**T.C.**
**DOKUZ EYLÜL UNIVERSITY**

**IZMIR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE**

# EPIGENETICS OF THE MESENCHYMAL TO EPITHELIAL TRANSITION

**İNCİ YAPRAK**

**DEPARTMENT OF GENOMICS AND MOLECULAR BIOTECHNOLOGY**

**Molecular Biology and Genetics**

**MASTER OF SCIENCE THESIS**

**IZMIR-2024**

**THESIS CODE**: DEU.IBG.MSc -2020850025

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Supervisor: Assoc. Prof. Dr. Hani Alotaibi

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## LIST OF ABBREVIATIONS

EMT: Epithelial-Mesenchymal Transition

MET: Mesenchymal-Epithelial Transition

TGFβ: Transforming Growth Factor Beta

TFs: Transcription Factors

ECM: Extracellular Matrix

miRNA: Micro RNA

circRNA: Circular RNA

LncRNA: Long Noncoding RNA

NMuMG: Normal Murine Mammary Gland Cell Line

MEF: Mouse Embryonic Fibroblast

MMPs: Matrix Metalloproteinases

ESRP1: Epithelial-specific regulatory protein 1

ESRP2: Epithelial-specific regulatory protein 2

HDAC1: Histone deacetylase 1

HDAC2: Histone deacetylase 2

BF: Bright Field

gDNA: Genomic DNA

BSA: Bovine Serum Albumin

Cdh1: E-Cadherin

Cdh2: N-Cadherin

Cebpα: CCAAT Enhancer Binding Protein Alpha

Fn1: Fibronectin

FBS: Fetal bovine serum

Grhl3: Grainyhead like transcription factor 3

Hnf4α: Hepatocyte nuclear factor-4 alpha

Ovol2: Ovo Like Zinc Finger 2

Klf4: Krüppel-like factor 4

Elf3: E74-like factor 3

Snai1: Snail Family Transcriptional Repressor 1

Snai2: Snail Family Transcriptional Repressor 2

Vim: Vimentin

Zeb1: Zinc Finger E-Box Binding Homeobox 1

Zeb2: Zinc Finger E-Box Binding Homeobox 2

Dnmt1: DNA (cytosine-5)-methyltransferase 1

Dnmt3a: DNA (cytosine-5)-methyltransferase 3a

Dnmt3b: DNA-methyltransferase 3 beta

Dnmt3l: DNA (cytosine-5)-methyltransferase 3-like

Uhrf1: Ubiquitin Like With PHD And Ring Finger Domains 1

siRNA: Small interfering RNA

PBS: Phosphate Buffer Saline

RT: Reverse Transcriptase

DIP: DNA Immunoprecipitation

MeDIP: Methylated DNA Immunoprecipitation

qPCR: quantitative polymerase chain reaction

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# EPIGENETIC OF THE MESENCHYMAL TO EPITHELIAL TRANSITION

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## ABSTRACT

Epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) are two evolutionarily conserved fundamental processes that take place at different stages of morphogenesis and organogenesis. These programs are a popular area of investigation because they play a role in physiological events such as embryogenesis and development and pathological events such as organ fibrosis and cancer. Although EMT and MET processes are genetic determinants of cellular plasticity, EMT is the first process that stands out. DNA methylation, histone modifications, histone variants, and non-coding RNAs; Epigenetic mechanisms such as lncRNAs, miRNAs, and circRNAs are also known to affect gene expression. Recent evidence suggests that MET is a dynamically coordinated process and not simply the opposite of EMT, progressing by the downregulation of EMT-inducing transcription factors and mesenchymal markers such as vimentin and N-cadherin and the upregulation of epithelial markers such as E-cadherin. MET has not been well investigated to date. MEF, a primary cell line with a low mutation rate and closer to in vivo conditions, was used to profile DNA methylation, one of the epigenetic mechanisms for the MET process. Grhl3 has been determined to be located in the promoters of DNA methylation enzymes and particularly prominent transcription factors. It shapes the chromatin organization by mediating the change of DNA methylation profile and the regulation of histone marks in the regulation of transcription of epithelial and mesenchymal genes. Therefore, integrating gene regulation with epigenetic markers stands out in terms of obtaining new gains for the MET process.

**Keywords:** EMT, MET, Grhl3, DNA methylation, Histone Marks, Epigenetics

# MEZENKİMAL EPİTELYAL DÖNÜŞÜMÜN EPİGENETİĞİ

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## ÖZET

Epitelyalden mezenkimal geçiş (EMT) ve mezenkimalden epitelyal geçiş (MET), morfogenez ve organogenezin farklı aşamalarında meydana gelen, evrimsel olarak korunan iki temel süreçtir. Bu programlar, embriyogenez ve gelişim gibi fizyolojik olayların yanı sıra organ fibrozisi ve kanser gibi patolojik olaylarda da rol oynaması nedeniyle popüler bir araştırma alanıdır. EMT ve MET süreçleri hücresel plastisitenin genetik belirleyicileri olsa da EMT öne çıkan ilk süreçtir. DNA metilasyonu, histon modifikasyonları, histon varyantları ve kodlanmayan RNA'lar; lncRNA'lar, miRNA'lar, circRNA'lar gibi epigenetik mekanizmaların da gen ekspresyonunu etkilediği bilinmektedir. Son kanıtlar MET'in dinamik olarak koordine edilmiş bir süreç olduğunu ve EMT'yi indükleyen transkripsiyon faktörlerinin ve vimentin ve N-kadherin gibi mezenkimal belirteçlerin aşağı regülasyonu ve E-kadherin gibi epitelyal belirteçlerin yukarı regülasyonu ile ilerleyen EMT'nin tam tersi olmadığını göstermektedir. MET bugüne kadar yeterince araştırılmamıştır.MET sürecinin epigenetik mekanizmalarından biri olan DNA metilasyonunun profilini çıkarmak için düşük mutasyon oranına sahip ve in vivo koşullara daha yakın bir birincil hücre çizgisi olan MEF kullanıldı. Grhl3'ün, DNA metilasyon enzimlerinin promotörlerinde ve özellikle belirgin transkripsiyon faktörlerinde yer aldığı belirlendi. Epitelyal ve mezenkimal genlerin transkripsiyonunun düzenlenmesinde DNA metilasyon profilinin değişmesine ve histon işaretlerinin düzenlenmesine aracılık ederek kromatin organizasyonunu şekillendirir. Bu nedenle gen regülasyonunun epigenetik belirteçlerle entegre edilmesi MET süreci için yeni kazanımlar elde edilmesi açısından öne çıkmaktadır.

