoma cells (181) increased metastasis and decreased the host survival. Endogenous MARCKS overexpression and siRNA against MARCKS increased cell attachment and reduced cell attachment, respectively. Furthermore, in an experiment where cholangiocarcinoma cells were transfected with siRNA control and siRNA against MARCKS followed by treated of PKC inhibitor and TPA (translational inducer). The results showed inhibition of cell migration compared to pretreatment with TPA alone. This experiment suggested that MARCKS is need for migration and metastasis of cholangiocarcinoma cells in a PKC phosphorylation dependent manner (182).

As CELF1 is well-known for its post-transcriptional role in various cancer cells, we are particularly interested in how CELF1 controls mRNA translation in oral cancer cells. First, we have adapted a pSILAC technique to understand the proteome-wide translational effect of CELF1. From our preliminary data, we have identified a novel target, MARCKS. Second, MARCKS involvement in cell migration lead us to the following hypothesis that overexpression of CELF1 controls cell motility in oral cancer cells through controlling the expression of MARCKS. To support our hypothesis, we have designed the following specific aims: **1)** we will determine whether CELF1 regulates MARCKS translation in oral cancer cells and **2)** we will determine the biological role of MARCKS in oral cancer cells.

Chapter 2

Methods and Materials

Cell Culture

Cell cultures were maintained in humidified atmosphere with 5% CO_2 at 37°C. The human oral cancer cell lines (HOK, UM74B, UM11A, UM11B, UM22A and UM22B) were maintained in Dulbecco's modified Eagle medium (DMEM-Hyclone, Logan, UT) containing 10% fetal bovine serum (FBS) (Seradigm, Randor, PA) with 100 U/ml penicillin and 100 µg/ml streptomycin (Hyclone, Logan, UT). Normal human oral keratinocyte cells (HOKs) (ScienCell, Carlsbad, CA) and non-malignant immortalized oral keratinocytes (OHKC/OKF6-TERT) were grown in keratinocyte serum-free medium supplemented with Bovine pituitary extract (BPE) and epidermal growth factor (EGF) (Gibco, Grand Island, NY).

Preparation of shRNA lentivirus particles

Cells were plated and after 6-8 hrs of incubation, shcontrol and shRNA directed against CELF1 were transduced at 50 MOI (multiplicity of infection) (viral particles were prepared by Dr. Reniqua House) in DMEM medium with 1 µg/mL polybrene final concentration. After 16-18hrs of incubation, the medium was replaced with fresh media. Cell were further incubated for 96-120 hrs and collected for further analysis.

Pulsed stable isotope labeling of amino acids in cell culture (pSILAC)

Twenty-four hours after transfection of UM74B cells with siRNA, the medium was changed to DMEM containing L-Lysine-2HCl and L-Arginine-HCl (light amino acids) for 48 hours (Pierce-DMEM SILAC quantitation kit). After 48 hours in the presence of light amino acids the medium on siControl treated cells was changed to DMEM containing L-Lysine-2HCl, 4,4,5,5-D4 (medium amino acids) and the medium

on siCELF1 treated cells was changed to DMEM containing L-Lysine-2HCl, 13C6, 15N2 (heavy amino acids) for 24 hours. Cells were lysed in 9 M Urea and 20 mM HEPES, pH 8.0 and rocked for 4hours at 4ºC. Lysates were combined at a 1:1 ratio (200µg of each sample) and send for mass spectrometry analysis (Unpublished observation from *House et al*) (183).

LC MS/MS Analysis—Orbitrap Elite w/ETD

Peptides were separated with a linear gradient of 5–50% buffer B (95% ACN and 0.2% formic acid) at a flow rate of 200nL/min on a C18-reversed phase column (75 mm ID _15 cm) packed in-house with Waters YMC-ODS C18-AQ 5 mm resin in buffer A (0.2% formic acid/95% water/5% Acetonitrile). A Dionex U3000 nano-LC chromatography system (Thermo Scientific) was on-line coupled to the Orbitrap Elite instrument (Thermo Scientific) via a Nanospray Flex Ion Source (Thermo Scientific). MS data were acquired in a data-dependent strategy selecting the fragmentation events based on the precursor abundance in the survey scan (400–1700 Th). The resolution of the survey scan was 60,000 at *m*/*z* 400 Th with a target value of 1e6 ions and 1 microscan. Low resolution CID MS/MS spectra were acquired with a target value of 5000 ions in normal CID scan mode. MS/MS acquisition in the linear ion trap was partially carried out in parallel to the survey scan in the Orbitrap analyzer by using the preview mode (first 192 ms of the MS transient). The maximum injection time for MS/MS was 100 ms. Dynamic exclusion was 120 s and early expiration was enabled. The isolation window for MS/MS fragmentation was set to 2 Th.

Database Search and Quantitation

The three triple pSILAC experiments were searched and quantitated together as 3 experiments using Maxquant v 1.4.1.2. The Human IPI v3.72 database was used. Static modification of carbomidomethyl on cysteines and variable modifications of methionine oxidation were included. All protein identifications were determined with a 1% FDR as determined by Maxquant.

Polysome gradient analysis

Cells were lysed in hypotonic lysis buffer (100 nM Tris-Cl pH 7.4, 5 mM MgCl2, 1% Triton X-100, 0.5% deoxycholate, 2 mM DTT, prepared in DEPC-treated water) and equivalent amount of protein were overlayed onto a sucrose gradient (10-50% (w/v) (increment of 10%), 100 nM KCl, 5 mM MgCl2, 2 mM DTT, 20 mM HEPES-KOH pH 7.4, prepared in DEPC-treated water). Gradients were centrifuged at 35,000 rpm for 3 hours at 4°C in an ultracentrifuge using Ti-40 rotor. Twenty to twenty-five 600uL fractions were collected and RNA was extracted using TRIzol® reagent (Ambion, Grand Island, NY) according to the manufacturer's protocol. Extracted RNA was run on a 0.8% agarose gel (10 mL of 10x MOPS, 62 mL of DEPC treated water and add 18 mL of 37% formaldehyde once the agarose is dissolved in the solution) (Sigma, St. Louis, MO). Afterwards, based on separation of tRNA, 40S, 60S, 80S, and multiple polysome (18S and 28S ribosome subunits), fractions were combined and run on a 1% agrose gel. Complementary DNA was synthesized for combined fractions, using Tetro cDNA Synthesis kit oligo dT primers (BIOline, Taunton, MA). Semi quantitative RT-PCR was run for the top 18 genes associated with CELF1 (Table 1). Polysome fractionation analysis was standardized

with help of Dr. Phil Howe's laboratory in the Department of Biochemistry and Molecular Biology.

Semi quantitative RT-PCR reaction of RNA isolated from polysome fractions

Total RNA was isolated from cells using Trizol according to manufacturer's protocol (Ambion, Grand Island, NY). Reverse transcription was performed using the Tetro cDNA Synthesis kit (BIOline, Taunton, MA). Quantitative PCR was performed using the Eppendorf vepo-protect instrument and cycle conditions were set as follow: 1st step: Initial 95° of denaturation for 10min, 2nd step: additional 5min of denaturation at 95°, 3rd step: annealing of primers at 64° for 30 seconds, 4th step: primer extension at 72° for 30sec and steps 2-4 repeats for 30 times). Briefly, reactions were prepared using 10 ul of total RNA from each pooled fraction, MY Taq mix (Bioline, Taunton, MA) and human-specific primers for MARCKS (IDT Technology, Coralville, IA) (Sense: 5′- CCC ACA GAT CCC ATC TCA AAT C-3′, anti-sense: 5′-GAG AAA CAA GGC AGA GGA AGA A -3′). All samples were run in triplicate and normalized to GAPDH (Sigma, St. Louis, MO) (Sense: 5′-GGT GGT CTC CTC TGA CTT CAA CA-3′, anti-sense: 5′-GTT GCT GTA GCC AAA TTC GTT GT-3′). Briefly, 1μL of cDNA, 4μL primer (1μM final concentration), 5μl DEPC treated water and 10μl of 2X MyTaq mix for 20ul final reaction.

siRNA transfection (MARCKS)

Cells were plated at twenty percent confluency. After incubating for 6-8 hours, either siControl (20nM; GTTCAATTGTCTACGCTA) or on-TARGETplus SMARTpool siRNA-targeting MARCKS (20nM; GAG AAG GCG GUG AGG CUG A, GAA GGU AAA CGG CGA CGC U, CAU AGG AACU UUU CACU UA and AAA UUG AAG

UGG UGC AUA A) (Dharmacon RNAi Technologies) was transfected (17 hrs) using HiPerfect as the transfection reagent (Qiagen, Valencia, CA), following the manufacturer's protocol.

Western Blot analysis

Cells were lysed on ice by vortexing 4–5 times in RIPA buffer [2 mM TRIS-HCl, 30 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, complete protease inhibitor cocktail (Boston Scientific, Marlborough, MA), 1% NP-40, pH 7.6] at 5 min intervals, followed by centrifugation at 12,000 × *g* for 30 min at 4°C. Supernatants were mixed with an equal volume of 2X Laemmli's buffer and heated for 5 min at 95°C. Total protein concentration was estimated using a Bradford assay. Forty to fifty micrograms of total protein was resolved on 10% SDS-PAGE gels for 1 hour at 110 volts and transferred onto a PVDF membrane (EMDmillipore, Billerica, MA) for 45 minutes at 15 volts using a semidry transfer system. Membranes were pre-incubated for 1 hr with 5% skim milk (block) prior to incubation with primary antibody against the target protein overnight at 4°C. After overnight incubation, the membrane was washed three times with tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with 1% skim milk containing 1:5,000 diluted HRP-conjugated secondary antibodies for 1 hr at room temperature. After additional washing with TBST, immune complexes were visualized using the ECL system (Pierce). Blots were re-probed with anti-β-actin antibody as described above. Western blot analyses were performed by using antibodies specific to CELF1 (1:1000) (EMDmillipore, Billerica, MA), MARCKS (Cell Signaling, Danvers, MA) (1:1000) and β-actin (Sigma, St. Louis, MO) (1:5000).

RNA extraction and Quantitative Real-time PCR (qRT-PCR)

Total RNA was prepared from HNSCC cell lines using the RNeasy mini kit (Qiagen, Valencia, CA)). qRT-PCR for all mRNA targets were performed using an Applied Biosystems StepOne Plus system with the iScript cDNA synthesis kit (BioRad). Briefly, one microgram of total RNA was used for cDNA synthesis. cDNA, was diluted 1:5 for qPCR. Quantative PCR reaction was as follows: 1µL of cDNA (from 1:5 diluted stock), 1µL of primer (5uM stock primers) and 5µL of 2X Syber green dye (Final concentraiton of 1X) (Qiagen, Valencia, CA). CELF1 primers sequecne as follow: sense: 5′-CCA GAC AAC CAG ATC TTG ATG CT-3′, anti-sense: 5′-AGG TTT CAT CTG TAT AGG GTG ATG-3′. MARCKS and GAPDH sequences are identical to sequences that are mentioned in the semi quantitative RT-PCR reaction section.

Ribonucleoprotein immunoprecipitation (RNP- IP)

CELF1 antibody *(EMDmillipore) (*4 µg /1mg cell extract) was coated onto 50-60 µL protein G agarose beads per 1 mg of whole cell extract in a total of 1mL NT2 buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, and 0.5% NP-40). UM74B cell pellets were resuspened in polysome lysis buffer (100mM HEPES pH7, 1 M KCl, 50 mM MgCl2, 0.25 M EDTA, and 5% NP-40) and placed in -80°C overnight. Antibody-bead complexes were allowed to form overnight at 4°C on a rotating device. Next day, lysates were cleared by centrifugation at 14,000 rpm for 10 min. Antibody coated beads were washed 4-6 times with NT2 buffer. For each IP, the antibody coated beads were resuspended in 850 µL NET2 buffer (850 µL NT2, 10 µL 0.1 M DTT, 30 µL 0.5 M EDTA, 2.5 µl RNase OUT, and 5 µL SuperaseIN) and 100 µL RNP lysate

was added, centrifuged briefly, and a 100µl aliquot was removed for input purpose. The RNP lysate-bead complexes were rotated overnight at 4°C. On day three, the IP material was centrifuged at 3000g for 3 min and the beads were washed with NT2 buffer 4-6 times. To dissociate the RNP complex from the antibody coated beads, the beads were resuspended in 100µl of NET2 and 100µl of 2X proteinase K buffer (20 mM Tris-Cl pH 7.5, 20 mM EDTA, 100 mM NaCl, 2% SDS, stored at room temperature), after which 3µl of proteinase K (Sigma, St. Louis, MO) was added and the solution was incubated at 55°C for 30min with occasional mixing. Afterwards, the supernatant was transferred into new tubes. Finally, RNA was isolated using Trizol (Life Technologies, Carlsbad, CA).

Overexpression of GFP-tagged MARCKS

The GFP-tagged MARCKS plasmid was purchased from OriGene (OriGene, Rockville, MD). UM74B cells ($1x10^5$) were seeded in a 24-well dish and transfected with 0.25 µg of MARCKS-GFP tag or GFP empty vector using 0.5-1uL of liptofactamine 2000 (Life Technologies, Carlsbad, CA).

Wound healing assay (scratch assay)

OHKC, OHKC CELF1 Flag tag (stable cell lines), UM74B MARCKS-GFP expressing cells were seeded in a 6-well plate. Wounds were created on cell monolayers using a 10µL pipette tip. At 0hr and 24hrs, images were captured using Nikon Eclipse TS100 with NIS-Element Br3.2 software (Melville, NY). Wound closure was quantified using ImageJ software.

Transwell migration assay

Fifty thousand cells of shRNA control plus GFP, depleted CELF1 plus GFP and depleted CELF1 with plus MARCKS-GFP were resuspended in 5 µL of serum-free medium containing 100 U/mL penicillin and 100 µg/mL streptomycin and plated into the upper well of a 24-well transwell (Corning). Transwells were placed into a 24-well plate containing complete medium. Cells were allowed to migrate for 24 hrs. Cells were stained with Hoechst 33442 for 30 minutes and the transwell membranes were mounted on to slides. Migrated cells were counted in 4 random fields of view at 10x magnification using a fluorescent microscope. Percent migrated cells were calculated by ImageJ software.

Statistical Analysis

All the experiments were conducted in triplicate analysis with mean and standard deviation was calculated. A student t-test was performed for most of the studies, with two-tailed distribution and a two-sample unequal variance. A p-value of < 0.05, indicating significant change, denoted by asterisk (*).

Chapter 3

CELF1 controls proteome-wide gene expression patterns

Introduction:

RNA-binding proteins are known to be involved in the regulation of gene expression at post-transcriptional levels, but they do not act alone, instead they function as a ribonucleoprotein complex with RBPs and mRNAs. CELF1 coordinately control the expression of multiple target genes at the posttranscriptional level. CELF1 was formerly demonstrated as an multifunctional protein that regulates many post-transcriptional processes including alternative splicing, mRNA deadenylation, decay and some extend translation (184). Several reports indicated that CELF1 play a key role in mRNA turnover, but its role in global mRNA translation remains largely unidentified.

Previous work from our laboratory reported that overexpression of CELF1 in head and neck tumor tissues compared to normal adjacent tissue samples (185), suggesting that overexpressed CELF1 play a role in oral cancer progression. In addition, unpublished observations from our laboratory indicate that CELF1 is capable of controlling the splicing and turnover of approximately 1500 mRNAs and translation of over 1350 mRNAs. Since there are no studies showing CELF1's role in global mRNA translation in cancer model, we were particularly interested in understanding CELF1's role in mRNA translation in OSCC cells. To identify the CELF1-mediated global mRNA translation control, we have adapted a novel proteomics approach called the pulsed stable isotope labeling of amino acids in cell culture (pSILAC) using oral cancer cells (Schematic 1). pSILAC is a technique based on mass spectrometry that detects difference in protein abundance among different conditioned samples using non-radioactive isotopic labeling (186). Using pSILAC we

have identified approximately 1350 genes that were controlled by CELF1. Further analysis of the pSILAC data revealed a novel gene; myristoylated alanine-rich C-kinase substrate (MARCKS) which was significantly affected by the absence of CELF1.Hence, we wish to determine how CELF1 controls MARCKS at the post-transcriptional level in OSCC cells.

Results:

Specific Aim #1: To determine whether CELF1 controls MARCKS translation in oral cancer cells.

Task 1: Determine the mRNA and protein expressions of CELF1 and MARCKS in multiple OSCC cells (UM74B, UM11A, UM11B, UM22A & UM22B) by qRT-PCR and Western blotting analysis.

Task 2: Determine if CELF1 expression proportionately correlates with MARCKS expression through Western blot analysis in UM74B and UM22B cells.

Task 3: Verification of the pSILAC data using polysome gradient analysis and semi-quantitative PCR followed by agarose gel analysis.

Task 4: Establish if CELF1 specifically controls MARCKS expression at the mRNA translational level, by measuring *MARCKS* mRNA in CELF1 depleted cells.

Task 5: Determine the association between CELF1 and *MARCKS* mRNA through RNA- immunoprecipitation (RNA-IP) assay.

First, unpublished results from our laboratory revealed that CELF1 controls the translation of several target mRNAs in oral cancer cells. Second, we have used the following criteria to select a subset of proteins from our pSILAC experiment for further evaluation; 1) top altered proteins identified based on statistical analysis, 2) mRNAs that contain GRE's at their 5′ or 3′ UTRs, and 3) top proteins that are involved in cancer cell biological processes. Figure 1 demonstrates that upon depletion of CELF1 in UM74B cells, only 56 proteins were up-regulated, but 1294 proteins were down-regulated in comparison to control cells, suggesting that CELF1 is a global translation regulator in OSCC cells. Third, enrichment analysis of pSILAC data indicate that diverse sets of genes are controlled by CELF1, and their coding proteins are involved in biological functions such as mRNA surveillance, RNA binding and translation (Figure 2). Based on the pSILAC proteome-wide analysis, we have identified top18 proteins coding mRNAs contains canonical GRE's at their UTRs (Table 1). Using Kyoto encyclopedia of genes and genomes (KEGG) pathway for biological function, UCSC genome browser database for GRE sequence search, and polysome fractionation gradient analysis for mRNA translation, we have identified MARCKS as a target of CELF1 in oral cancer cells (Table 1 and Figure 8). Thus, the specific aim-1 of this thesis is planned to test whether CELF1 regulates MARCKS mRNA translation in oral cancer cells. Therefore, to determine the expression level of CELF1 and MARCKS in oral cancer cells, first, we have tested the protein levels of MARCKS and CELF1 in oral cancer cells in comparison with primary normal human oral keratinocytes (HOK). Our Western blot analysis revealed that the protein expression-fold change of MARCKS and CELF1 are greater than 2

fold in UM74B, UM11A, UM11B, UM22A, and UM22B oral cancer cells compare to HOK cells (Figure 3). Interestingly, most of the oral cancer cells did not exhibit significant differences in CELF1 and *MARCKS* mRNA expression compared to HOK cells (Figure 4), exceptions are UM11B and UM22A cell lines. Altogether the results obtained from Figures 3 and 4 indicated that MARCKS protein expression was relatively higher compared to its mRNA levels in OSCC cells. Which allows us to conclude that MARCKS may be regulated at the post-transcriptional level, in particular at the mRNA translation step in oral cancer cells.