**Anahtar kelimeler:** EMT, MET, Grhl3, DNA metilasyonu, Histon İşaretleri, Epigenetik

# 1. INTRODUCTION

## 1.1. Statement and Importance of the Problem

The cell acquires its identity through a complex network of autonomous and non-autonomous factors. The acquisition and sustainability of this identity are dynamically controlled and regulated to ensure homeostasis, depending on the microenvironment with which it interacts. EMT and MET processes are genetic determinants of cellular plasticity. Therefore, each cell's specific function requires the integration of genetic and epigenetic mechanisms controlled by gene expression patterns. Control of gene expression is a key process for various biological events such as development and differentiation, as well as being necessary for interpreting the complex nature of pathological processes. Since it is known that epigenetic mechanisms such as DNA methylation, histone modifications, histone variants, and long non-coding RNAs (lncRNA) also affect gene expression, MET is a dynamically coordinated process that reflects the opposite of its classical definition.

For this reason, vimentin is much more than the downregulation of mesenchymal markers such as N-cadherin and upregulation of epithelial markers such as E-cadherin. Although it has been reported that several transcription factors (TFs) function as molecular switches in regulating MET in the establishment of cellular plasticity regulated by epigenetic mechanisms, information is still insufficient when all known regulatory mechanisms are considered. A primary cell line with a low mutation rate and closer to in vivo conditions was used to profile DNA methylation, one of the epigenetic mechanisms, for the MET process. Integrating the transcription factors involved in the induction of this process in MEF cells and epigenetic markers in gene regulation is important in achieving gains for the MET process.

## 1.2. Aim of Study

This thesis aims to overexpress the Grhl3 transcription factor, one of the transcription factors that play a role in establishing epithelial characteristics, and to determine its effects on DNA methylation, one of the auxiliary mechanisms involved in regulating chromatin structure. In in vitro studies, MEF primary cell line was used to evaluate Grhl3 induction in terms of the MET process, and NMuMG was used as a positive control in comparison.

**Aim:**

To determine the capacity of Grhl3 to initiate MET in MEF cells and to study the accompanying epigenetic modulations.

**Objectives:**

1. To investigate the capacity to induce MET by overexpressing epithelial transcription factors in MEF cells exhibiting fibroblastic characteristics.
2. To examine the effect of transcription factors on protecting the MET process by using the RNAi-mediated silencing method.
3. To determine the methylation changes in the promoter regions of epithelial and mesenchymal markers with the overexpression of Grhl3, one of the transcription factors with a high capacity to direct MET.
4. To determine the activity of Grhl3, one of the transcription factors we highlighted in the MET process is in the promoters of DNA methylation enzymes, specifically prominent transcription factors, and the histone marks are located around the promoter.

### 1.3. Hypothesis of Study

Acute changes in the expression of the adhesion molecules E-cadherin and N-cadherin, in addition to the differentiation of the gene expression profile and the coordinated reorganization of the cytoskeleton, are the hallmarks of EMT and MET events.

Therefore, after the mature mRNA transcript obtained after the activation of the Cdh1 locus is folded and subjected to modifications following the translation process, cooperation is established with the relevant molecules and the systems they are involved in for its transport to the membrane. At this stage, we only focus on the factors that play a role in the rapid activation of the Cdh1 locus in the MET process and the maintenance of epithelial character. One of these factors, Grhl3, is a protein expressed at low levels in MEF cells. When this factor, which increases Grhl3 and Cdh1 transcript levels when overexpressed, is knocked down, the cell can no longer maintain the MET state. The balance of division, differentiation, and apoptosis of cells is disrupted. The same situation is seen in NMuMG cells used as a positive control after the knockdown of Grhl3 during MET. Cells become trapped in the mesenchymal state. Grhl3, one of the epithelial markers, is thought to play a role in reprogramming the cell in terms of metabolism and reshaping its chromatin. In this context, the presence of Grhl3 is associated with the methylation of epithelial and mesenchymal genes associated with MET.

# 2. GENERAL INFORMATION

## 2.1. Cellular Plasticity

Epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) appear as two evolutionarily conserved, fundamental processes that direct the anatomy, physiology, and behavior of metazoan organisms and take place at different stages of morphogenesis and organogenesis, shaping tissue and tissue organization. These programs play a role in physiological events such as embryogenesis and development and pathological events such as organ fibrosis and cancer. Morphogenesis requires spatial and temporal control of embryo mechanics to facilitate cell movement and conformational changes. Organogenesis is the process of development of the organism from fetus to embryo. It is essential to elucidate these two fundamental processes, which are at the basis of the formation of many tissues and organs during development, from a general perspective. Only in this way is it possible to accurately interpret the complex action-response mechanisms that cause deviations from the characteristic function. In order to capture this perspective, one should start with the cell, the smallest unit of the living thing that can display structural and functional features.

Although they contain the same set of genes and cell types, each has its characteristics and different bodily functions. This variability is determined by genetic and epigenetic mechanisms that respond differently in each cell, controlled by gene expression patterns. Therefore, controlling gene expression is a key process for various biological events such as development and differentiation. In addition to genetic mechanisms, epigenetic mechanisms such as DNA methylation, histone modifications, histone variants, and long non-coding RNAs (lncRNA) are also known to have an impact on gene expression, especially at the transcription level (Gibney and Nolan 2010). Cellular plasticity refers to a cell's ability to adapt to changing conditions. Adaptation may be reversible, or the cell may acquire an irreversible identity. EMT and MET processes are genetic determinants of cellular plasticity. Therefore, understanding how a cell's identity is determined and regulated in different cell types is crucial for manipulating this plasticity.