Next, we sought out to validate our pSILAC data using the Western blotting technique. First, CELF1 was depleted in UM74B and UM22B cell lines using shRNA directed against CELF1 with appropriate shRNA control lentiviral particles. Ninety-six hours of post-viral infection, cells were collected and proteins were extracted for Western blot analyses. The data obtained from this study indicated that both UM74B and UM22B cells exhibited reduced MARCKS expression in CELF1 depleted cells compared to control shRNA treated cells (Figure 5). We observed 80% depletion of CELF1 reduced MARCKS expression by 48% in UM74B cells (Figure 5) and a 60% reduction in CELF1 in UM22B cells resulted in a 58% decrease in MARCKS expression (Figure 6). Thus, the data obtained from these studies demonstrated that reduction of CELF1 reduced the expression of MARCKS at the protein level in oral cancer cells.

To further confirm the data obtained from pSILAC and Western blot analysis of MARCKS, we have utilized polysome fraction gradient analysis to understand CELF1's role in mRNA translation of MARCKS. First, we treated the UM74B cells

with either control shRNA or shRNA against CELF1 to knockdown CELF1. Next, we have extracted the protein lysates for polysome gradient analysis. The fractions obtained from polysome gradient were subjected to RNA extraction to test the relative expression levels of different RNA species. As expected, we have observed a separation of tRNA (free flowing RNA), 40S (ribosomal subunit), 60S (ribosomal subunit), 80S/monosome subunits (complex of 40S and 60S ribosomal subunits) and multiple polysomes in shRNA control and shRNA CELF1-treated cell polysome fractions (Figure 7, top panel). The bottom panel in Figure 7 illustrates the schematics of the polysome gradient profiles. Next, we used semi-quantitative PCR analysis of combined polysome fractions to estimate the mRNAs undergoing active translation in oral cancer cells. The RNA extracted from polysome fractions were subjected to estimate MARCKS mRNA by using gene specific primers. The cDNA was prepared from total RNA and subjected to semi-quantitative PCR for measuring MARCKS mRNA level. The PCR products were separated by using agarose gel and the relative expression of MARCKS mRNA was measured. The results obtained from this experiment indicated that there is reduction of MARCKS mRNA in active translation fractions of shRNA CELF1 treated samples in comparison to active translation fractions in cells treated with shRNA control (Figure 8). Thus, the data obtained from this section of the study indicated that CELF1 directly controls the expression of MARCKS at the mRNA translation level.

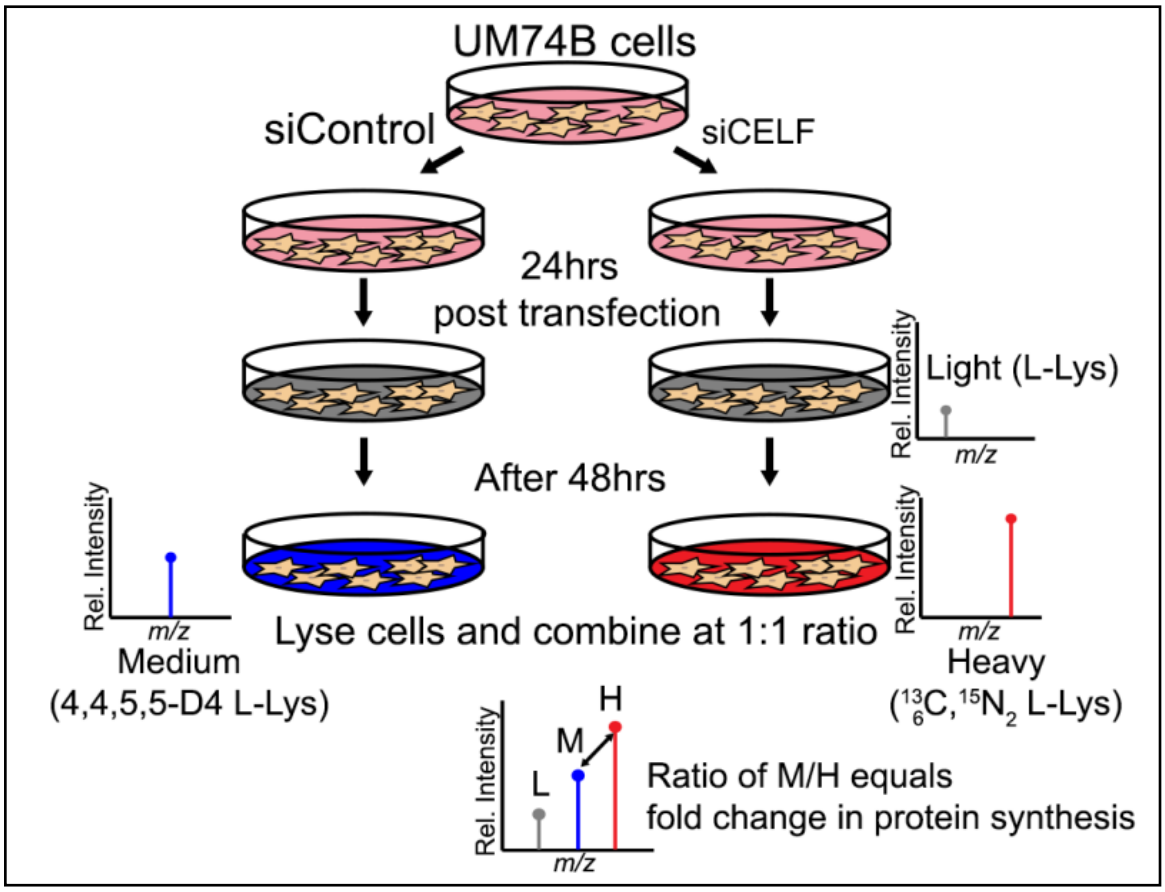
Next, to test whether CELF1 is controlling MARCKS translation through its mRNA levels, we measured the relative expression level of *MARCKS* mRNA in CELF1 depleted UM74B and UM22B cell lines. Both cell lines were treated with

either shRNA control or shRNA against CELF1 viral particles and 96 hours post-transduction the lysates were prepared for RNA extraction and analyzed by qRT-PCR. The results indicated that reduction of CELF1 in UM74B and UM22B cells did not significantly alter *MARCKS* mRNA levels compared to shRNA control cells (Figure 9 and 10). This observation clearly demonstrated that CELF1 controls MARCKS protein expression at the mRNA translation level but not at the expression of mRNA. Finally, to determine if CELF1 directly bound to *MARCKS* mRNA and control its translation, we performed RNP-IP assays using an anti-CELF1 antibody and an IgG control. We have observed a 3.8 fold enrichment of *MARCKS* mRNA in CELF1 immunolysates (Figure 11) compared with IgG control beads. These data suggest that CELF1 directly binds *MARCKS* mRNA and promotes its translation in oral cancer cells.

In specific aim-1, we have now established that overexpression of CELF1 appears to regulates MARCKS expression in various oral cancer cell lines. Specifically, depletion of CELF1 in oral cancer cells reduced MARCKS protein expression, but did not changing the level of mRNA. Furthermore, RNP-IP assays revealed that CELF1 directly associated with MARCKS mRNA. In summary, taking all of these data in specific aim-1, we have now demonstrated that CELF1 controls MARCKS mRNA translation in oral cancer cells.

Proteins		GRE Clusters	
Up Regulated	**Fold Change**	**Cluster**	**3′ UTR GRE**
EPS8	2.27	Cluster 4	TTGTTTGTTTGTA
COL5A3	2.86		-
PTPN11	1.59		-
SPEN	1.96	Cluster 3	TTTTGTTTGTTTG
Down Regulated			
SNRPD3	-4.53	-	-
SUMF2	-4.42	-	-
LUZP1	-4.36	-	-
SFRS2	-3.88	-	-
AKR1D1	-3.53	Cluster 5	TGGTGTTTGTTTC
NCAPG	-3.40	Cluster 5	TTCTGTTTGTTGG
ATF7IP	-2.92	Cluster 5	GGGTGTTTGTGTG
ERCC5	-2.89	Cluster 5	TTGTGTTTGATGG
ADNP	-2.73	Cluster 5	TTTTGTTTGTTTT
EPS15	-2.65	Cluster 5	TTATGTTTGTTGT
COPZ1	-2.65	Cluster 5	TTCTGTTTGTGGT
STK10	-2.65	Cluster 5	TTGTGTTTGTTGT
MARCKS	-2.64	Cluster 4	GTTTGTTTGTTGG
CREBBP	-2.52	Cluster 5	TTTTGTTTGGGG

Table 1: Top genes showing significant differences in expression patterns by pSILAC analysis. These genes were selected based on their GRE motif in their 3' UTR.



Schematic 1: Illustration of pulsed stable isotope labeling of amino acids in cell culture (pSILAC) using UM74B oral cancer cells.

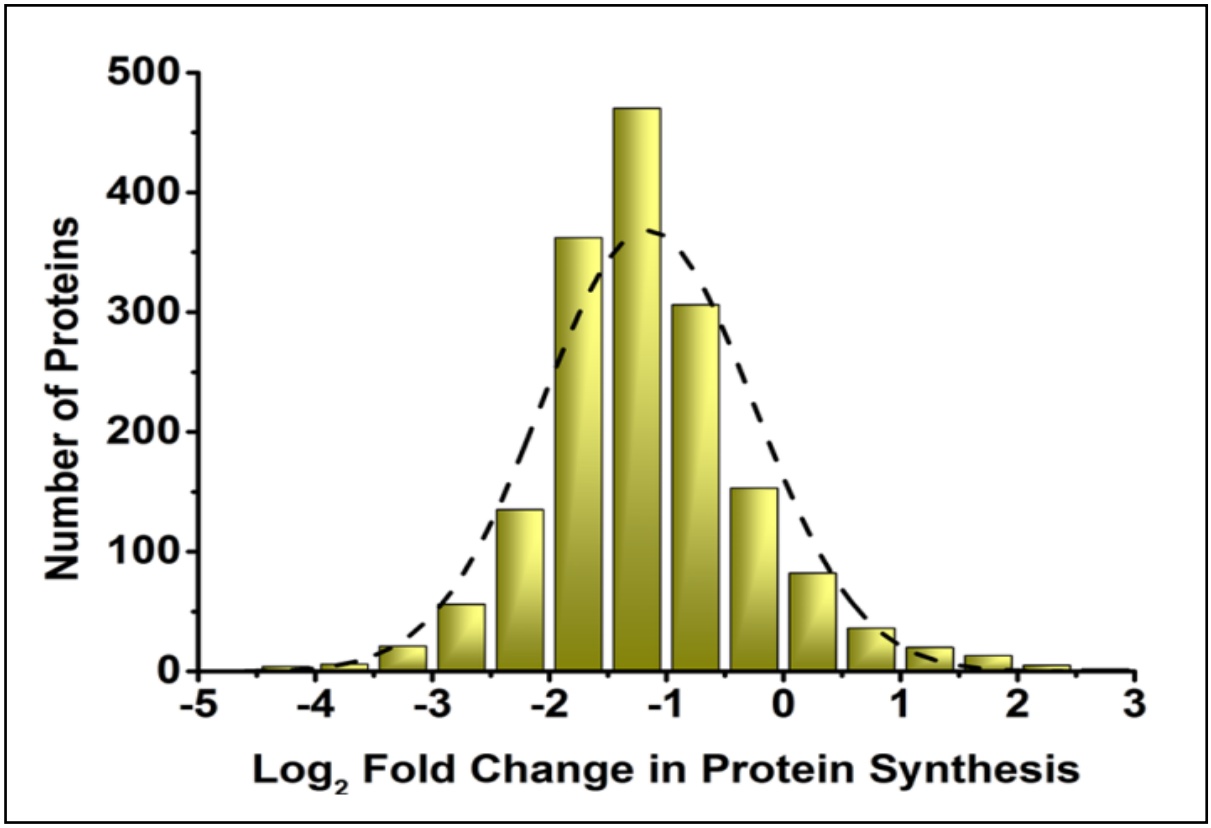


Figure 1: Histogram of log_2 fold change versus expression of proteins altered in the absence of CELF1 in UM74B oral cancer cells. In absence of CELF1, reduced global mRNA translation has been observed.

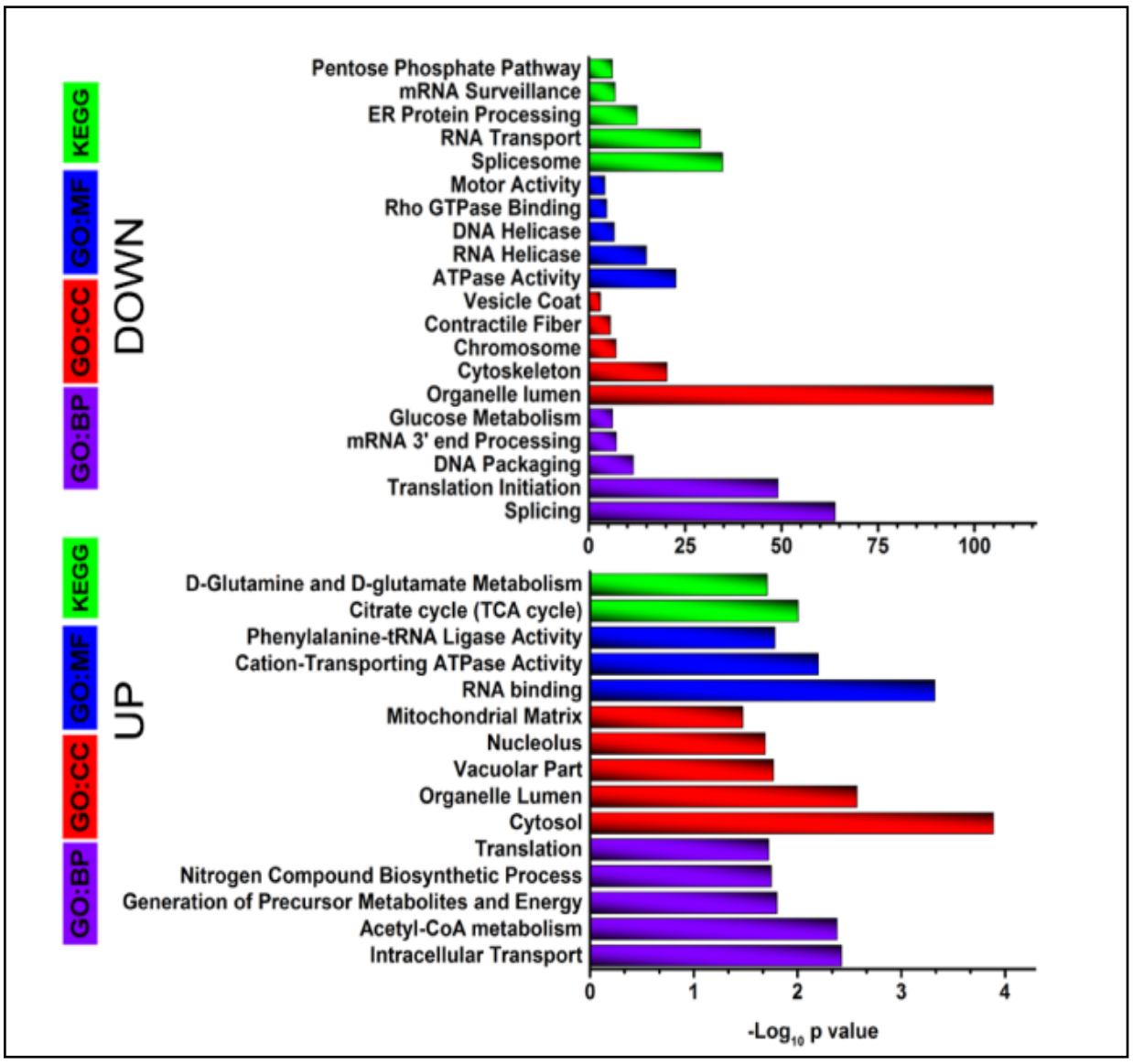


Figure 2: Enrichment analysis of CELF1 regulated proteins using cluego and cluepedia (cytoscape). The enrichment analysis of pSILAC has identified CELF1's role in various cell processes, including mRNA surveillance, cytoskeleton reorganization, mRNA 3' end processing and translation. The three triple pSILAC experiments were searched and quantitated together as 3 experiments using Maxquant.

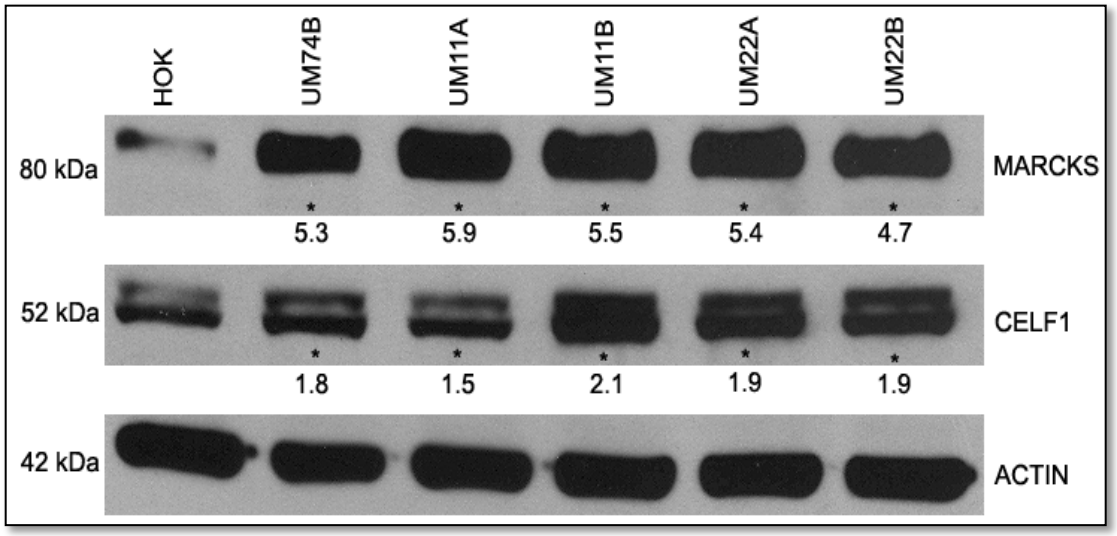


Figure 3: Western blot analysis of MARCKS and CELF1 in oral cancer cell lines in comparison to normal human oral keratinocytes. Beta-actin serves as a loading control. Double band of CELF1 reflects possible post-translational modification. N=2, *$p<0.05$.