In addition to the coordinated cooperation towards the differentiation of the gene expression profile and the rearrangement of the cytoskeleton, the acute change in the expression of the adhesion molecules E-cadherin and N-cadherin is the distinguishing feature of EMT and MET events. Although EMT is understood through a comprehensive approach, the regulation of MET is still far from understood. Therefore, it is necessary to correlate such factors with Cdh1 expression to identify the mechanisms involved in the regulation of MET. Just because adaptation is reversible does not mean it is a simple process. MET should be considered a fundamental program on its own, rather than just the reversal of EMT, because MET initiation, although associated with the

downregulation of EMT inducers, is tightly regulated by transcriptional and post-transcriptional mechanisms. Since the reorganization of the plasticity that the cell gains as a reflection of its response to the effects of stimuli is aimed at changing the existing situation, it does not mean that the mechanisms that direct the reversible situation work with the same coordination. Reflecting the dynamic regulation of MET by transcriptional and post-transcriptional mechanisms is Cdh1. We speculate that the process from the initiation of expression of Cdh1 to the eventual presence of E-cadherin at the plasma membrane is necessary for proper entry into MET.

In this context, we focused on DNA methylation to interpret the induction and post-induction changes of MET from an epigenetics perspective.

### 2.2. Epithelial-Mesenchymal Transition (EMT)

Epithelial–mesenchymal transition or EMT is a biological process by which cuboidal, tightly packed, and non-motile epithelial cells adopt a loosely organized mesenchymal or fibroblast-like phenotype with properties such as reduced intercellular adhesion, loss of apical-basal polarity, a gain of motility and invasive ability, increased resistance to apoptosis, and enhanced ability of ECM production (Debnath et al., 2022). Depending on the tissue and signaling contexts, epithelial cells may lose only some characteristics or may show some epithelial and mesenchymal properties; this can be considered as partial EMT. Epithelial plasticity covers changes in differentiation and cell behavior, with complete epithelial integrity at one end and EMT as the full realization of a plasticity response at the other end (Huang et al., 2012). EMT (type I) was initially identified as a critical program during early embryonic morphogenesis and was found to be involved in various developmental stages such as gastrulation, neural crest formation, and heart morphogenesis. EMT (type II) was found to be induced in response to inflammation, for example, during wound healing, tissue regeneration, and fibrosis. EMT (type III) program was shown to be activated during metastasis (Figure 1), which is the primary cause of mortality in cancer patients (Debnath et al., 2022; Lee et al., 2006). Also, EMT has been found to induce other properties, such as the acquisition of stem cell-like phenotype, resistance to chemotherapy, immune evasion, etc, in cancer cells, thus making these cells difficult to eradicate (Debnath et al., 2022). The fact that cells sometimes exhibit hybrid properties expressing both epithelial and mesenchymal markers reflects the complexity of the EMT mechanism.

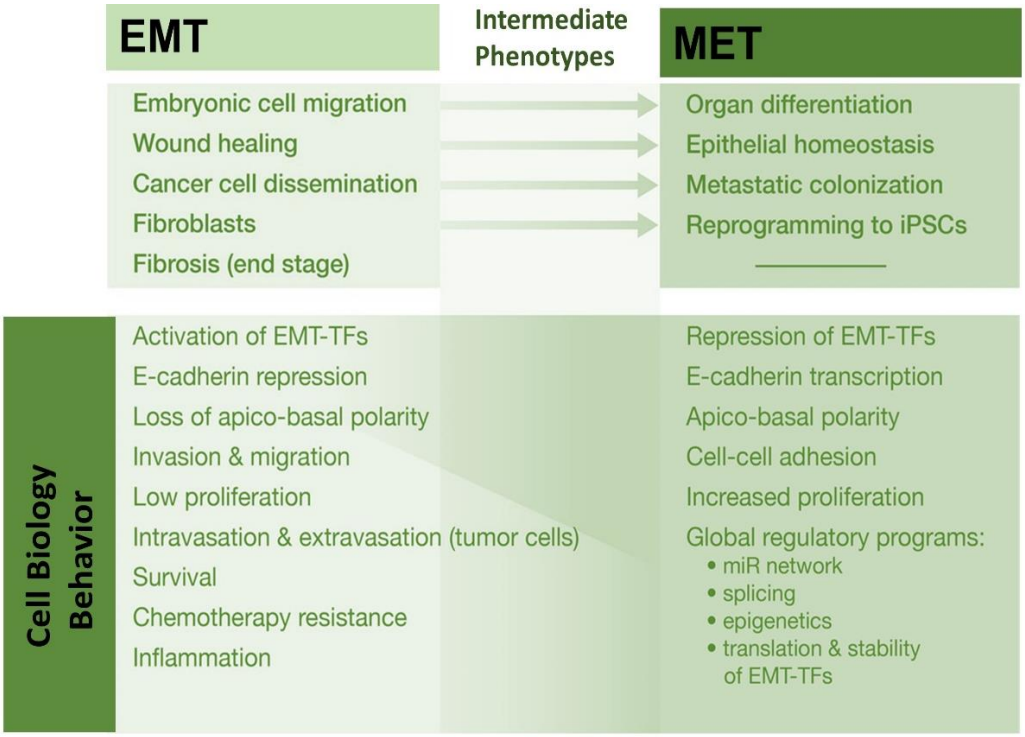


**Figure 1. The control of epithelial plasticity in development and disease.** ( Adapted from Nieto, 2013).

### *2.2.1. Cellular changes during EMT*

Coordinated collaborations of protein structures on the cell surface play a major role in connecting epithelial cells to establish epithelial integrity. Vertebrate cells contact each other through subapical tight junctions, adherens junctions and desmosomes at lateral surfaces, and scattered gap junctions at lateral surfaces (Huang et al., 2012; Lamouille et al., 2014). Induction of EMT requires disruption of the structure of protein complexes involved in the maintenance of epithelial integrity and subsequent rearrangement of their localization. During the EMT process, tight junctions dissolve, claudin and occludin expression levels decrease, and zonula occludin 1 diffuses from cell-cell contacts. Adherent junctions are cleaved through a process that culminates in the cleavage and subsequent degradation of the epithelial cadherin (E-cadherin) at the plasma membrane (Figure 2).

Consequently, β catenin can no longer interact with E-cadherin, and it is either degraded or protected from degradation so that it can act in transcription (Niehrs, 2012). p120 catenin also accumulates in the nucleus and participates in transcription following a decrease in E-cadherin levels (Kourtidis et al., 2013). In order to maintain EMT, the expression of junctional proteins must be transcriptionally suppressed, thus stabilizing the formation of epithelial junctions. The steps involved in the activation of EMT can be summarized as follows. First, the epithelial tissue must be rearranged in a morphogenic manner. Disruption of the interaction between epithelial cells and the

basement membrane must then occur through proteolytic degradation. The migrating cells must then separate from the epithelial layer. Finally, the cells must differentiate into a mesenchymal phenotype, changing cell-ECM interactions, cytoskeletal organization, and fundamental aspects of cellular metabolism. Therefore, EMT requires a comprehensive organization covering the entire epithelial tissue.

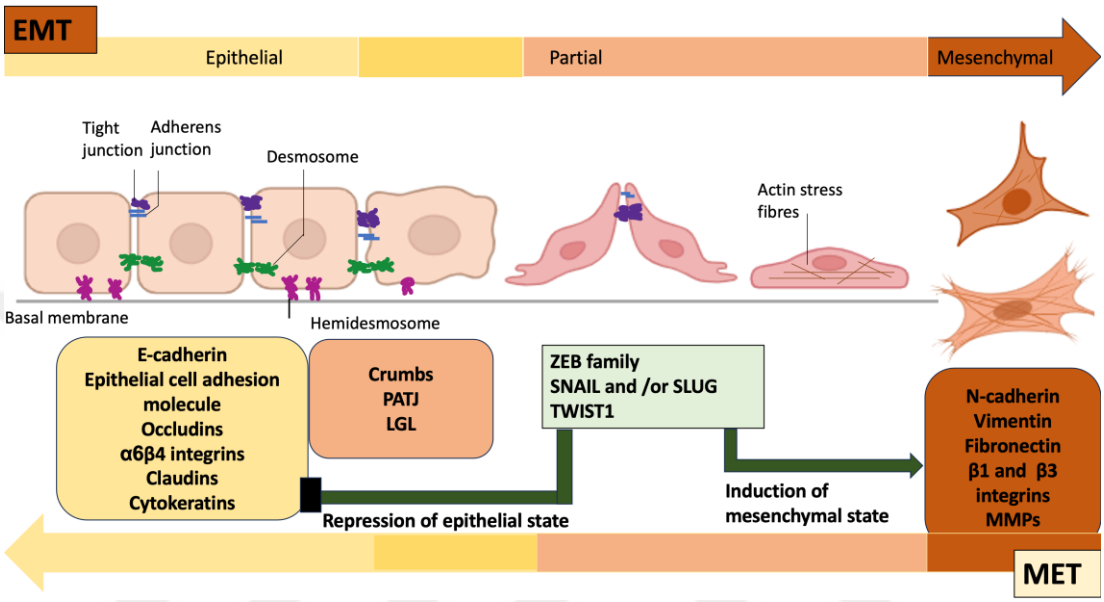


**Figure 2. Outline of a typical EMT program** (Adapted from Dongre & Weinberg, 2019).

### *2.2.2. EMT's Regulatory Networks*

EMT is one of the regulation processes of cell plasticity. In this process, all known regulatory mechanisms function in coordination. The entire function of the protein synthesized following regulations such as epigenetic modifications, transcriptional controls, and alternative splicing depends on many factors. Post-translational regulations, the compartment in which it is active within the cell, and the microenvironment in which it is located have a complex relationship in regulating signal communication. In order to fully understand any mechanism within the cell, the change to be examined is evaluated in isolation. This isolation is insufficient to summarize and accurately evaluate the complex process. Therefore, reflecting the mechanism involved in the developmental process may not have the same effect in the pathological situation. Despite efforts to identify common regulatory networks in normal development and disease, it is clear that some pathways may be specific to a particular EMT event in a particular tissue or tumor subset (Nieto et al., 2016).

The EMT-TFs that have major roles in orchestrating EMT programs include the zinc-finger E-box binding homeobox factors ZEB1 and ZEB2, SNAIL, SLUG, and the basic helix–loop–helix factors TWIST1 and TWIST2

(Dongre & Weinberg, 2019). These transcription factors, which play a role in the EMT transition, are responsible for regulating each other and, in general, activating or repressing many genes that cause changes in the cell's behavior. The acquisition of a fully mesenchymal state is related to the dynamics within the cell of the regulations that mediate the suppression of the epithelial state. E-cadherin and N-cadherin, which are adhesion proteins affected by changes in EMT and MET processes following fluctuations in gene expression, are proteins that play a role in the observable reflection of the regulation of the cytoskeleton on morphology. Downregulation of E-cadherin is crucial for successful EMT (Wells et al., 2008). These transcription factors bind to various E-boxes in the Cdh1 promoter and cause repression of E-cadherin expression (Bolós et al., 2016; Serrano-Gomez et al., 2016). SNAIL represses the E-cadherin-encoding gene, CDH1, by binding to E boxes in the CDH1 promoter and recruiting the polycomb repressive complex (Herranz et al., 2008).EMT-TFs, SNAIL, and ZEB1 also directly control the expression of several genes associated with cell polarity (Spaderna et al., 2008). Additionally, SNAIL and ZEB2 activate the expression of matrix metalloproteinases (MMPs) that facilitate the degradation of the basement membrane, promoting cell invasion (Miyoshi et al., 2005).

Understanding of EMT has evolved to explain how their transcripts are expressed and processed by miRNAs (Lamouille et al., 2013) or alternative splicing (Warzecha & Carstens, 2012). It is unrealistic for EMT inducers to dominate this entire system by simply binding to the promoter of the Cdh1 gene. ZEB1 and SNAI1, as well as miR-200 and miR-34 (Puisieux et al., 2014), play a role in regulating each other by negative feedback to maintain epithelial homeostasis under normal conditions. Proteins involved in cell-cell adhesion, cell motility, and cell-matrix adhesion are regulated by alternative splicing mediated by epithelial-specific regulatory proteins 1 and 2 (ESRP1 and ESRP2) (Warzecha et al., 2009) to ensure maintenance of epithelial properties. Epithelial markers such as E-cadherin and claudins are the downstream targets of EMT-inducing factors that play a role in acquiring mesenchymal character and all system components involved in processing transcripts and their transformation into mature forms.