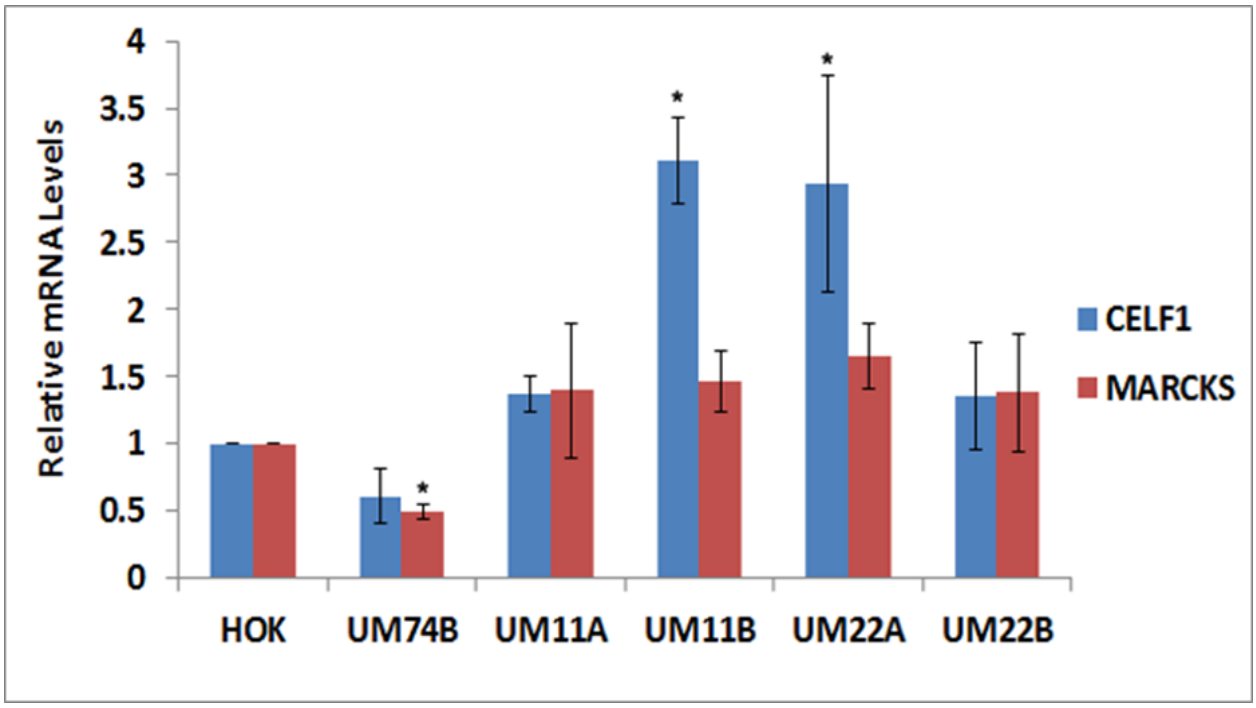
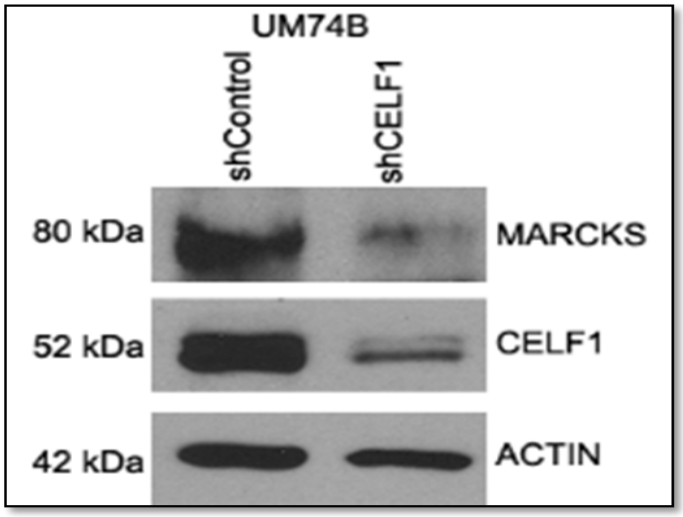


Figure 4: Relative quantity of mRNAs measured with qRT-PCR in oral cancer cells. CELF1 mRNA expression was significantly altered in UM11B and UM22A; additionally, MARCKS mRNA expression was also significantly altered in UM74B in comparison to HOK cells. N=3, MARCKS: *p<0.027 (UM74B); CELF1; CELF1: *p<0.005 (UM11B), *p<0.014 (UM22A).



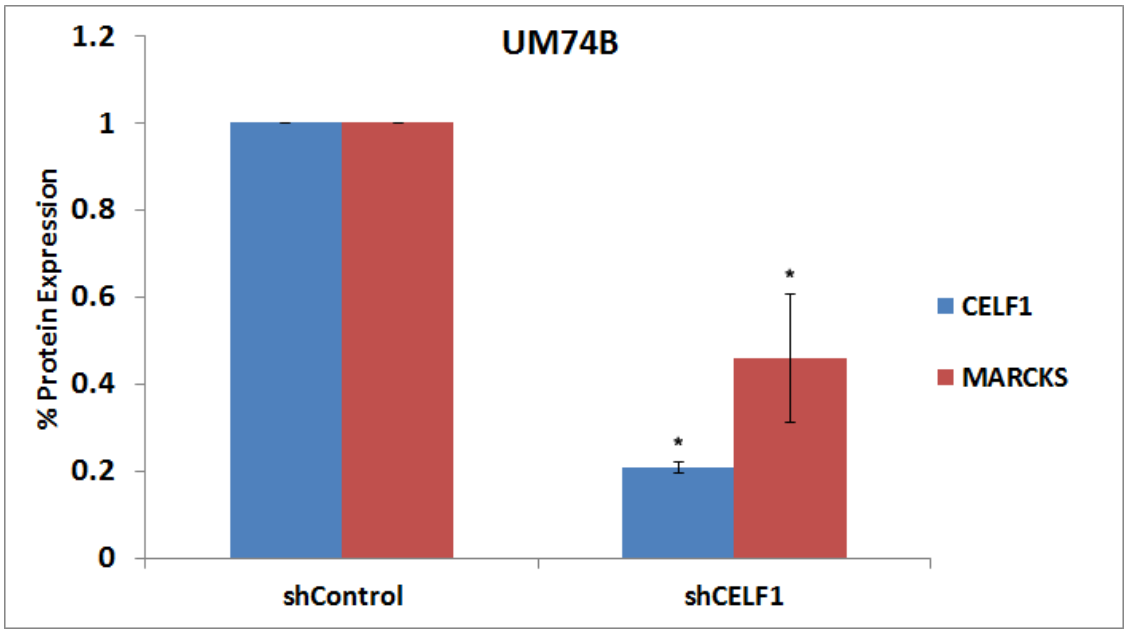
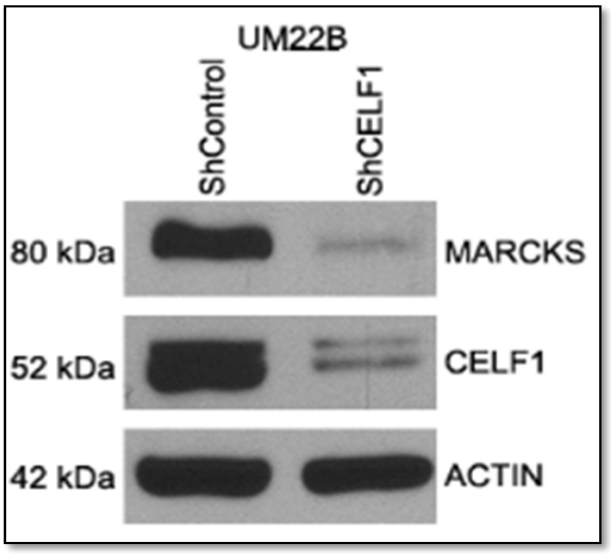


Figure 5: Reduction of CELF1 in UM74B cells influences MARKCS protein expression. Western blot analysis of CELF1 and MARCKS in oral cancer cells, and beta-actin serves as a loading control. Bottom graph depicts quantitative values of representative Western blots expression levels of CELF1 and MARCKS. N=3, *p<0.001 CELF1, *p< 0.011 MARCKS.



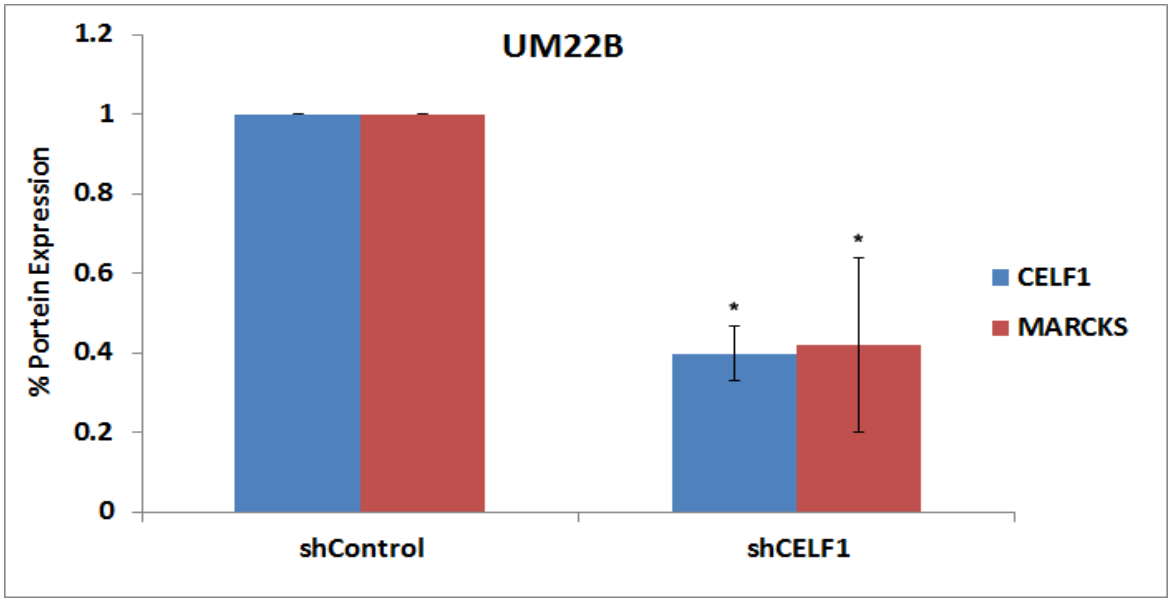


Figure 6: Reduction of CELF1 in UM22B cells influences MARKCS protein expression. Western blot analysis of CELF1 and MARCKS in oral cancer cells, and beta-actin serves as a loading control. Bottom graph depicts quantitative values of representative Western blots expression levels of CELF1 and MARCKSN=3, *p<0.018 CELF1, *p<0.001.

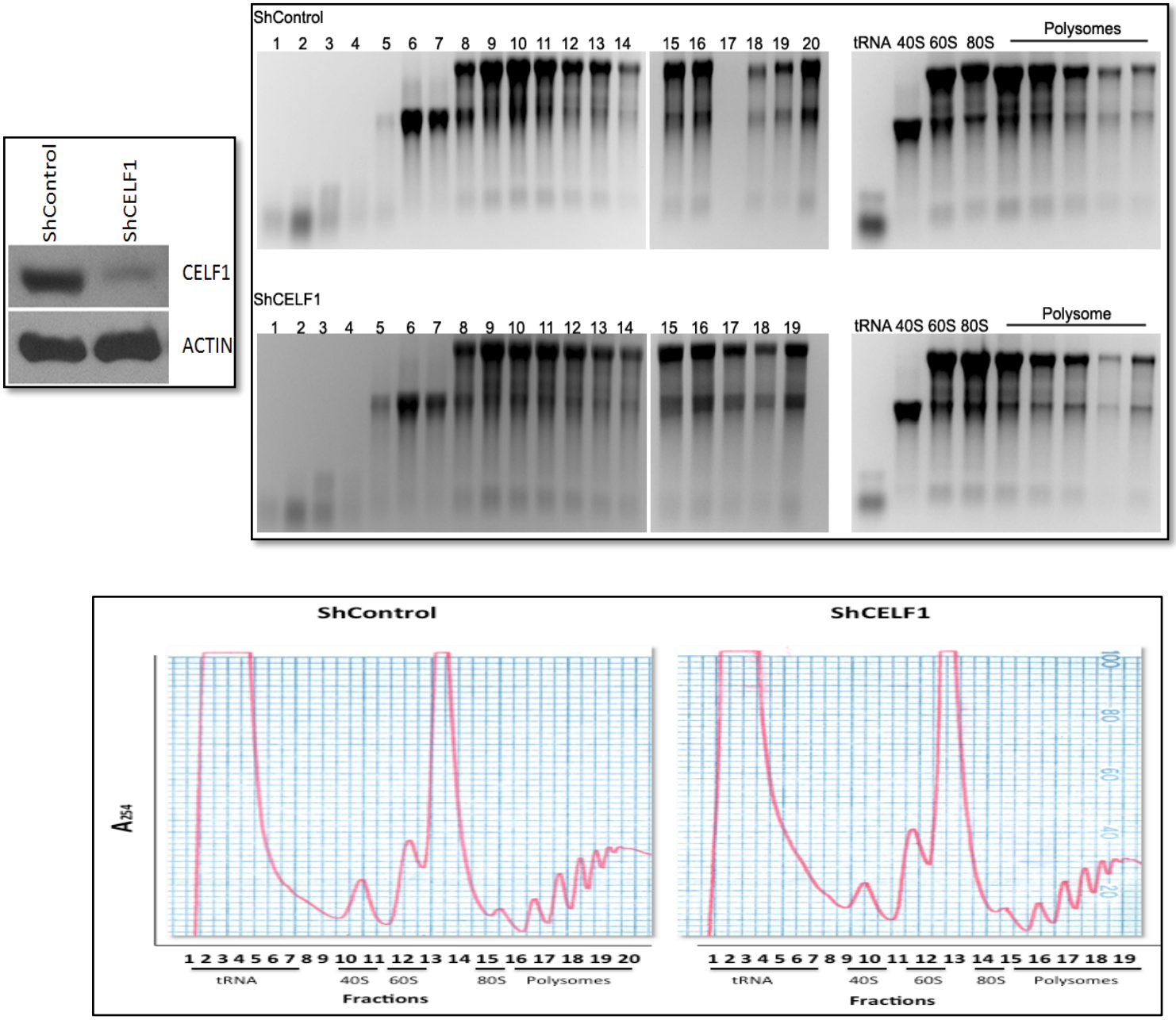


Figure 7, top: Polysome gradient fractionation analysis of CELF1 depleted cells. The standardization of polysome fractionation analysis have shown separation of free flowing RNA, 40S ribosome subunits, 60S ribosomes subunits, 80S subunits (non-active translation) and polysome (active translation). N=2.

Figure 7, bottom: Schematic of polysome fractionation profile analysis.

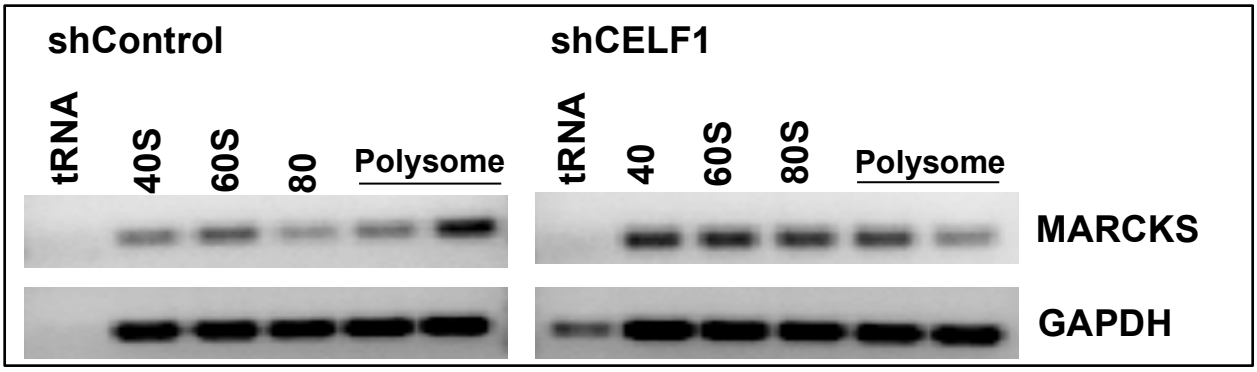


Figure 8: Semi-quantitative PCR analysis of CELF1 depleted UM74B polysome fractionations. The control samples shows lesser amount of MARCKS mRNA present in the non-active translation (free flowing tRNA, 40S, 60S and 80S fractions) in comparison to active translation (polyosme fractions). In the depleted samples, more MARCKS mRNA present in the non-active translation fractions compared to active translation fractions. (N=2).