Upstream and downstream epigenetic controls primarily affect the EMT miRNA-SNAIL-ZEB networks, as these EMT transcriptional drivers are involved in a complex epigenetic regulatory loop (Bedi et al., 2014) SNAI1 and ZEB1 recruit chromatin modifiers, histone deacetylase 1 (HDAC1), (HDAC2).

Spatial and temporal regulation of many proteins is achieved by post-translational modification. At the protein level, EMT is also regulated by post-translational modifications, including the ubiquitin-mediated degradation of critical effectors, such as that following the phosphorylation of Snai1 and Twist1 by GSK3b or MAP kinases, respectively (Hong et al., 2011).

EMT can be induced by intracellular signaling mechanisms when epithelial cells are exposed to signals released by cells in their

microenvironment. Various signaling networks, including hypoxia, TGFβ, Wnt, Hedgehog, and Notch signaling (Thiery et al., 2009), mediate broad transcriptional regulation by recruiting epigenetic regulatory proteins and activating EMT-inducing transcription factors.

### 2.3. Mesenchymal to Epithelial Transition (MET)

Mesenchymal cells can revert to an epithelial state, known as mesenchymal-epithelial transition (MET). MET is employed to generate epithelia at different developmental stages. During MET, mesenchymal cells progressively establish apicobasal polarity through an evolutionarily conserved group of proteins and distinct but well-defined mechanisms: polarity-related cell-surface receptors generate different membrane domains and become stabilized with the assembly of junctional complexes, leading to the formation of tight junctions at the apex of the lateral domain and the organization of cytoskeletal structures and organelles (Pei et al., 2019). EMT and MET also regulate embryonic stem cell differentiation, induced pluripotency, and cancer stem cell behavior (Lamouille et al., 2014).

Until recently, MET was viewed solely as an inverse process of EMT; this dynamic process has been constrained by known EMT regulators. Although the reduction of E-cadherin and suppression of EMT inducers such as Zeb1/2 and Snai1/2 are hallmarks of EMT (Adhikary et al., 2014), they are not limited to EMT inducers. Several transcription factors (TFs) also work as molecular switches to maintain cellular plasticity regulated by epigenetic mechanisms (Skrypek et al., 2017), suggesting that MET is not simply the reversal of the EMT process but rather a fundamental process governed by distinct regulatory cues. Different studies show that the reduction in expression of key EMT regulators and their dissociation from the Cdh1 promoter region is insufficient for rapid activation of the Cdh1 locus during the MET process (Alotaibi et al., 2015). The Cdh1 promoter alone, including all known E-box elements, is insufficient to provide unequivocal cell type specificity. In contrast, intron 2 carries sufficient information for proper E-cadherin expression (Stemmler et al., 2005). Although the second intron has emerged as a key regulator of Cdh1 expression (Alotaibi et al., 2015; Stemmler et al., 2005; Stemmler et al., 2003), little is known about the molecular determinants that control the transcriptional activity of the locus.

The pluripotent embryonic stem (ES) cells in the inner mass of the blastocyst have epithelial characteristics (Eastham et al., 2007). In gastrulation, pluripotent epithelial epiblast cells ingress to form, through EMT, the primary mesoderm (Acloque et al., 2009). EMT thus represents an initial differentiation event in the generation of the three germ layers from pluripotent cells. Illustrating the importance of EMT in early differentiation, ES cell or epiblast cell colonies in culture give rise to peripheral cells with a mesenchymal phenotype, as judged by loss of epithelial cadherin (E cadherin) expression and the expression of vimentin and neural cadherin (N-cadherin) (Ullmann et al., 2007).

Somatic cell reprogramming by a combination of Oct4, Sox2, Klf4, and Myc (Takahashi & Yamanaka, 2006) provides an ideal in vitro model to investigate the regulation of cell fate changes (Figure 3). Accumulating evidence indicates that MET is essential during early somatic cell reprogramming in mouse embryonic fibroblasts (MEFs) and human fibroblasts(Subramanyam et al., 2011) and that blocking this step can impair reprogramming efficiency. In the initial stages of MEF reprogramming, Sox2, Oct4, and Myc suppress TGFβ-Snail signaling, whereas Klf4 induces the epithelial program (R. Li et al., 2010), with a net effect of downregulating mesenchymal proteins and upregulating epithelial proteins, as confirmed by proteomics analyses (Hansson et al., 2012).

At the stage when fibroblast cells are programmed into induced pluripotent stem cells with various factors, the MET transformation of the cells has already begun. Reducing the effects of the factors that induce EMT and the signaling mechanisms involved will not be sufficient to eliminate all effects involved in the maintenance of initial plasticity. Therefore, even if a good knowledge of EMT helps interpret this entire process, its effect on the knowledge of the processes that manage MET is still insufficient in the literature.

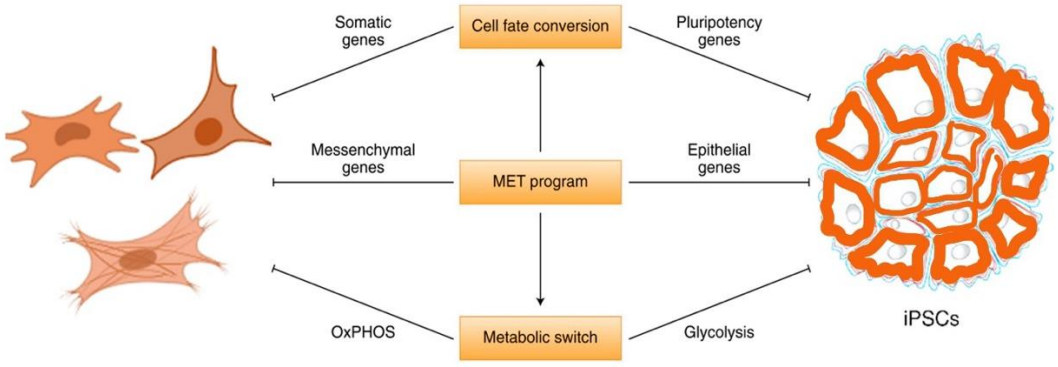


**Figure 3. Sequential EMT and MET for reprogramming of somatic cells**. Significant changes in the somatic reprogramming of MEFs. The OSKM factors silence somatic genes in MEFs and induce pluripotency genes during the late stages of reprogramming. A metabolic switch from oxidative phosphorylation (OxPHOS) to glycolysis occurs during reprogramming. MET is an essential early step in reprogramming, as it remodels the mesenchymal cytoskeleton and cell-surface protein expression to adopt an epithelial-like morphology and facilitates metabolic and cell fate changes (Adapted from Pei et al., 2019).

### *2.3.1. Regulation of MET*

The ability of a gene to be expressed and function under certain conditions depends on many conditions. The formation of the chromatin infrastructure in the presence of both histone modifications and histone variants, transcription factors responsible for the activation or repression of the gene regulate each other and the relevant gene through physical interaction,

regulations carried out by miRNA, lncRNA, alternative splicing, cell metabolism is reprogrammed as a result of all these processes.

Identifying the components and functions involved in the dynamics of the MET process to ensure the homeostasis of the cell is important for understanding developmental and pathological conditions. Both of these processes are quite comprehensive. Although there is a lot of data on EMT, the data on MET are still insufficient. Due to the urgency of the conditions, the main aim has brought to the fore studies on pathological conditions affected by many mutations and factors instead of their function in the physiological process. Investigation of MET, which lags behind EMT and becomes more critical with metastasis, will provide better information about how it contributes to essential biological events such as regulating chromatin infrastructure, epigenetics, pluripotency, cellular homeostasis, and metastasis.

### *2.3.2. Transcription Factors*

While many publications containing many mechanisms regarding the regulation of EMT attract attention in the literature, only the factors that induce EMT to stand out from the regulation of MET. Although the change in binding affinities is emphasized, the intermediate steps are missing information about why it is associated with EMT factors and the regulation of the molecules with which it interacts. Therefore, there are very few publications investigating the mechanisms underlying MET regulation.

However, studies conducted in recent years draw attention to factors such as Grhl2/3, Hnf4α, and Ovol1/2, which are involved in dynamic transcriptional processes during EMT-MET (Alotaibi et al., 2015).

#### *2.3.2.1. Grainy head-like proteins*

Grainy head transcription factors are conserved factors that regulate epidermal barrier formation (Yu et al., 2009). In vertebrates, genes similar to Drosophila grainy head are called Grhl (grainy head-like) genes, and there are three known members: Grhl1, Grhl2, and Grhl3(Chalmers et al., 2006). It is known that the expression of these genes is specific for tissue and development and is expressed in many ectodermal and endodermal cell types during embryonic development and in adult skin (Mlacki et al., 2015).

GRHL proteins protect epidermal integrity by changing the chromatin infrastructure (Sundararajan et al., 2020). The most common function of Grh and GRHL family members is epidermal differentiation (Yu et al., 2009). They also show common features during developmental and pathological events, such as epigenetic regulation, chromatin remodeling, and differentiation (Sundararajan et al., 2020) (Figure 4).

GRHL3 (grainy head-like 3), previously referred to as GET1, MGR, LBP-32, SOM, and BOM, is a highly conserved transcription factor responsible for epidermal differentiation and barrier formation during mouse embryogenesis

(Chalmers et al., 2006). Spina bifida, a disease that occurs as a developmental defect of the neural tube, is observed in Grhl3 knock-out mice (De Castro et al., 2018). Mice lacking Grhl3 cannot form a suitable skin barrier and die from dehydration as soon as they are born (Goldie et al., 2019). GRHL3 is a critical factor for the development and homeostasis of the surface ectoderm in many species (Kimura-Yoshida et al., 2018). Its origin is based on the fruit fly (Drosophila) grainy head (grh) gene. Grh is a protein that plays a critical role in cuticle formation and repair mechanism in Drosophila and is predominantly expressed in the surface ectoderm. Grainyhead-mutated embryos fail to hatch and have loose cuticles, a vascularized and discontinuous skeleton, and an irregular tracheal tube (Gustavsson et al., 2008).

Grh orthologs are known to be required for many different functions in animal epithelial cells (Hislop et al., 2008). One of the conserved functions in mice and fruit flies is the transcriptional activation of epidermal barrier cross-linking genes and the regeneration of this epidermal barrier after wounding (Mace et al., n.d.) Studies conducted in recent years also emphasize the place of GRHL3 in cancer studies. It is known that GRHL factors are associated with cancer types such as skin squamous cell carcinoma, breast cancer, stomach cancer, hepatocellular carcinoma, colorectal cancer, prostate and cervical (Kotarba et al., 2020).