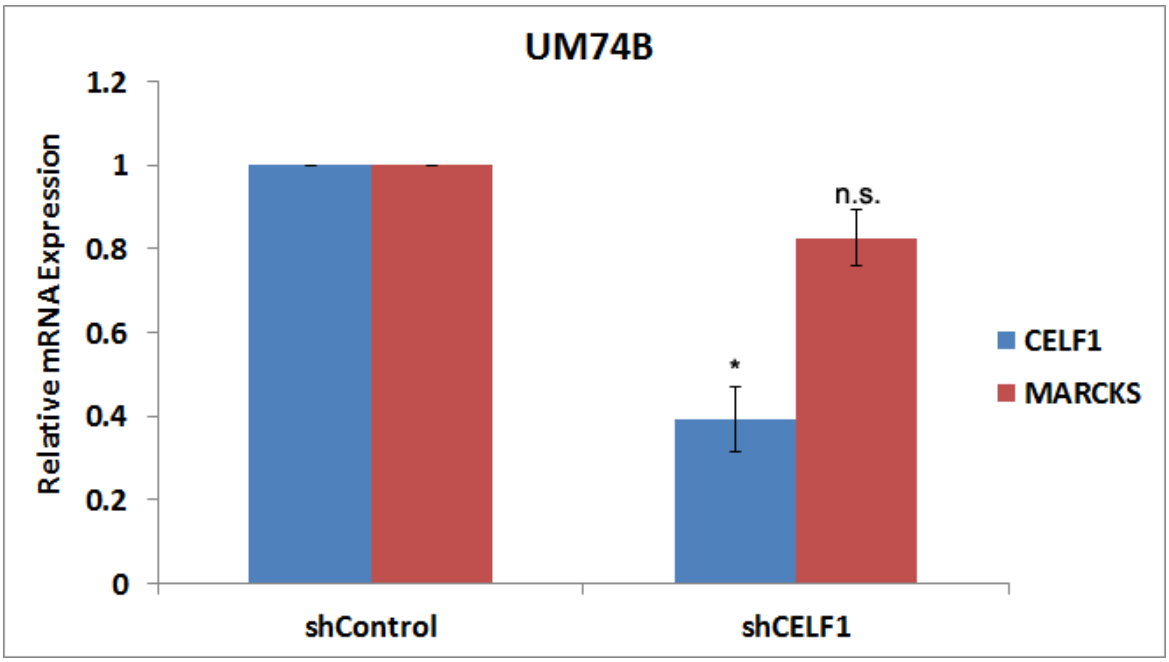


Figure 9: Depletion of CELF1 did not alter MARCKS mRNA in UM74B cells. qRT-PCR analysis of CELF1 and MARCKS in the absence of CELF1 oral cancer cells. Depletion of CELF1 does not alter MARCKS mRNA in UM74B. N=3, *p<0.004 CELF1, *p>0.528 MARCKS; n.s.= non-significant.

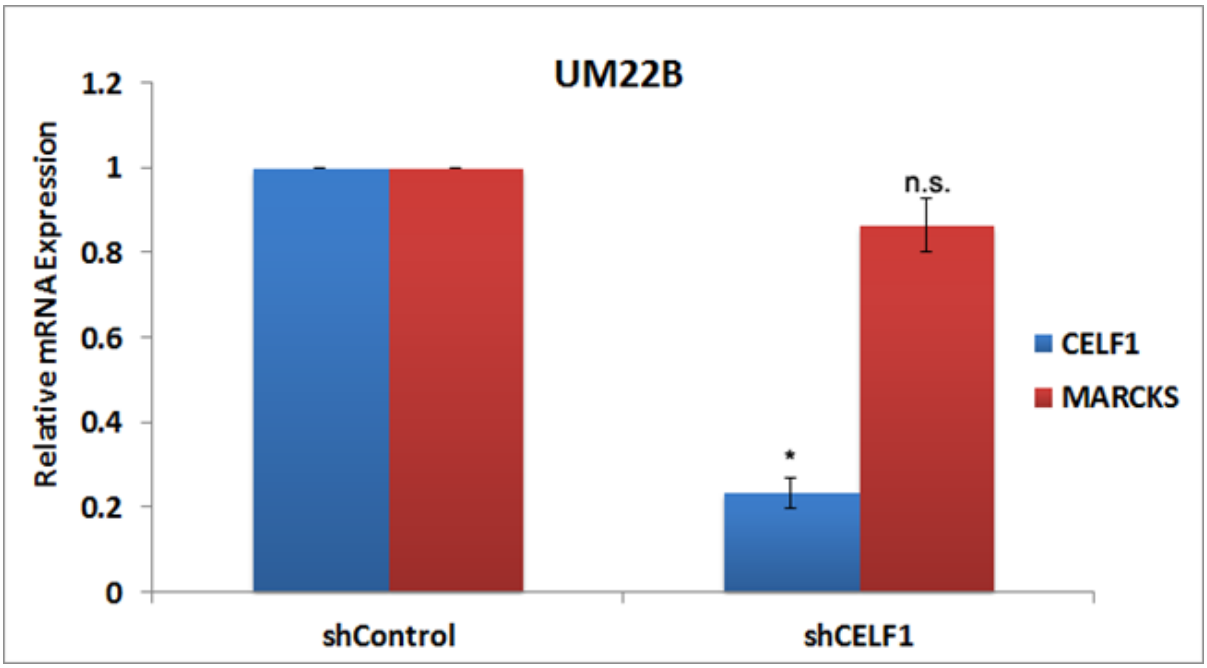


Figure 10: Knockdown of CELF1 did not alter the expression of MARCKS mRNA in UM22B cells. qRT-PCR analysis of CELF1 and MARCKS in the absence of CELF1 oral cancer cells. Depletion of CELF1 does not alter MARCKS mRNA in UM22B. N=3, *p<0.029 CELF1, p> 0.684 MARCKS; n.s.= non-significant.

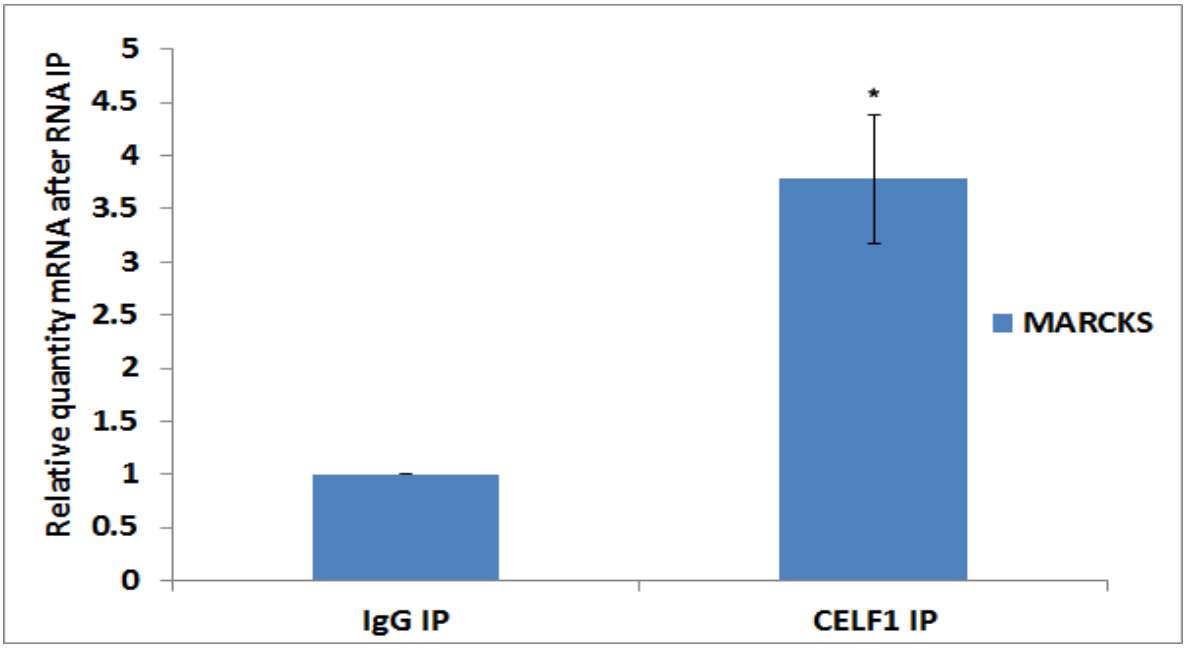


Figure 11: CELF1 associated with MARCKS mRNA identified by RNA-IP. The UM74B cell lysate was immunoprecipitated with IgG or anti-CELF1 antibody. The RNAs isolated from the total lysate and the immunoprecipitates (IP) were then anlauyzed by qRT-PCR. The qRT-PCR showed 3.8 fold enrichment in the Anti-CELF1 antibody in comparison to IgG control antibody. N=3, *p<0.05.

Discussion:

Gene expression is a tightly regulated and adaptive process that encompasses co- and post–transcriptional modifications and ultimately determines the fate of the mRNA. The mRNA translation is a key post-transcriptional regulatory process in eukaryotes, and deciphering CELF1-mediated protein translation provides an opportunity to dissect this pathway in cancer cells. Interestingly, at different stages of development, CELF1 can activate the translation of various mRNAs (102). For example, in *Drosophila* CELF1 protein called Bru forms a complex with protein CUP and the 5′ cap binding initiation factor, eIF4E, and initiates circularization of mRNA and inhibit its translation (104). In addition, interaction of CELF1 with hnRNP H at the two ends of the SHMT (folate-dependent enzyme) transcripts also aid in circularization and facilitate translation from an internal ribosome entry site (IRES) by increasing ribosome recycling (112, 113). Altogether, these studies support CELF1 as a regulator of mRNA translation in different cell systems.

From our pSILAC analysis we have identified MARCKS as a target of CELF1 protein at the mRNA translational level. Notably, CELF1 and MARCKS are overexpressed in oral cancer cells compared to normal oral keratinocytes (Figure 3), which demonstrate the importance in the coexistence of these proteins in oral cancer cells. Surprisingly, we did not see significant changes in the level of mRNA (Figure 4) encoding MARCKS in the absence of CELF1, indicate that MARCKS protein expression levels are post-transcriptionally regulated at the translation level.

Thus, CELF1's role in mRNA translation has been established, and the target MARCKS has been identified in oral cancer cells.

Specific deletion of CELF1 followed by measurement of MARCKS protein expression in UM74B and UM22B cell lines using Western blot analysis clearly established a relationship between CELF1 and MARCKS (Figure 5 and 6). In both of the cell lines, we have observed that deletion of CELF1 protein leads to significant reduction of MARCKS protein levels. In contrast to this observation, recent reports have indicated that CELF1 along with another RBP, HuR (Hu Antigen-R) are shown to regulate translation of *occluin* mRNA by binding at its 3′ UTR (187). Overexpression of CELF1 decreased the HuR binding to occluin mRNA and repressed its translation, whereas HuR overexpression inhibited CELF1 association with occluin mRNA and promoted its translation (187). These data suggests that RBPs copete with each other for binding and involved in the regulation of mRNA translation depending on the transcript. We assume that CELF1 possibly associates with the 3′ and/or 5′ UTR of *MARCKS* and controls its translation. Utilizing the UCSC genome browser database query, we were able to observe that the 3′ UTR of *MARCKS* contains the canonical GRE sequence UGUUUGUUUGU and the 5′ UTR contain several GU and U stretches, where CELF1 thought to bind and regulate its protein expression levels. Moreover, our RNP-IP analysis clearly demonstrated that CELF1 is strongly associated with *MARCKS* mRNA. As CELF1 was shown to bind to mRNAs containing UGU triplets (188), we have observed several triplets of UGU at the MARCKS 3′ UTR. In addition, CELF1 enhanced the translation of cyclin A2 through binding consensus sequences in its 3′ UTR, indicating that CELF1 also

targets the 3′ UTR of mRNAs to regulate its translation (102). Collectively, other reports mentioned above and our data suggests that CELF1 interact with MARCKS and controls its translation in oral cancer cells. Currently, to delineate the exact mechanism of CELF1 binding with MARCKS mRNA, we are investigating the *MARCKS* 3′ UTR association with CELF1 using the 3′ UTR reporter assays.

In order to strengthen the pSILAC data, we have adapted polysome fractionation analysis. The separation of tRNA (free flowing RNA), 40S (ribosomal subunit), 60S (ribosomal subunit), 80S/monosome subunits (complex of 40S and 60S ribosomal subunits) and multiple polysomes in shRNA control and shRNA CELF1 depleted cells were achieved (Figure 7, top panel). The separation of tRNA, 40S and 60S ribosome, 80S ribosome and multiple polysome fractions are important to determine the active vs. non-active translation of particular mRNA, with inactive translated mRNAs predominately found in the free flowing RNA, 40S, 60S and 80S and actively translated mRNAs found in multiple polysome fractions. The fractions were subjected to semi-quantitative PCR analysis and showed significant reduction of *MARCKS* mRNA in polysome fractions, of cells treated with shCELF1 compared to cells treated with shcontrol (Figure 8). The reduction of *MARCKS* mRNA in polysome fractions demonstrated that it was not undergoing active translation in the absence of CELF1. It has been shown that a model for the IRES-mediated translation of SHMT1 (serine hydroxymethyltransferase 1), whereby the circularization of the mRNA typically provided by the eukaryotic initiation factor (eIF) 4G/PABP/poly(A) tail interaction is achieved through the hnRNP H2/CELF1-mediated interaction of the 5′ and 3′ UTR of the SHMT1 transcripts (112). Our

observations indicate that CELF1 might interact with either the 3′ or 5′ UTR of MARCKS and possibly forms a circularization with mRNA and regulate its translation.

As CELF1- is controlling the expression of MARCKS, the changes in *MARCKS mRNA* turnover needs to be addressed. Interestingly, in the absence of CELF1 both UM74B and UM22B cells did not exhibit significant changes in the expression of *MARCKS* mRNA (Figure 10 and 11), instead it exhibits reduced MARCKS protein level, demonstrated that the CELF1 solely act on the mRNA translational level and promoted the expression of MARCKS. Under certain circumstances, the absence of one RBP might recruit additional RBPs to associate with target mRNAs. For example, a recent report indicated that CELF1 could bind to the 3′ UTR of *Myc* mRNA repressing its translation without affecting total Myc mRNA levels. Interestingly, HuR interacted with the same Myc 3′ UTR element, and increasing the level of HuR, decreased CELF1 binding to *Myc* mRNA (189). It has been shown that the 3′ UTR of MARCKS contains canonical AU-rich sequences (190), which are known to interact with HuR, but this observation needs further investigation. Thus, RBPs can interplay between each other to bind with mRNAs and regulate its translation in mammalian cells.

Chapter 4

CELF1 Regulates Cell Migration through MARCKS expression

Introduction:

Cell migration is an important for physiological processes that depend on regulated movement of cells, including immune responses, embryonic development and tissue maintenance and repair. In addition, alteration in cell motility plays a role in driving disease states such as; vascular disease, chronic inflammation and tumor metastasis (191). Tremendous efforts have been made to understand these aberrant changes in diseases. These efforts uncovered the current concept of cell migration, which is a cycle of several tightly regulated steps (191, 192). For example, directional movement of the cell is initiated by polarization of cells to migratory cues (191). Cell polarization, allows for the segregation of machinery that regulates the multiple states of migratory cycle (191). A migratory cycle consists of (1) the cell extending an actin cytoskeletal-rich protrusion that occurs in the leading edge of the cell toward the migratory cue, (2) the leading edge attaches to the extracellular matrix (ECM) through-integrin mediated adhesion complexes, (3) the actin-myosin cytoskeleton generates force to move the bulk of the cell forward and lastly, the adhesive contacts at the rear of the cell are disassembled to complete the cycle (191). Thus, any irregularity in the cycle mentioned above can lead to altered cell motility and potentially promote diseases.

Interestingly, CELF1s role in cell migration has not been addressed so far. However, based on our pSILAC proteomic analysis and gene ontology, we were able to identify CELF1-targetted genes that are involved in cell migration and motility. Therefore, for specific aim-2 of this study, we will examine how CELF1 controls MARCKS to regulate cell migration in oral cancer cells.

Results:

Specific Aim 2: Determine the biological role of MARCKS in oral cancer cells.

Task 1: Determine if CELF1 modulates cell migration in UM74B cells by using a wound scratch healing assay.

Task 2: Confirm whether overexpression of CELF1 promotes the expression of MARCKS in non-malignant human oral keratinocytes and enhances cell motility.

Task 3: Understand if MARCKS directly controls cell motility in UM74B cells using a cell migration assay.

Task 4: Establish if ectopic expression of MARCKS in CELF1 depleted UM74B cells rescues the cell migration phenotype.

Although pSILAC and gene ontology analysis suggest that CELF1 could play a role in cell-cell communication and regulate extra cellular matrix proteins in oral cancer cells (Figure 2), its cell migratory functions were never tested in a cancer cell model. To address whether depletion of CELF1 in UM74B cells modulate cell motility, we employed two separate methods to quantify cell migration: a wound healing assay and a transwell migration assay. First, to measure the cell migration in UM74B cells, we created a scratch 72 hours post-transduction shRNA control and shRNA against CELF1, a wound was created as described in materials and methods. Next, the percent migration was calculated by measuring wound closure at 0 and 24-hours post wounding for control and CELF1 depleted cells. Compared to control shRNA treated cells, we have observed a 56% reduction in wound closure in the CELF1 knockdown cells (Figure 12), suggesting that CELF1 promotes cell migration in oral cancer cells.

To further confirm CELF1's role as a cell migratory protein, we tested its function by overexpressing CELF1 in non-malignant oral keratinocytes (OHKC), which express low levels of CELF1 and measured cell migration. By using wound scratch assay, we have observed OHKC CELF1 overexpressing cells to exhibits 49% enhancement in cell migration compared to control OHKC (Figure 13). This observation indicated that CELF1 enhanced cell migration, possibly through its associated gene network.

Next, to test whether overexpression of CELF1 in OHKC cells promotes MARCKS protein expression, we have used Western blot analysis. CELF1 overexpressing OHKC cells exhibited a 2.7 fold increase in MARCKS protein

compared to control vector-expressing cells (Figure 14, left panel). This observation clearly indicated that overexpression of CELF1 promoted MARCKS expression in these cells. To further confirm overexpression of CELF1 directly promotes the translation of MARCKS, we quantified *MARCKS* mRNA levels using qRT-PCR in OHKC and OHKC CELF1 overexpressing cells. As expected, we did not observe a significant difference in MARCKS mRNA level in OHKC cells relative to OHKC-CELF1 cells (Figure 14, right panel). Altogether, these two key experimental results confirmed that overexpression of CELF1 promotes MARCKS expression at the mRNA translational level, but not at the mRNA turnover level.

Although depletion of CELF1 reduces cell migration in UM74B cells, the question remains, whether CELF1 or MARCKS alone or in combination to control the function of cell migration. To confirm if MARCKS independently controls cell migration in oral cancer cells, we have reduced MARCKS expression in UM74B cells using siRNA against *MARCKS* mRNA (Figure 15, top left panel) and measured cell migration using the wound healing assay. As expected, compared to control siRNA transfected cells, we observed a 40% decrease in wound closure in MARCKS siRNA treated cells (Figure 15, bottom panel), suggesting that MARCKS directly controls cell motility in oral cancer cells.

In addition, to determine if CELF1 controls cell migration through MARCKS, we ectopically expressed GFP-tagged MARCKS in CELF1 depleted cells and tested whether expression of MARCKS alone enhances or rescues cell migration in oral cancer cells. The ectopic expression of MARCKS in CELF1 depleted cells was tested by Western blot and is shown in Figure 16, bottom left. Due to severe cell

death after CELF1 depletion combined with MARCKS overexpression, we were unable to perform wound scratch assay. Consequently, we have utilized a transwell migration assay to measure cell motility (Figure 16). As expected in CELF1 depleted cells, cell migration was reduced in comparison to shRNA control treated cells; whereas cells overexpressing GFP-MARCKS in the absence of CELF1 did not exhibits a significant change in migration compared to shRNA control treated cells. Thus, the data indicate that CELF1 phenotype was rescued. In another word the cell migration phenotype was rescued. In conclusion, the results obtained from specific aim-2 positively demonstrated that CELF1 controls cell migration through MARCKS protein, which was confirmed by gain- and/or loss-of-function studies of CELF1 and MARCKS in oral cancer cells.

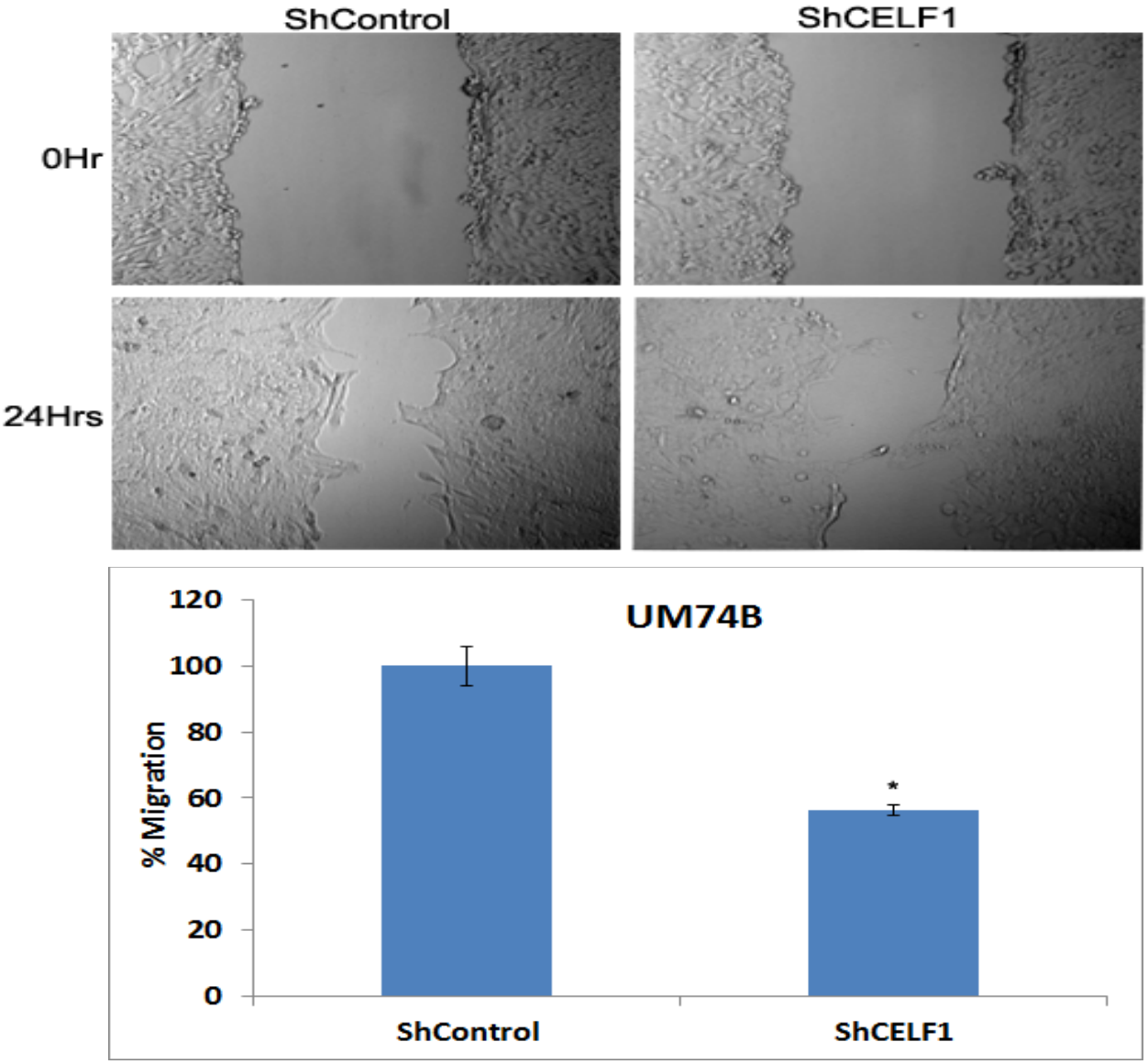


Figure12: CELF1 influences cell migration in UM74B cells. Reduction of CELF1 reduces cell migration by 54%. Monolayer cells were wounded and photographs were taken immediately after wound induction and 24 hours later. The scratch creation was performed as described in materials and methods. Additionally, the quantification of wound closer was measured using ImageJ software, the protocol is descried in methods and materials sections. N=4, *p<0.002.

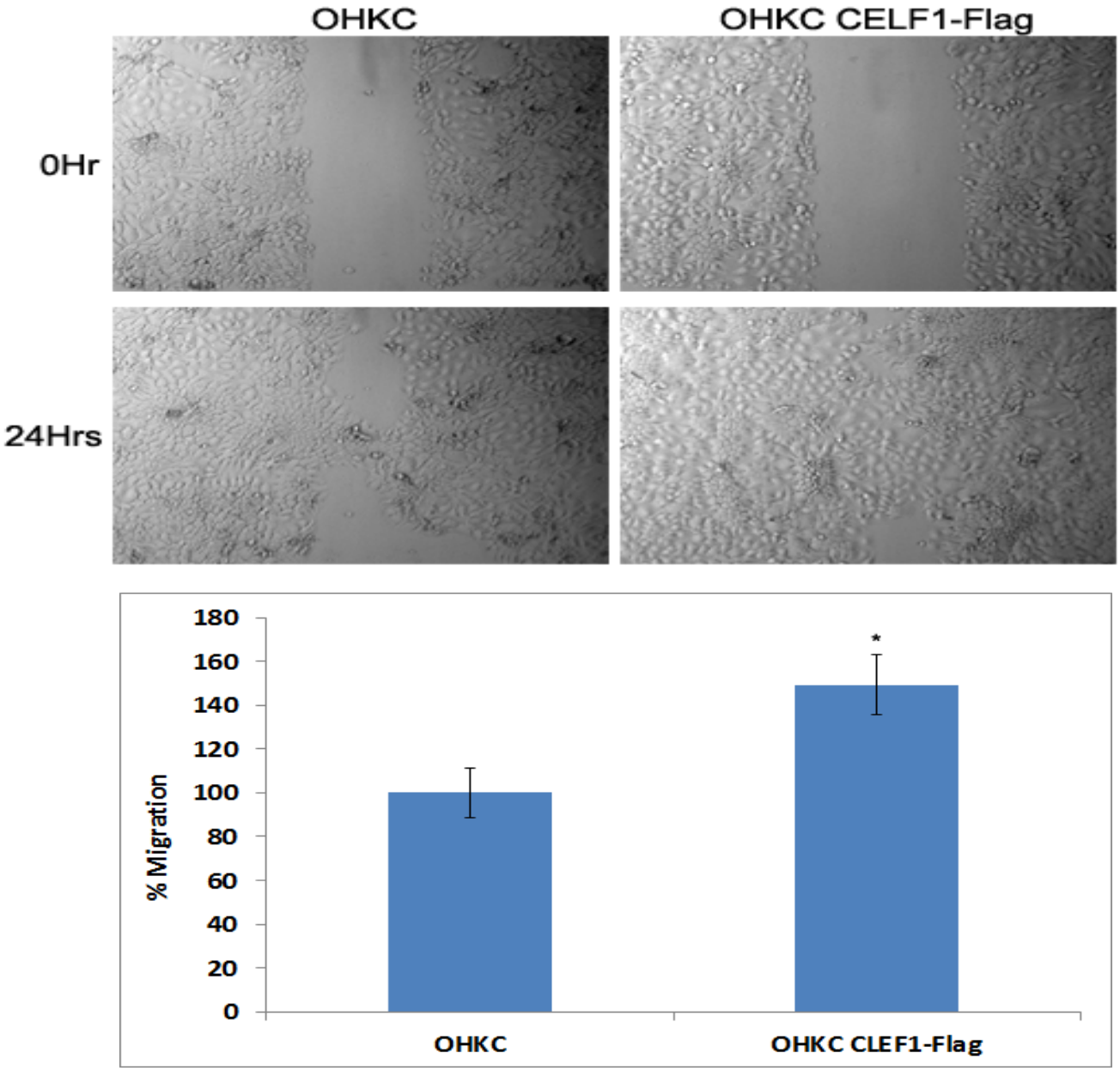


Figure 13: Overexpression of CELF1 in non-malignant human oral keratinocytes (OHKC-CELF1). Overexpression of CELF1 in OHKC enhances cell migration by 49% in compared to OHKC. Monolayer cells were wounded and photographs were taken immediately after wound induction and 24 hours post wound creation. The scratch creation and quantification of wound closer protocols are described in methods and materials. Additionally, the quantification was measured by ImageJ software. N=3, *p<0.038.

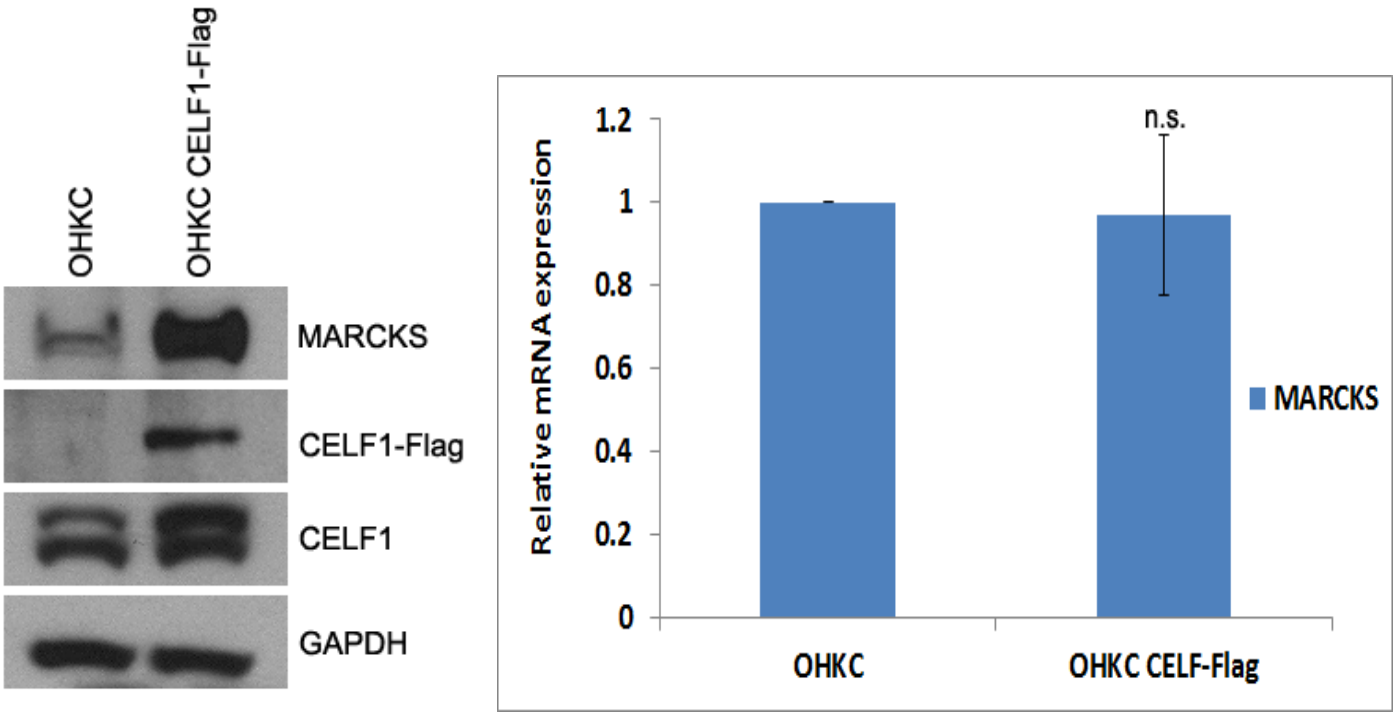


Figure 14, left: Overexpression of CELF1 in OHKC CELF1 Flag tag leads to upregulation of MARCKS protein expression. The Western blot of total protein was extracted from OHKC cells and OHKC CELF1-Flag tag cells. The CELF1 is overexpressed in the OHKC CELF-Flag tag cell line in comparison to OHKC. Additionally, the western blot also shows overexpression of MARCKS in OHKC CELF1-Flag tag cells in comparison to OHKC cells.

Figure 14, right: MARCKS mRNA levels remain unchanged in CELF1 overexpressing OHKC compared to normal OHKC. N=3, $p > 0.895$; n.s.= non-significant.

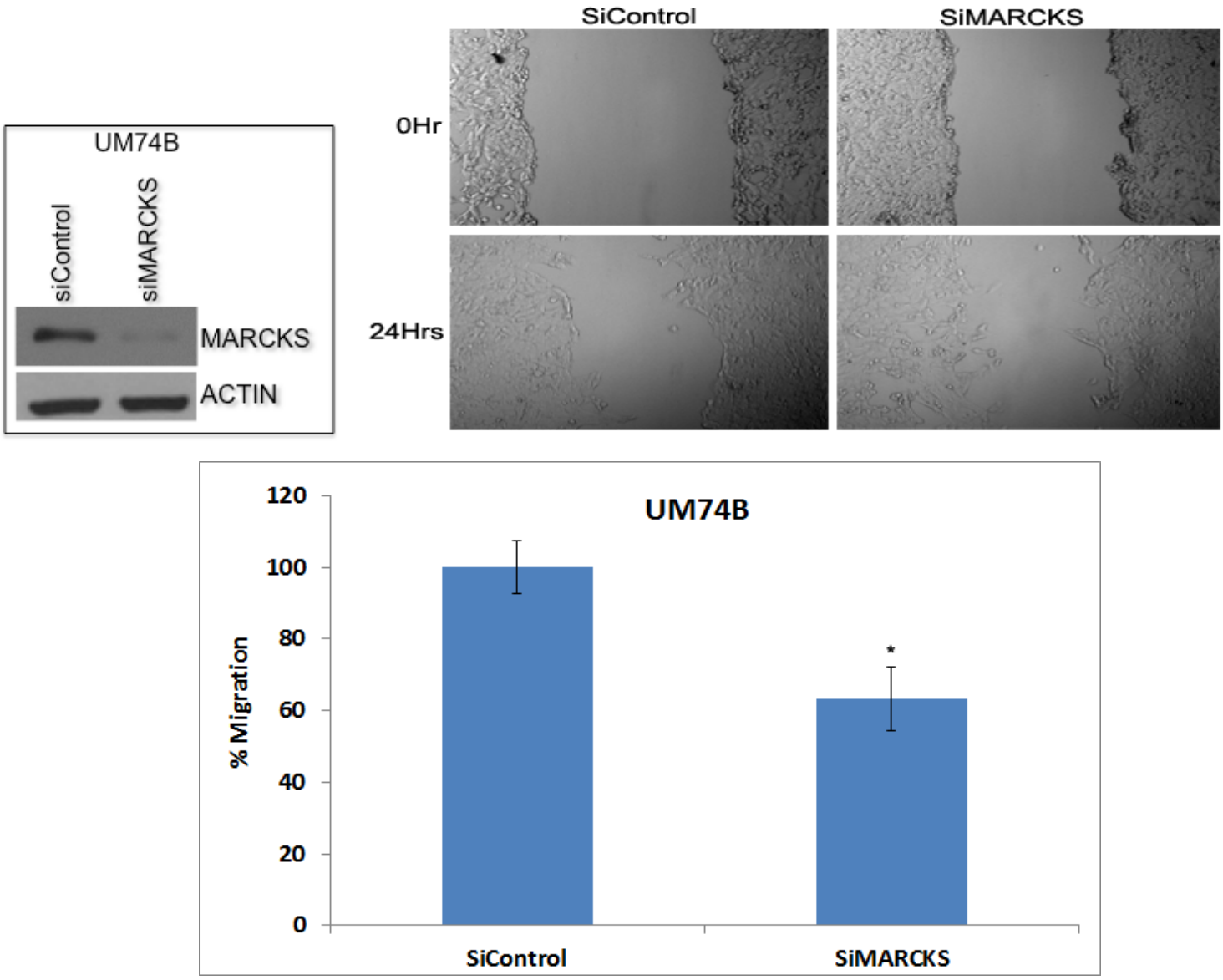
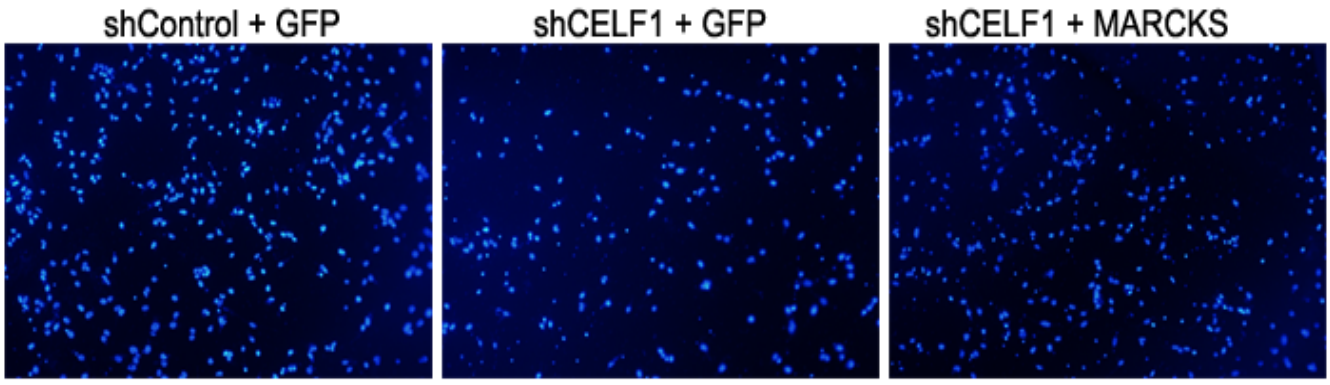
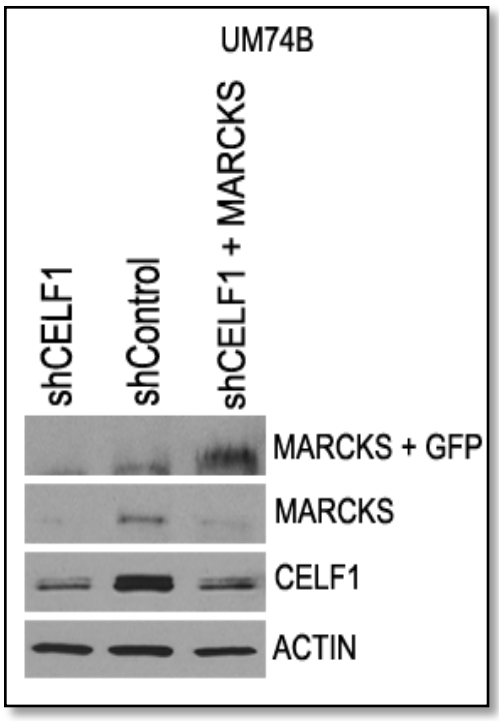


Figure 15: MARCKS influences OSCC cell migration in a scratch wound healing assay. Treatment of siRNA against MARCKS reduces cell migration by 40% in UM74B. Monolayer of cells were wounded and photographs were taken immediately after wound induction and 24 hours post wound creation. The scratch creation and quantification of wound closer protocols are described in methods and materials. Additionally, the quantification of scratch wound healing assay was measured by ImageJ software. N=3, *p<0.0005.