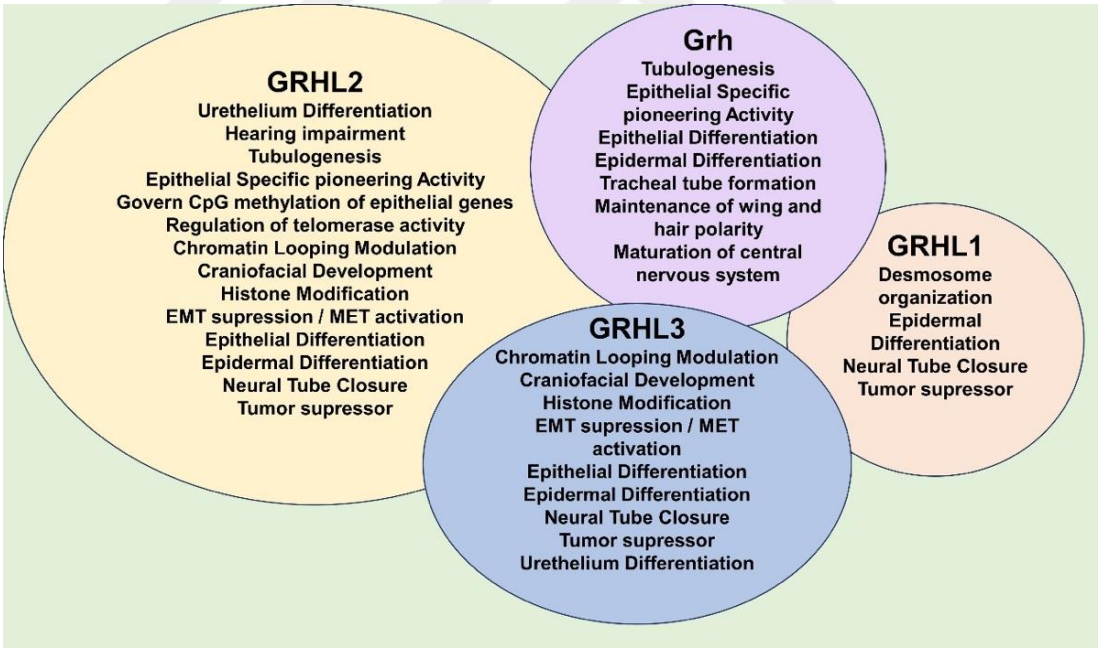


**Figure 4. Common features of Grh and GRHL family members**. (Adapted from Sundararajan et al., 2020).

### *2.3.2.2. Hnf4α*

Hepatic differentiation factor Hnf4α is a transcription factor belonging to the transcriptional nuclear receptor superfamily that is abundantly expressed

in the liver, kidney, and intestine in a tissue-specific manner (Z. Wang et al., 2020). It is necessary for maintaining cellular homeostasis, maintaining inflammation, and regulating the epithelial phenotype and lipid and glucose metabolisms (Darsigny et al., 2009). The balance between EMT coregulators and Hnf4α affects EMT-MET events (Moore et al., 2016).

Grhl3/Hnf4α interaction is responsible for the stabilization of the epithelial infrastructure through suppression of the mesenchymal program. According to previous studies performed at the Alotaibi Laboratory, Grhl3 was determined to contribute to the regulation of Hnf4α during MET in NMuMG cells (Alotaibi et al., 2015) (Figure 5).

The dual function of Hnf4α as a key factor in reprogramming transcriptional factors and modulating epigenetic changes is required for the complex nature of EMT-MET cellular programs.

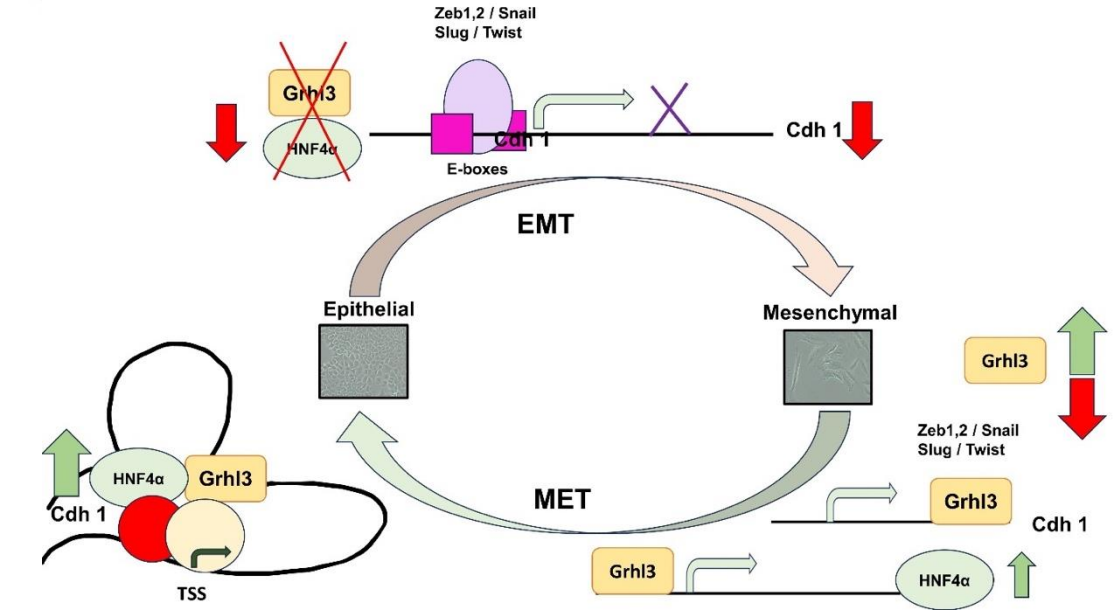


**Figure 5. Proposed model of Grhl3 and Hnf4α mediated looping and activation of the Cdh1 locus during MET**. (Adapted from Alotaibi et al., 2015).

### *2.3.2.3. Elf3*

Ets transcription factors are one of the largest transcription factor families, consisting of approximately 30 members (27 in humans and 26 in mice) that can be expressed ubiquitously and tissue-specific. Ets transcription factors are characterized by conserved DNA binding domains called ETS domains, which bind to a typical 5'-GGA (A/T)-3 DNA sequence (Cooper et al., 2014). Members of this family are trans-acting phosphoproteins that can act as upstream and downstream effectors of many signaling pathways, including MAP kinases, Erk1/2, p38, and JNK (Otero et al., 2012). Therefore, these factors have important roles in many biological processes, such as

differentiation, development, proliferation, apoptosis, tissue remodeling, and EMT/MET (Hsu et al., 2004).

ELF3 is involved in the regulation of MET by changing the expected localization of E-cadherin in the plasma membrane. Again, in the MET process, Grhl3 can regulate the expression of Grhl3 by binding to the protected regions on the promoter region. Loss of Elf3 expression disrupts the initiation of MET and causes cells to remain in a mesenchymal state (Sengez et al., 2019). Considering all the data, a positive feedback network involving Grhl3 and Elf3 can be mentioned.

#### *2.3.2.4. Cebpα*

CCAAT/enhancer-binding protein Cebpα is a member of the bZIP (basic leucine zipper) transcription factor family (Tsukada et al., 2011). Cebpα is an intronless gene containing three transactivation domains, a regulatory region, and a bZIP region (Birkenmeier et al., 1989). Cebpα protein has two isoforms, 42 kDa and 30 kDa long, namely p42 and p30, respectively (Reckzeh & Cammenga, 2010).