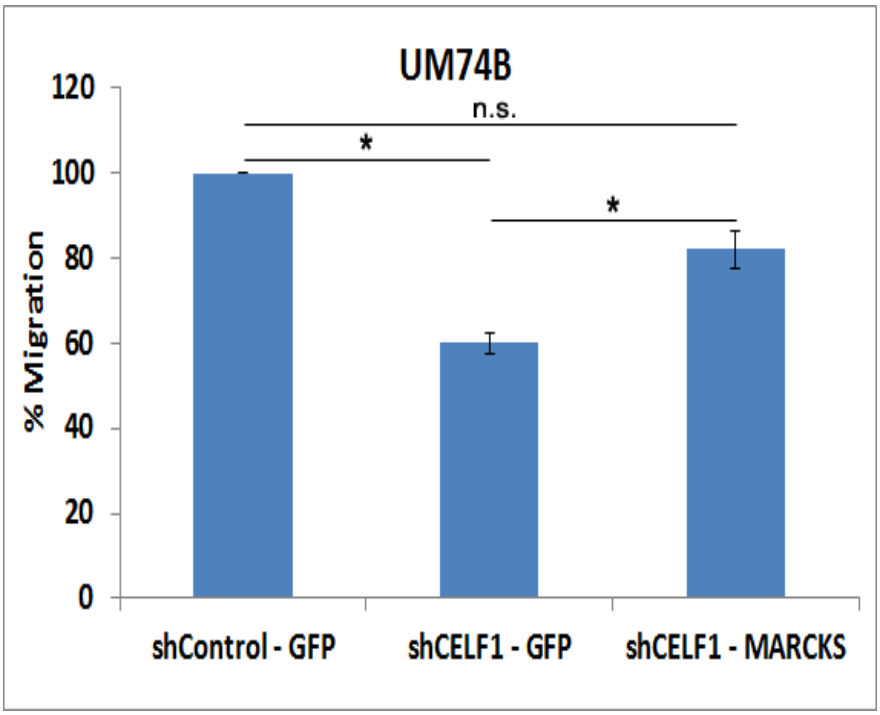


Figure 16, top panel: Transwell migration assay of MARCKS overexpression in CELF1 depleted UM74B cells.

Figure 16, bottom left panel: Confirmation of overexpression of MARCKS in CELF1 depleted UM74B cells.

Figure 16, bottom right panel: Migration through transwell in UM74B. Migration was performed as described in methods and materials. The N=3; shControl-GFP/ shCELF 1- GFP, *p<0.002; shCELF1-GFP/ shCELF-MARCKS, *p<0.025; shContro-GFP;shCELF1-MARCKS; p>.050, n.s.= non-significant.

Discussion:

RNA-binding proteins regulate gene expression in mammalian cells mostly at a global level through coordinated gene network and control over several cell biological processes. For example, systems-level mRNA:protein (mRNP) biology has begun to emerge from quantitative genomic, proteomic, and microscopy-based investigations (193). These approaches have yielded information about mRNA and protein state, protein-protein, protein-nucleic acid interactions, protein localization, and dynamics of mRNP systems and processes. Although large-scale proteomic studies have generally focused on whole proteome interactions rather than those of mRNP systems in particular, our approach in this thesis primarily focuses on proteomic and functional investigation of CELF1 in gene expression and cell migration in oral cancer cells.

Interestingly, published network analysis of CELF1 and its associated targets in cervical cancer cells (HeLa) showed genes that are specifically involved in cell migration (194). However, biological studies associated with this observation is reported. Based on our pSILAC analysis, we have identified several proteins targeted by CELF1 implicated for their role in cell migration and motility. Based on our identification of key proteins involved in cell migration, MARCKS becomes a prominent protein shown to be associated with cell migration in a variety of cell systems (177). Thus, CELF1 associated proteome network analysis followed by target validation for functional studies of MARCKS suggest that the systems approach on understanding biological function of CELF1 can be very successful.

The results obtained in specific aim-2, for example, depletion of CELF1 reduces cell migration (Figure 12) and overexpression of CELF1 in non-malignant cells enhances cell migration (Figure 13), clearly demonstrate that CELF1 is a critical regulator of cell motility. RBPs are well-known for controlling cell migration in variety of other systems, for example, HuR has been shown in HeLa cells to stabilize the β-*actin* mRNA by associating with a uridine-rich element within its 3′ UTR and controlling cell adhesion, invasion and migration (195). Thus, our data in-line with the above mentioned report and suggest that CELF1's control of MARCKS could be part of the regulatory mechanisms responsible for cell migration in oral cancer cells.

MARCKS is a well-known protein factor involved in cell migration that is regulated by several different cellular pathways, such as epigenetic modifications, transcription and signaling pathways. It is very interesting to add an additional layer of regulation for MARCKS through mRNA translation machinery, which controls its expression in cancer cells. Based on the findings in this thesis, we are first to report that MARCKS is controlled at the mRNA translational level in cancer cells.

It is interesting to note that we have previously reported that depletion of CELF1 in UM74B cells leads to reduction of cell growth and increased apoptosis (185). But, specific deletion of CELF1 in OHKC did not alter cell growth or apoptosis (57) suggesting that CELF1 function are cancer cell-specific and in cancer cells the function of CELF1 is different than normal cells. But overexpression of CELF1 in OHKC cells promotes cancer like phenotype. Based on the data obtained from our study (Figure 14), we were able to establish that overexpression of CELF1 in non-malignant cells promotes the expression of MARCKS and subsequently enhances

cell motility. Thus, CELF1 and its associated gene network are important regulators of cell migration in oral cancer cells.

It is widely accepted that signal transduction pathways affect cancer cell motility through phosphorylation of targeted proteins. For example, PDGF-BB is a known mitogen and chemoattractant for fibroblasts that signals through tyrosine kinases and the PDGF-BB receptor (PDGFR-BB). PDGF-BB signaling results in increased intracellular Ca^{2+} concentrations and subsequent activation of protein kinase C (PKCε), which phosphorylates MARCKS (196, 197). The resulting phosphorylated MARCKS is unable to bind with actin filaments and cell migration is induced. Thus, kinase activation plays a critical role in MARCKS cell migratory activity. Surprisingly, CELF1 was shown to be phosphorylated upon activation of PKC (79), which could impact the binding and promotion of MARCKS expression in oral cancer cells. Both CELF1 and MARCKS proteins were overexpressed in oral cancer cells (Figure 3), and possibly phosphorylated by PKC, but this proposed model requires further investigation. It would it interesting to see if activation and inactivation of PKC can increase and decrease cell migration through phosphorylation of CELF1 and MARCKS, respectively.

Chapter 6

Summary and Future Directions

Summary:

Post-transcriptional regulation of gene expression plays an important role in disease progression. RNA binding proteins with the help of other accessory proteins and mRNAs are capable of regulating multiple steps within the post-transcriptional regulatory process. CELF1, a member of CELF family RBPs is known to regulate pre-mRNA splicing, mRNA degradation, and mRNA translation (61, 184). CELF1's role in cancer has emerged within the last five years; however, its precise cancer biological function has not been established. Here, we have utilized a global proteome-wide analysis and identified the protein MARCKS as a target of CELF1. We have also established that cell migration is controlled by CELF1 through translational regulation of MARCKS in oral cancer cells.

Our proteome-wide analysis revealed that approximately 1350 proteins are translationally controlled by CELF1. The biological enrichment of these proteins discovered a vast variety of genes that are controlled by CELF1 including RNA transport, mRNA 3′ end processing, translation initiation, and cytoskeleton remodeling. Moreover, based on statistical analysis of top altered proteins in the absence of CELF1, we were able to identify the top 18 genes that were associated with CELF1 (Table. 1). Continued analysis of identified genes through KEGG pathway, USCS genome browser database and multiple rounds of polysome fractionation gradient analysis, we found that MARCKS was a target of CELF1. Given the significance of MARCKS role in cell migration, we have identified that overexpression of CELF1 controls cell motility in oral cancer cells through regulation and expression of the cytoskeleton associated protein MARCKS.

Future Directions:

In this thesis, we have demonstrated that CELF1 and MARCKS are overexpressed in multiple oral cancer cells. Furthermore, we have observed upon depletion of CELF1, protein expression of MARCKS is significantly reduced. Using RNA-IP we were able to establish CELF1 is directly associated with *MARCKS* mRNA and controls its translation. However, we have not demonstrated the exact molecular mechanism of how CELF1 binds to *MARCKS* mRNA. However, we have observed that the MARCKS 3′ UTR contains canonical GRE sequences, where CELF1 can bind and potentially regulate its translation. At the same time, we cannot disregard the 5′ UTR of MARCKS, which contains GU stretches that can also be controlled by CELF1. Therefore, future experiments are needed to determine how CELF1 control MARCKS translation through association with 5′ or 3′ UTRs. To address this, we plan to use a luciferase reporters constructs with MARCKS 3′ or 5′ UTRs to test the expression level of these constructs by the gain- and loss-of-function of CELF1. This experiment will provide additional confirmation of CELF1's control over MARCKS expression in oral cancer cells and may be suitable for other cell systems.

Conclusion:

Overall, the work presented in this thesis has further strengthened our understanding of CELF1 function at the post-transcriptional gene regulation level and establishes the molecular mechanism of mRNA translation of MARCKS. We were able to provide evidence to support MARCKS as a target of CELF1-regulated post-transcriptional regulation. Furthermore, in specific aim-2, we were able to

demonstrate that CELF1 controls cell migration through MARCKS in oral cancer cells. Interestingly, reduction of CELF1 in oral cancer cells down-regulates protein synthesis of MARCKS, but not the *MARCKS* mRNA, further validating that CELF1 is controlling *MARCKS* at the mRNA translational level. Finally, gain-and loss-of-function of CELF1 and MARCKS in oral cancer cells demonstrated that both proteins play a key role in cell migration. The down-regulation of CELF1 resulted in decreased MARCKS gene expression, which in turn resulted in decreased cell motility of oral cancer cells. Thus, based on all of the data presented in this thesis, we conclude that CELF1 mediated cell migration occurs through the post-transcriptional regulation of MARCKS protein.

References:

1. Friedman JM, Stavas MJ, Cmelak AJ. Clinical and scientific impact of human papillomavirus on head and neck cancer. World journal of clinical oncology. 2014;5(4):781-91.
2. Martin D, Abba MC, Molinolo AA, Vitale-Cross L, Wang Z, Zaida M, et al. The head and neck cancer cell oncogenome: a platform for the development of precision molecular therapies. Oncotarget. 2014;5(19):8906-23.
3. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA: a cancer journal for clinicians. 2014;64(1):9-29.
4. Warnakulasuriya S. Causes of oral cancer--an appraisal of controversies. British dental journal. 2009;207(10):471-5.
5. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(32):4294-301.
6. Chung CH, Gillison ML. Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. Clinical cancer research : an official journal of the American Association for Cancer Research. 2009;15(22):6758-62.
7. Crick F. Central dogma of molecular biology. Nature. 1970;227(5258):561-3.
8. Dixon DA, Tolley ND, King PH, Nabors LB, McIntyre TM, Zimmerman GA, et al. Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. The Journal of clinical investigation. 2001;108(11):1657-65.
9. Heinonen M, Bono P, Narko K, Chang SH, Lundin J, Joensuu H, et al. Cytoplasmic HuR expression is a prognostic factor in invasive ductal breast carcinoma. Cancer research. 2005;65(6):2157-61.
10. Vlasova IA, McNabb J, Raghavan A, Reilly C, Williams DA, Bohjanen KA, et al. Coordinate stabilization of growth-regulatory transcripts in T cell malignancies. Genomics. 2005;86(2):159-71.
11. Sandri-Goldin RM, Mendoza GE. A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. Genes & development. 1992;6(5):848-63.
12. Noah DL, Twu KY, Krug RM. Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAS. Virology. 2003;307(2):386-95.
13. Dolken L, Perot J, Cognat V, Alioua A, John M, Soutschek J, et al. Mouse cytomegalovirus microRNAs dominate the cellular small RNA profile during lytic infection and show features of posttranscriptional regulation. Journal of virology. 2007;81(24):13771-82.
14. Jenal M, Elkon R, Loayza-Puch F, van Haaften G, Kuhn U, Menzies FM, et al. The poly(A)-binding protein nuclear 1 suppresses alternative cleavage and polyadenylation sites. Cell. 2012;149(3):538-53.
15. Bhakar AL, Dolen G, Bear MF. The pathophysiology of fragile X (and what it teaches us about synapses). Annual review of neuroscience. 2012;35:417-43.

16. Guhaniyogi J, Brewer G. Regulation of mRNA stability in mammalian cells. Gene. 2001;265(1-2):11-23.
17. Ross J. mRNA stability in mammalian cells. Microbiological reviews. 1995;59(3):423-50.
18. Stoeckle MY. Removal of a 3' non-coding sequence is an initial step in degradation of gro alpha mRNA and is regulated by interleukin-1. Nucleic acids research. 1992;20(5):1123-7.
19. Lee SW, Tsou AP, Chan H, Thomas J, Petrie K, Eugui EM, et al. Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mRNA. Proceedings of the National Academy of Sciences of the United States of America. 1988;85(4):1204-8.
20. Peppel K, Vinci JM, Baglioni C. The AU-rich sequences in the 3' untranslated region mediate the increased turnover of interferon mRNA induced by glucocorticoids. The Journal of experimental medicine. 1991;173(2):349-55.
21. Hamalainen L, Oikarinen J, Kivirikko KI. Synthesis and degradation of type I procollagen mRNAs in cultured human skin fibroblasts and the effect of cortisol. The Journal of biological chemistry. 1985;260(2):720-5.
22. Keene JD. RNA regulons: coordination of post-transcriptional events. Nat Rev Genet. 2007;8(7):533-43.
23. Shyu AB, Wilkinson MF, van Hoof A. Messenger RNA regulation: to translate or to degrade. The EMBO journal. 2008;27(3):471-81.
24. Tenenbaum SA, Carson CC, Lager PJ, Keene JD. Identifying mRNA subsets in messenger ribonucleoprotein complexes by using cDNA arrays. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(26):14085-90.
25. Tenenbaum SA, Carson CC, Atasoy U, Keene JD. Genome-wide regulatory analysis using en masse nuclear run-ons and ribonomic profiling with autoimmune sera. Gene. 2003;317(1-2):79-87.
26. Keene JD, Tenenbaum SA. Eukaryotic mRNPs may represent posttranscriptional operons. Molecular cell. 2002;9(6):1161-7.
27. Hogan DJ, Riordan DP, Gerber AP, Herschlag D, Brown PO. Diverse RNA-binding proteins interact with functionally related sets of RNAs, suggesting an extensive regulatory system. PLoS biology. 2008;6(10):e255.
28. Garneau NL, Wilusz J, Wilusz CJ. The highways and byways of mRNA decay. Nature reviews Molecular cell biology. 2007;8(2):113-26.
29. Goldstrohm AC, Wickens M. Multifunctional deadenylase complexes diversify mRNA control. Nature reviews Molecular cell biology. 2008;9(4):337-44.
30. Wu X, Brewer G. The regulation of mRNA stability in mammalian cells: 2.0. Gene. 2012;500(1):10-21.
31. van Hoof A, Frischmeyer PA, Dietz HC, Parker R. Exosome-mediated recognition and degradation of mRNAs lacking a termination codon. Science. 2002;295(5563):2262-4.
32. Raghavan A, Ogilvie RL, Reilly C, Abelson ML, Raghavan S, Vasdewani J, et al. Genome-wide analysis of mRNA decay in resting and activated primary human T lymphocytes. Nucleic acids research. 2002;30(24):5529-38.

33. Vlasova IA, Tahoe NM, Fan D, Larsson O, Rattenbacher B, Sternjohn JR, et al. Conserved GU-rich elements mediate mRNA decay by binding to CUG-binding protein 1. Mol Cell. 2008;29(2):263-70.
34. Vlasova-St Louis I, Dickson AM, Bohjanen PR, Wilusz CJ. CELFish ways to modulate mRNA decay. Biochimica et biophysica acta. 2013;1829(6-7):695-707.
35. Moraes KC, Wilusz CJ, Wilusz J. CUG-BP binds to RNA substrates and recruits PARN deadenylase. Rna. 2006;12(6):1084-91.
36. Paillard L, Omilli F, Legagneux V, Bassez T, Maniey D, Osborne HB. EDEN and EDEN-BP, a cis element and an associated factor that mediate sequence-specific mRNA deadenylation in Xenopus embryos. The EMBO journal. 1998;17(1):278-87.
37. Delaunay J, Le Mee G, Ezzeddine N, Labesse G, Terzian C, Capri M, et al. The Drosophila Bruno paralogue Bru-3 specifically binds the EDEN translational repression element. Nucleic acids research. 2004;32(10):3070-82.
38. Vlasova-St Louis I, Bohjanen PR. Coordinate regulation of mRNA decay networks by GU-rich elements and CELF1. Current opinion in genetics & development. 2011;21(4):444-51.
39. Choi DK, Ito T, Tsukahara F, Hirai M, Sakaki Y. Developmentally-regulated expression of mNapor encoding an apoptosis-induced ELAV-type RNA binding protein. Gene. 1999;237(1):135-42.
40. Li D, Bachinski LL, Roberts R. Genomic organization and isoform-specific tissue expression of human NAPOR (CUGBP2) as a candidate gene for familial arrhythmogenic right ventricular dysplasia. Genomics. 2001;74(3):396-401.
41. Good PJ, Chen Q, Warner SJ, Herring DC. A family of human RNA-binding proteins related to the Drosophila Bruno translational regulator. The Journal of biological chemistry. 2000;275(37):28583-92.
42. Kress C, Gautier-Courteille C, Osborne HB, Babinet C, Paillard L. Inactivation of CUG-BP1/CELF1 causes growth, viability, and spermatogenesis defects in mice. Molecular and cellular biology. 2007;27(3):1146-57.
43. Choi DK, Yoo KW, Hong SK, Rhee M, Sakaki Y, Kim CH. Isolation and expression of Napor/CUG-BP2 in embryo development. Biochemical and biophysical research communications. 2003;305(3):448-54.
44. Yang Y, Mahaffey CL, Berube N, Maddatu TP, Cox GA, Frankel WN. Complex seizure disorder caused by Brunol4 deficiency in mice. PLoS genetics. 2007;3(7):e124.
45. Wu J, Li C, Zhao S, Mao B. Differential expression of the Brunol/CELF family genes during Xenopus laevis early development. The International journal of developmental biology. 2010;54(1):209-14.
46. Mankodi A, Takahashi MP, Jiang H, Beck CL, Bowers WJ, Moxley RT, et al. Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. Molecular cell. 2002;10(1):35-44.
47. Ho TH, Bundman D, Armstrong DL, Cooper TA. Transgenic mice expressing CUG-BP1 reproduce splicing mis-regulation observed in myotonic dystrophy. Human molecular genetics. 2005;14(11):1539-47.
48. Suzuki H, Jin Y, Otani H, Yasuda K, Inoue K. Regulation of alternative splicing of alpha-actinin transcript by Bruno-like proteins. Genes to cells : devoted to molecular & cellular mechanisms. 2002;7(2):133-41.

49. Roberts R, Timchenko NA, Miller JW, Reddy S, Caskey CT, Swanson MS, et al. Altered phosphorylation and intracellular distribution of a (CUG)n triplet repeat RNA-binding protein in patients with myotonic dystrophy and in myotonin protein kinase knockout mice. Proceedings of the National Academy of Sciences of the United States of America. 1997;94(24):13221-6.
50. Paillard L, Legagneux V, Beverley Osborne H. A functional deadenylation assay identifies human CUG-BP as a deadenylation factor. Biology of the cell / under the auspices of the European Cell Biology Organization. 2003;95(2):107-13.
51. Paillard L, Legagneux V, Maniey D, Osborne HB. c-Jun ARE targets mRNA deadenylation by an EDEN-BP (embryo deadenylation element-binding protein)-dependent pathway. The Journal of biological chemistry. 2002;277(5):3232-5.
52. Timchenko LT, Miller JW, Timchenko NA, DeVore DR, Datar KV, Lin L, et al. Identification of a (CUG)n triplet repeat RNA-binding protein and its expression in myotonic dystrophy. Nucleic acids research. 1996;24(22):4407-14.
53. Caskey CT, Swanson MS, Timchenko LT. Myotonic dystrophy: discussion of molecular mechanism. Cold Spring Harbor symposia on quantitative biology. 1996;61:607-14.
54. Timchenko LT, Timchenko NA, Caskey CT, Roberts R. Novel proteins with binding specificity for DNA CTG repeats and RNA CUG repeats: implications for myotonic dystrophy. Human molecular genetics. 1996;5(1):115-21.
55. Philips AV, Timchenko LT, Cooper TA. Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. Science. 1998;280(5364):737-41.
56. Kim-Ha J, Kerr K, Macdonald PM. Translational regulation of oskar mRNA by bruno, an ovarian RNA-binding protein, is essential. Cell. 1995;81(3):403-12.
57. Milne CA, Hodgkin J. ETR-1, a homologue of a protein linked to myotonic dystrophy, is essential for muscle development in Caenorhabditis elegans. Current biology : CB. 1999;9(21):1243-6.
58. Hashimoto Y, Suzuki H, Kageyama Y, Yasuda K, Inoue K. Bruno-like protein is localized to zebrafish germ plasm during the early cleavage stages. Gene expression patterns : GEP. 2006;6(2):201-5.
59. Brimacombe KR, Ladd AN. Cloning and embryonic expression patterns of the chicken CELF family. Developmental dynamics : an official publication of the American Association of Anatomists. 2007;236(8):2216-24.
60. Ladd AN, Charlet N, Cooper TA. The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. Molecular and cellular biology. 2001;21(4):1285-96.
61. Tsuda K, Kuwasako K, Takahashi M, Someya T, Inoue M, Terada T, et al. Structural basis for the sequence-specific RNA-recognition mechanism of human CUG-BP1 RRM3. Nucleic acids research. 2009;37(15):5151-66.
62. Tripsianes K, Sattler M. Repeat recognition. Structure. 2010;18(10):1228-9.
63. Edwards J, Malaurie E, Kondrashov A, Long J, de Moor CH, Searle MS, et al. Sequence determinants for the tandem recognition of UGU and CUG rich RNA elements by the two N--terminal RRMs of CELF1. Nucleic acids research. 2011;39(19):8638-50.
64. Teplova M, Song J, Gaw HY, Teplov A, Patel DJ. Structural insights into RNA recognition by the alternate-splicing regulator CUG-binding protein 1. Structure. 2010;18(10):1364-77.

65. Mori D, Sasagawa N, Kino Y, Ishiura S. Quantitative analysis of CUG-BP1 binding to RNA repeats. Journal of biochemistry. 2008;143(3):377-83.
66. Salisbury E, Sakai K, Schoser B, Huichalaf C, Schneider-Gold C, Nguyen H, et al. Ectopic expression of cyclin D3 corrects differentiation of DM1 myoblasts through activation of RNA CUG-binding protein, CUGBP1. Experimental cell research. 2008;314(11-12):2266-78.
67. Takahashi N, Sasagawa N, Suzuki K, Ishiura S. The CUG-binding protein binds specifically to UG dinucleotide repeats in a yeast three-hybrid system. Biochemical and biophysical research communications. 2000;277(2):518-23.
68. Timchenko NA, Welm AL, Lu X, Timchenko LT. CUG repeat binding protein (CUGBP1) interacts with the 5' region of C/EBPbeta mRNA and regulates translation of C/EBPbeta isoforms. Nucleic acids research. 1999;27(22):4517-25.
69. Marquis J, Paillard L, Audic Y, Cosson B, Danos O, Le Bec C, et al. CUG-BP1/CELF1 requires UGU-rich sequences for high-affinity binding. The Biochemical journal. 2006;400(2):291-301.
70. Goraczniak R, Gunderson SI. The regulatory element in the 3'-untranslated region of human papillomavirus 16 inhibits expression by binding CUG-binding protein 1. The Journal of biological chemistry. 2008;283(4):2286-96.
71. Lee JE, Lee JY, Wilusz J, Tian B, Wilusz CJ. Systematic analysis of cis-elements in unstable mRNAs demonstrates that CUGBP1 is a key regulator of mRNA decay in muscle cells. PLoS One. 2010;5(6):e11201.
72. Wu C, Alwine JC. Secondary structure as a functional feature in the downstream region of mammalian polyadenylation signals. Molecular and cellular biology. 2004;24(7):2789-96.
73. Mooers BH, Logue JS, Berglund JA. The structural basis of myotonic dystrophy from the crystal structure of CUG repeats. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(46):16626-31.
74. Lee JE, Cooper TA. Pathogenic mechanisms of myotonic dystrophy. Biochemical Society transactions. 2009;37(Pt 6):1281-6.
75. Bonnet-Corven S, Audic Y, Omilli F, Osborne HB. An analysis of the sequence requirements of EDEN-BP for specific RNA binding. Nucleic acids research. 2002;30(21):4667-74.
76. Masuda A, Andersen HS, Doktor TK, Okamoto T, Ito M, Andresen BS, et al. CUGBP1 and MBNL1 preferentially bind to 3' UTRs and facilitate mRNA decay. Scientific reports. 2012;2:209.
77. Zhang L, Lee JE, Wilusz J, Wilusz CJ. The RNA-binding protein CUGBP1 regulates stability of tumor necrosis factor mRNA in muscle cells: implications for myotonic dystrophy. The Journal of biological chemistry. 2008;283(33):22457-63.
78. Wang GS, Kuyumcu-Martinez MN, Sarma S, Mathur N, Wehrens XH, Cooper TA. PKC inhibition ameliorates the cardiac phenotype in a mouse model of myotonic dystrophy type 1. The Journal of clinical investigation. 2009;119(12):3797-806.
79. Kuyumcu-Martinez NM, Wang GS, Cooper TA. Increased steady-state levels of CUGBP1 in myotonic dystrophy 1 are due to PKC-mediated hyperphosphorylation. Molecular cell. 2007;28(1):68-78.

80. Timchenko LT, Salisbury E, Wang GL, Nguyen H, Albrecht JH, Hershey JW, et al. Age-specific CUGBP1-eIF2 complex increases translation of CCAAT/enhancer-binding protein beta in old liver. The Journal of biological chemistry. 2006;281(43):32806-19.
81. Welm AL, Mackey SL, Timchenko LT, Darlington GJ, Timchenko NA. Translational induction of liver-enriched transcriptional inhibitory protein during acute phase response leads to repression of CCAAT/enhancer binding protein alpha mRNA. The Journal of biological chemistry. 2000;275(35):27406-13.
82. Timchenko NA, Wang GL, Timchenko LT. RNA CUG-binding protein 1 increases translation of 20-kDa isoform of CCAAT/enhancer-binding protein beta by interacting with the alpha and beta subunits of eukaryotic initiation translation factor 2. The Journal of biological chemistry. 2005;280(21):20549-57.
83. Huichalaf C, Sakai K, Jin B, Jones K, Wang GL, Schoser B, et al. Expansion of CUG RNA repeats causes stress and inhibition of translation in myotonic dystrophy 1 (DM1) cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2010;24(10):3706-19.
84. Panasenko OM, Chekanov AV, Vlasova, II, Sokolov AV, Ageeva KV, Pulina MO, et al. [A study of the effect of ceruloplasmin and lactoferrin on the chlorination activity of leukocytic myeloperoxidase using the chemiluminescence method]. Biofizika. 2008;53(4):573-81.
85. Wang ET, Sandberg R, Luo S, Khrebtukova I, Zhang L, Mayr C, et al. Alternative isoform regulation in human tissue transcriptomes. Nature. 2008;456(7221):470-6.
86. Ladd AN, Cooper TA. Finding signals that regulate alternative splicing in the post-genomic era. Genome biology. 2002;3(11):reviews0008.
87. Voelker RB, Berglund JA. A comprehensive computational characterization of conserved mammalian intronic sequences reveals conserved motifs associated with constitutive and alternative splicing. Genome research. 2007;17(7):1023-33.
88. Dembowski JA, Grabowski PJ. The CUGBP2 splicing factor regulates an ensemble of branchpoints from perimeter binding sites with implications for autoregulation. PLoS genetics. 2009;5(8):e1000595.
89. Kalsotra A, Xiao X, Ward AJ, Castle JC, Johnson JM, Burge CB, et al. A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart. Proceedings of the National Academy of Sciences of the United States of America. 2008;105(51):20333-8.
90. Savkur RS, Philips AV, Cooper TA. Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. Nature genetics. 2001;29(1):40-7.
91. Charlet BN, Savkur RS, Singh G, Philips AV, Grice EA, Cooper TA. Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. Molecular cell. 2002;10(1):45-53.
92. Ladd AN, Taffet G, Hartley C, Kearney DL, Cooper TA. Cardiac tissue-specific repression of CELF activity disrupts alternative splicing and causes cardiomyopathy. Molecular and cellular biology. 2005;25(14):6267-78.
93. Ladd AN, Nguyen NH, Malhotra K, Cooper TA. CELF6, a member of the CELF family of RNA-binding proteins, regulates muscle-specific splicing enhancer-dependent alternative splicing. The Journal of biological chemistry. 2004;279(17):17756-64.

94. Gromak N, Matlin AJ, Cooper TA, Smith CW. Antagonistic regulation of alpha-actinin alternative splicing by CELF proteins and polypyrimidine tract binding protein. Rna. 2003;9(4):443-56.
95. Charlet BN, Logan P, Singh G, Cooper TA. Dynamic antagonism between ETR-3 and PTB regulates cell type-specific alternative splicing. Molecular cell. 2002;9(3):649-58.
96. Kino Y, Washizu C, Oma Y, Onishi H, Nezu Y, Sasagawa N, et al. MBNL and CELF proteins regulate alternative splicing of the skeletal muscle chloride channel CLCN1. Nucleic acids research. 2009;37(19):6477-90.
97. Dujardin G, Buratti E, Charlet-Berguerand N, Martins de Araujo M, Mbopda A, Le Jossic-Corcos C, et al. CELF proteins regulate CFTR pre-mRNA splicing: essential role of the divergent domain of ETR-3. Nucleic acids research. 2010;38(20):7273-85.
98. Barron VA, Zhu H, Hinman MN, Ladd AN, Lou H. The neurofibromatosis type I pre-mRNA is a novel target of CELF protein-mediated splicing regulation. Nucleic acids research. 2010;38(1):253-64.
99. Koebis M, Ohsawa N, Kino Y, Sasagawa N, Nishino I, Ishiura S. Alternative splicing of myomesin 1 gene is aberrantly regulated in myotonic dystrophy type 1. Genes to cells : devoted to molecular & cellular mechanisms. 2011;16(9):961-72.
100. Berger DS, Moyer M, Kliment GM, van Lunteren E, Ladd AN. Expression of a dominant negative CELF protein in vivo leads to altered muscle organization, fiber size, and subtype. PloS one. 2011;6(4):e19274.
101. Terenzi F, Brimacombe KR, Penn MS, Ladd AN. CELF-mediated alternative splicing is required for cardiac function during early, but not later, postnatal life. Journal of molecular and cellular cardiology. 2009;46(3):395-404.
102. Horb LD, Horb ME. BrunoL1 regulates endoderm proliferation through translational enhancement of cyclin A2 mRNA. Developmental biology. 2010;345(2):156-69.
103. Fujimura K, Kano F, Murata M. Dual localization of the RNA binding protein CUGBP-1 to stress granule and perinucleolar compartment. Experimental cell research. 2008;314(3):543-53.
104. Nakamura A, Sato K, Hanyu-Nakamura K. Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Developmental cell. 2004;6(1):69-78.
105. Bae EJ, Kim SG. Enhanced CCAAT/enhancer-binding protein beta-liver-enriched inhibitory protein production by Oltipraz, which accompanies CUG repeat-binding protein-1 (CUGBP1) RNA-binding protein activation, leads to inhibition of preadipocyte differentiation. Molecular pharmacology. 2005;68(3):660-9.
106. Karagiannides I, Thomou T, Tchkonia T, Pirtskhalava T, Kypreos KE, Cartwright A, et al. Increased CUG triplet repeat-binding protein-1 predisposes to impaired adipogenesis with aging. The Journal of biological chemistry. 2006;281(32):23025-33.
107. Jin J, Wang GL, Timchenko L, Timchenko NA. GSK3beta and aging liver. Aging. 2009;1(6):582-5.
108. Iakova P, Wang GL, Timchenko L, Michalak M, Pereira-Smith OM, Smith JR, et al. Competition of CUGBP1 and calreticulin for the regulation of p21 translation determines cell fate. The EMBO journal. 2004;23(2):406-17.

109. Lian XJ, Gallouzi IE. Oxidative Stress Increases the Number of Stress Granules in Senescent Cells and Triggers a Rapid Decrease in p21waf1/cip1 Translation. The Journal of biological chemistry. 2009;284(13):8877-87.
110. Timchenko NA, Iakova P, Cai ZJ, Smith JR, Timchenko LT. Molecular basis for impaired muscle differentiation in myotonic dystrophy. Molecular and cellular biology. 2001;21(20):6927-38.
111. Timchenko NA, Patel R, Iakova P, Cai ZJ, Quan L, Timchenko LT. Overexpression of CUG triplet repeat-binding protein, CUGBP1, in mice inhibits myogenesis. The Journal of biological chemistry. 2004;279(13):13129-39.
112. Fox JT, Stover PJ. Mechanism of the internal ribosome entry site-mediated translation of serine hydroxymethyltransferase 1. The Journal of biological chemistry. 2009;284(45):31085-96.
113. Woeller CF, Fox JT, Perry C, Stover PJ. A ferritin-responsive internal ribosome entry site regulates folate metabolism. The Journal of biological chemistry. 2007;282(41):29927-35.
114. Zheng Y, Miskimins WK. CUG-binding protein represses translation of p27Kip1 mRNA through its internal ribosomal entry site. RNA biology. 2011;8(3):365-71.
115. Wu WC, Walaas SI, Nairn AC, Greengard P. Calcium/phospholipid regulates phosphorylation of a Mr "87k" substrate protein in brain synaptosomes. Proceedings of the National Academy of Sciences of the United States of America. 1982;79(17):5249-53.
116. Stumpo DJ, Graff JM, Albert KA, Greengard P, Blackshear PJ. Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(11):4012-6.
117. Zolessi FR, Hellman U, Baz A, Arruti C. Characterization of MARCKS (Myristoylated alanine-rich C kinase substrate) identified by a monoclonal antibody generated against chick embryo neural retina. Biochemical and biophysical research communications. 1999;257(2):480-7.
118. Kim SS, Kim JH, Kim HS, Park DE, Chung CH. Involvement of the theta-type protein kinase C in translocation of myristoylated alanine-rich C kinase substrate (MARCKS) during myogenesis of chick embryonic myoblasts. The Biochemical journal. 2000;347 Pt 1:139-46.
119. Brooks SF, Herget T, Erusalimsky JD, Rozengurt E. Protein kinase C activation potently down-regulates the expression of its major substrate, 80K, in Swiss 3T3 cells. The EMBO journal. 1991;10(9):2497-505.
120. Carballo E, Colomer D, Vives Corrons JL, Blackshear PJ, Gil J. Phosphorylation of the MARCKS family of protein kinase C substrates in human B chronic lymphocytic leukemia cells. Leukemia. 1995;9(5):834-9.
121. Eckert RE, Neuder LE, Park J, Adler KB, Jones SL. Myristoylated alanine-rich C-kinase substrate (MARCKS) protein regulation of human neutrophil migration. American journal of respiratory cell and molecular biology. 2010;42(5):586-94.
122. Seykora JT, Ravetch JV, Aderem A. Cloning and molecular characterization of the murine macrophage "68-kDa" protein kinase C substrate and its regulation by bacterial

lipopolysaccharide. Proceedings of the National Academy of Sciences of the United States of America. 1991;88(6):2505-9.
123. Takashi S, Park J, Fang S, Koyama S, Parikh I, Adler KB. A peptide against the N-terminus of myristoylated alanine-rich C kinase substrate inhibits degranulation of human leukocytes in vitro. American journal of respiratory cell and molecular biology. 2006;34(6):647-52.
124. Erusalimsky JD, Morris C, Perks K, Brown R, Brooks S, Rozengurt E. Internal amino acid sequence analysis of the 80 kDa protein kinase C substrate from rat brain: relationship to the 87 kDa substrate from bovine brain. FEBS letters. 1989;255(1):149-53.
125. Graff JM, Young TN, Johnson JD, Blackshear PJ. Phosphorylation-regulated calmodulin binding to a prominent cellular substrate for protein kinase C. The Journal of biological chemistry. 1989;264(36):21818-23.
126. Blackshear PJ, Tuttle JS, Oakey RJ, Seldin MF, Chery M, Philippe C, et al. Chromosomal mapping of the human (MACS) and mouse (Macs) genes encoding the MARCKS protein. Genomics. 1992;14(1):168-74.
127. Arbuzova A, Schmitz AA, Vergeres G. Cross-talk unfolded: MARCKS proteins. The Biochemical journal. 2002;362(Pt 1):1-12.
128. Larsson C. Protein kinase C and the regulation of the actin cytoskeleton. Cellular signalling. 2006;18(3):276-84.
129. Denisov G, Wanaski S, Luan P, Glaser M, McLaughlin S. Binding of basic peptides to membranes produces lateral domains enriched in the acidic lipids phosphatidylserine and phosphatidylinositol 4,5-bisphosphate: an electrostatic model and experimental results. Biophysical journal. 1998;74(2 Pt 1):731-44.
130. James G, Olson EN. Myristoylation, phosphorylation, and subcellular distribution of the 80-kDa protein kinase C substrate in BC3H1 myocytes. The Journal of biological chemistry. 1989;264(35):20928-33.
131. George DJ, Blackshear PJ. Membrane association of the myristoylated alanine-rich C kinase substrate (MARCKS) protein appears to involve myristate-dependent binding in the absence of a myristoyl protein receptor. The Journal of biological chemistry. 1992;267(34):24879-85.
132. Swierczynski SL, Blackshear PJ. Myristoylation-dependent and electrostatic interactions exert independent effects on the membrane association of the myristoylated alanine-rich protein kinase C substrate protein in intact cells. The Journal of biological chemistry. 1996;271(38):23424-30.
133. Swierczynski SL, Blackshear PJ. Membrane association of the myristoylated alanine-rich C kinase substrate (MARCKS) protein. Mutational analysis provides evidence for complex interactions. The Journal of biological chemistry. 1995;270(22):13436-45.
134. Taniguchi H, Manenti S. Interaction of myristoylated alanine-rich protein kinase C substrate (MARCKS) with membrane phospholipids. The Journal of biological chemistry. 1993;268(14):9960-3.
135. Kim J, Blackshear PJ, Johnson JD, McLaughlin S. Phosphorylation reverses the membrane association of peptides that correspond to the basic domains of MARCKS and neuromodulin. Biophysical journal. 1994;67(1):227-37.

136. McLaughlin S, Aderem A. The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. Trends in biochemical sciences. 1995;20(7):272-6.
137. Seykora JT, Myat MM, Allen LA, Ravetch JV, Aderem A. Molecular determinants of the myristoyl-electrostatic switch of MARCKS. The Journal of biological chemistry. 1996;271(31):18797-802.
138. Newton AC, Johnson JE. Protein kinase C: a paradigm for regulation of protein function by two membrane-targeting modules. Biochimica et biophysica acta. 1998;1376(2):155-72.
139. Li Y, Martin LD, Spizz G, Adler KB. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells in vitro. The Journal of biological chemistry. 2001;276(44):40982-90.
140. Disatnik MH, Boutet SC, Lee CH, Mochly-Rosen D, Rando TA. Sequential activation of individual PKC isozymes in integrin-mediated muscle cell spreading: a role for MARCKS in an integrin signaling pathway. Journal of cell science. 2002;115(Pt 10):2151-63.
141. Chappell DS, Patel NA, Jiang K, Li P, Watson JE, Byers DM, et al. Functional involvement of protein kinase C-betaII and its substrate, myristoylated alanine-rich C-kinase substrate (MARCKS), in insulin-stimulated glucose transport in L6 rat skeletal muscle cells. Diabetologia. 2009;52(5):901-11.
142. Ohmori S, Sakai N, Shirai Y, Yamamoto H, Miyamoto E, Shimizu N, et al. Importance of protein kinase C targeting for the phosphorylation of its substrate, myristoylated alanine-rich C-kinase substrate. The Journal of biological chemistry. 2000;275(34):26449-57.
143. Nakaoka T, Kojima N, Ogita T, Tsuji S. Characterization of the phosphatidylserine-binding region of rat MARCKS (myristoylated, alanine-rich protein kinase C substrate). Its regulation through phosphorylation of serine 152. The Journal of biological chemistry. 1995;270(20):12147-51.
144. Yamaguchi H, Shiraishi M, Fukami K, Tanabe A, Ikeda-Matsuo Y, Naito Y, et al. MARCKS regulates lamellipodia formation induced by IGF-I via association with PIP2 and beta-actin at membrane microdomains. Journal of cellular physiology. 2009;220(3):748-55.
145. Ikenoya M, Hidaka H, Hosoya T, Suzuki M, Yamamoto N, Sasaki Y. Inhibition of rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho-kinase inhibitor. Journal of neurochemistry. 2002;81(1):9-16.
146. Li J, O'Connor KL, Greeley GH, Jr., Blackshear PJ, Townsend CM, Jr., Evers BM. Myristoylated alanine-rich C kinase substrate-mediated neurotensin release via protein kinase C-delta downstream of the Rho/ROK pathway. The Journal of biological chemistry. 2005;280(9):8351-7.
147. Shiraishi M, Tanabe A, Saito N, Sasaki Y. Unphosphorylated MARCKS is involved in neurite initiation induced by insulin-like growth factor-I in SH-SY5Y cells. Journal of cellular physiology. 2006;209(3):1029-38.
148. Tatsumi S, Mabuchi T, Katano T, Matsumura S, Abe T, Hidaka H, et al. Involvement of Rho-kinase in inflammatory and neuropathic pain through

phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS). Neuroscience. 2005;131(2):491-8.
149. Satoh K, Matsuki-Fukushima M, Qi B, Guo MY, Narita T, Fujita-Yoshigaki J, et al. Phosphorylation of myristoylated alanine-rich C kinase substrate is involved in the cAMP-dependent amylase release in parotid acinar cells. American journal of physiology Gastrointestinal and liver physiology. 2009;296(6):G1382-90.
150. Hofmann F, Dostmann W, Keilbach A, Landgraf W, Ruth P. Structure and physiological role of cGMP-dependent protein kinase. Biochimica et biophysica acta. 1992;1135(1):51-60.
151. Matsubara T, Okumura N, Okumura A, Nagai K. cGMP-dependent phosphorylation and degradation of myristoylated alanine-rich C-kinase substrate. Biochemical and biophysical research communications. 2005;326(4):735-43.
152. Schonwasser DC, Palmer RH, Herget T, Parker PJ. p42 MAPK phosphorylates 80 kDa MARCKS at Ser-113. FEBS letters. 1996;395(1):1-5.
153. Tzlil S, Murray D, Ben-Shaul A. The "electrostatic-switch" mechanism: Monte Carlo study of MARCKS-membrane interaction. Biophysical journal. 2008;95(4):1745-57.
154. Allen LA, Aderem A. Protein kinase C regulates MARCKS cycling between the plasma membrane and lysosomes in fibroblasts. The EMBO journal. 1995;14(6):1109-21.
155. Yamamoto H, Arakane F, Ono T, Tashima K, Okumura E, Yamada K, et al. Phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) by proline-directed protein kinases and its dephosphorylation. Journal of neurochemistry. 1995;65(2):802-9.
156. Disatnik MH, Boutet SC, Pacio W, Chan AY, Ross LB, Lee CH, et al. The bi-directional translocation of MARCKS between membrane and cytosol regulates integrin-mediated muscle cell spreading. Journal of cell science. 2004;117(Pt 19):4469-79.
157. Kim SS, Kim JH, Lee SH, Chung SS, Bang OS, Park D, et al. Involvement of protein phosphatase-1-mediated MARCKS translocation in myogenic differentiation of embryonic muscle cells. Journal of cell science. 2002;115(Pt 12):2465-73.
158. Clarke PR, Siddhanti SR, Cohen P, Blackshear PJ. Okadaic acid-sensitive protein phosphatases dephosphorylate MARCKS, a major protein kinase C substrate. FEBS letters. 1993;336(1):37-42.
159. Seki K, Chen HC, Huang KP. Dephosphorylation of protein kinase C substrates, neurogranin, neuromodulin, and MARCKS, by calcineurin and protein phosphatases 1 and 2A. Archives of biochemistry and biophysics. 1995;316(2):673-9.
160. Westermarck J, Li SP, Kallunki T, Han J, Kahari VM. p38 mitogen-activated protein kinase-dependent activation of protein phosphatases 1 and 2A inhibits MEK1 and MEK2 activity and collagenase 1 (MMP-1) gene expression. Molecular and cellular biology. 2001;21(7):2373-83.
161. Yarmola EG, Edison AS, Lenox RH, Bubb MR. Actin filament cross-linking by MARCKS: characterization of two actin-binding sites within the phosphorylation site domain. The Journal of biological chemistry. 2001;276(25):22351-8.

162. Bubb MR, Lenox RH, Edison AS. Phosphorylation-dependent conformational changes induce a switch in the actin-binding function of MARCKS. The Journal of biological chemistry. 1999;274(51):36472-8.
163. Tapp H, Al-Naggar IM, Yarmola EG, Harrison A, Shaw G, Edison AS, et al. MARCKS is a natively unfolded protein with an inaccessible actin-binding site: evidence for long-range intramolecular interactions. The Journal of biological chemistry. 2005;280(11):9946-56.
164. Toledo A, Arruti C. Actin modulation of a MARCKS phosphorylation site located outside the effector domain. Biochemical and biophysical research communications. 2009;383(3):353-7.
165. Tang JX, Janmey PA. The polyelectrolyte nature of F-actin and the mechanism of actin bundle formation. The Journal of biological chemistry. 1996;271(15):8556-63.
166. Sundaram M, Cook HW, Byers DM. The MARCKS family of phospholipid binding proteins: regulation of phospholipase D and other cellular components. Biochemistry and cell biology = Biochimie et biologie cellulaire. 2004;82(1):191-200.
167. Myat MM, Anderson S, Allen LA, Aderem A. MARCKS regulates membrane ruffling and cell spreading. Current biology : CB. 1997;7(8):611-4.
168. Bogoyevitch MA, Court NW. Counting on mitogen-activated protein kinases--ERKs 3, 4, 5, 6, 7 and 8. Cellular signalling. 2004;16(12):1345-54.
169. Estrada-Bernal A, Gatlin JC, Sunpaweravong S, Pfenninger KH. Dynamic adhesions and MARCKS in melanoma cells. Journal of cell science. 2009;122(Pt 13):2300-10.
170. Sechi AS, Wehland J. The actin cytoskeleton and plasma membrane connection: PtdIns(4,5)P(2) influences cytoskeletal protein activity at the plasma membrane. Journal of cell science. 2000;113 Pt 21:3685-95.
171. McLaughlin S, Wang J, Gambhir A, Murray D. PIP(2) and proteins: interactions, organization, and information flow. Annual review of biophysics and biomolecular structure. 2002;31:151-75.
172. Kim J, Shishido T, Jiang X, Aderem A, McLaughlin S. Phosphorylation, high ionic strength, and calmodulin reverse the binding of MARCKS to phospholipid vesicles. The Journal of biological chemistry. 1994;269(45):28214-9.
173. Wang J, Arbuzova A, Hangyas-Mihalyne G, McLaughlin S. The effector domain of myristoylated alanine-rich C kinase substrate binds strongly to phosphatidylinositol 4,5-bisphosphate. The Journal of biological chemistry. 2001;276(7):5012-9.
174. Glaser M, Wanaski S, Buser CA, Boguslavsky V, Rashidzada W, Morris A, et al. Myristoylated alanine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. The Journal of biological chemistry. 1996;271(42):26187-93.
175. Laux T, Fukami K, Thelen M, Golub T, Frey D, Caroni P. GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. The Journal of cell biology. 2000;149(7):1455-72.
176. Spizz G, Blackshear PJ. Overexpression of the myristoylated alanine-rich C-kinase substrate inhibits cell adhesion to extracellular matrix components. The Journal of biological chemistry. 2001;276(34):32264-73.

177. Rombouts K, Lottini B, Caligiuri A, Liotta F, Mello T, Carloni V, et al. MARCKS is a downstream effector in platelet-derived growth factor-induced cell motility in activated human hepatic stellate cells. Experimental cell research. 2008;314(7):1444-54.
178. Monahan TS, Andersen ND, Martin MC, Malek JY, Shrikhande GV, Pradhan L, et al. MARCKS silencing differentially affects human vascular smooth muscle and endothelial cell phenotypes to inhibit neointimal hyperplasia in saphenous vein. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2009;23(2):557-64.
179. Micallef J, Taccone M, Mukherjee J, Croul S, Busby J, Moran MF, et al. Epidermal growth factor receptor variant III-induced glioma invasion is mediated through myristoylated alanine-rich protein kinase C substrate overexpression. Cancer research. 2009;69(19):7548-56.
180. Herget T, Oehrlein SA, Pappin DJ, Rozengurt E, Parker PJ. The myristoylated alanine-rich C-kinase substrate (MARCKS) is sequentially phosphorylated by conventional, novel and atypical isotypes of protein kinase C. European journal of biochemistry / FEBS. 1995;233(2):448-57.
181. Loilome W, Yongvanit P, Wongkham C, Tepsiri N, Sripa B, Sithithaworn P, et al. Altered gene expression in Opisthorchis viverrini-associated cholangiocarcinoma in hamster model. Molecular carcinogenesis. 2006;45(5):279-87.
182. Techasen A, Loilome W, Namwat N, Takahashi E, Sugihara E, Puapairoj A, et al. Myristoylated alanine-rich C kinase substrate phosphorylation promotes cholangiocarcinoma cell migration and metastasis via the protein kinase C-dependent pathway. Cancer science. 2010;101(3):658-65.
183. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Molecular & cellular proteomics : MCP. 2002;1(5):376-86.
184. Vlasova IA, Bohjanen PR. Posttranscriptional regulation of gene networks by GU-rich elements and CELF proteins. RNA biology. 2008;5(4):201-7.
185. Talwar S, Balasubramanian S, Sundaramurthy S, House R, Wilusz CJ, Kuppuswamy D, et al. Overexpression of RNA-binding protein CELF1 prevents apoptosis and destabilizes pro-apoptotic mRNAs in oral cancer cells. RNA biology. 2013;10(2):277-86.
186. Schwanhausser B, Gossen M, Dittmar G, Selbach M. Global analysis of cellular protein translation by pulsed SILAC. Proteomics. 2009;9(1):205-9.
187. Yu TX, Rao JN, Zou T, Liu L, Xiao L, Ouyang M, et al. Competitive binding of CUGBP1 and HuR to occludin mRNA controls its translation and modulates epithelial barrier function. Molecular biology of the cell. 2013;24(2):85-99.
188. Lambert N, Robertson A, Jangi M, McGeary S, Sharp PA, Burge CB. RNA Bind-n-Seq: quantitative assessment of the sequence and structural binding specificity of RNA binding proteins. Molecular cell. 2014;54(5):887-900.
189. Liu L, Ouyang M, Rao JN, Zou T, Xiao L, Chung HK, et al. Competition between RNA-binding proteins CELF1 and HuR modulates MYC translation and intestinal epithelium renewal. Molecular biology of the cell. 2015.
190. Wein G, Rossler M, Klug R, Herget T. The 3'-UTR of the mRNA coding for the major protein kinase C substrate MARCKS contains a novel CU-rich element interacting

with the mRNA stabilizing factors HuD and HuR. European journal of biochemistry / FEBS. 2003;270(2):350-65.
191. Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. Cell. 1996;84(3):359-69.
192. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. Science. 2003;302(5651):1704-9.
193. Hieronymus H, Silver PA. A systems view of mRNP biology. Genes & development. 2004;18(23):2845-60.
194. Rattenbacher B, Beisang D, Wiesner DL, Jeschke JC, von Hohenberg M, St Louis-Vlasova IA, et al. Analysis of CUGBP1 targets identifies GU-repeat sequences that mediate rapid mRNA decay. Molecular and cellular biology. 2010;30(16):3970-80.
195. Dormoy-Raclet V, Menard I, Clair E, Kurban G, Mazroui R, Di Marco S, et al. The RNA-binding protein HuR promotes cell migration and cell invasion by stabilizing the beta-actin mRNA in a U-rich-element-dependent manner. Molecular and cellular biology. 2007;27(15):5365-80.
196. Andrae J, Gallini R, Betsholtz C. Role of platelet-derived growth factors in physiology and medicine. Genes & development. 2008;22(10):1276-312.
197. Bornfeldt KE, Raines EW, Graves LM, Skinner MP, Krebs EG, Ross R. Platelet-derived growth factor. Distinct signal transduction pathways associated with migration versus proliferation. Annals of the New York Academy of Sciences. 1995;766:416-30.