It has essential biological functions such as regulation of hepatogenesis, adipogenesis, and hematopoiesis in many cell types (Yuan et al., 2015). C/EBP proteins are also known to be in a complex with p300 (Dhawan et al., 2005). Cebpα has been detected in some cell types to associate with chromatin regulatory proteins p300 and HDAC1 (G. L. Wang et al., 2008).

#### *2.3.2.5. Klf4*

Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor first identified in a screen for transcription factors involved in growth regulation (Shields et al., 1996). It is associated with terminal differentiation and the homeostasis of various epithelial tissues, including its role in maintaining the stability of adherens junctions and establishing the barrier function of the skin (Cowan et al., 2010). Klf4 transcriptionally regulates proliferation, differentiation, apoptosis, and somatic cell reprogramming in various physiological and pathological processes (Ann Garrett-Sinha et al., 1996; Rowland & Peeper, 2006; Takahashi & Yamanaka, 2006). Given its stem cell-promoting activity and ability to regulate growth and differentiation during development, it is unsurprising that KLF4 also plays various roles in tumorigenesis. Klf4 plays a divergent role in various cancers, depending on different cell contexts.

At the molecular level, KLF4 has been shown to inhibit and be inhibited by both SNAIL (SNAI1) (Z. Li et al., 2018), two of the members of the SNAI superfamily that can induce EMT to varying degrees. There is increasing knowledge in the literature on the role of KLF4 in inhibiting EMT driving MET in different biological contexts. For instance, in the colon epithelial cell line RKO, KLF4 upregulates the levels of various epithelial-specific keratins, such

as KRT8 and KRT18 (Chen et al., 2003). Furthermore, E-cadherin is a direct transcriptional target of KLF4, and this regulation is thought to be essential for maintaining the epithelial phenotype in mammary epithelial cells (Yori et al., 2010). As a potential MET-TF, it is included as an additional transcription factor in our list, which includes Ovol1/2, Grhl1/2/3, Cebpα, Elf3, and Hnf4α.

### *2.3.3. Epigenetic Regulation of EMT/MET*

Epigenetic modifications are changes that are independent of changes arising from the primary DNA sequence and do not alter the corresponding DNA sequence. It creates heritable changes in the gene expression profile by influencing the outcome of the locus located in the chromosome. The epigenetic landscape, encompassing DNA methylation, histone code, non-coding RNA and nucleosome positioning, and DNA sequence, has become increasingly complex (Jin et al., 2011) (Figure 6).

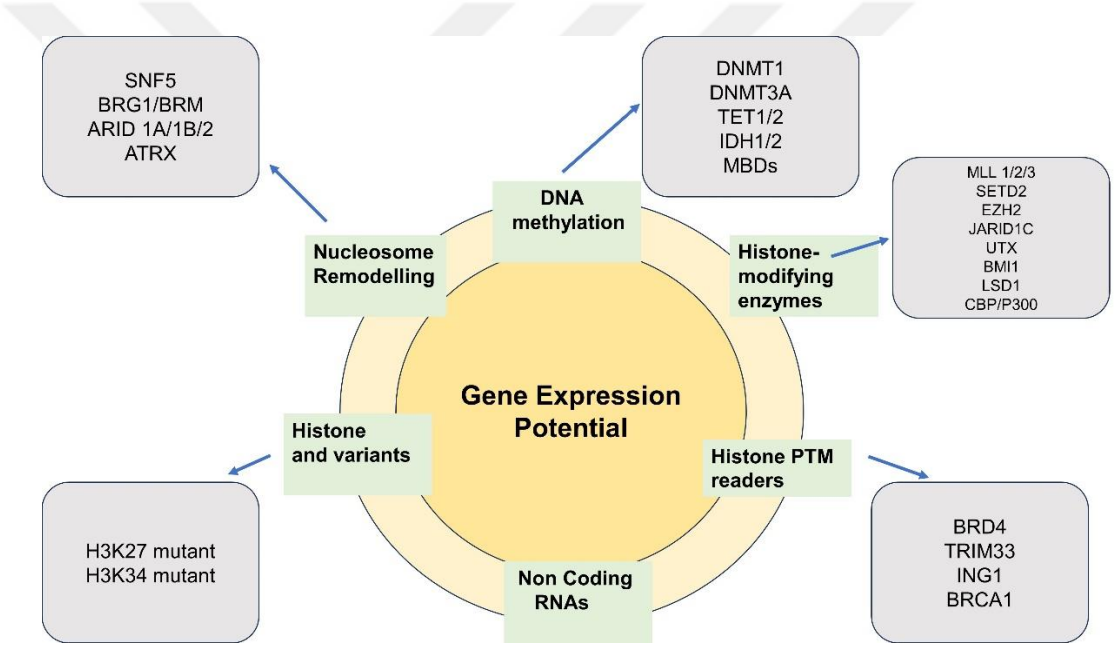


**Figure 6. The drawing shows the input of epigenetic processes in specifying gene expression patterns**. (Adapted from You & Jones, 2012).

These modifications are tightly regulated by several enzymes, which may act isolated or in chromatin remodeling complexes and grouped according to function. These epigenetic enzymes include DNA methyltransferases (DNMTs) and demethylases (TETs), histone methyltransferases (HTMs) and demethylases (HDMs), histone acetyltransferases (HATs) and deacetylases (HDACs), and histone ubiquitin ligases (UbLs) and deubiquitinases (dUbs) (Baylin & Jones, 2016).

DNA methyltransferases (DNMTs), responsible for the transfer of a methyl group from the universal methyl donor, *S*-adenosyl-L-methionine (SAM), to the 5-position of cytosine residues in DNA, are essential for

mammalian development (Robertson, 2005). In plants, cytosines are methylated in symmetrical (CG or CHG) or asymmetrical (CHH, where H is A, T, or C) contexts. In mammals, DNA methylation occurs at cytosines in any context of the genome (Kareta et al., 2006). Most DNA methylation is essential for normal development, and it plays a crucial role in a number of key processes, including genomic imprinting, X-chromosome inactivation, and suppression of repetitive element transcription and transposition and, when dysregulated, contributes to diseases like cancer (Jin et al., 2008; Robertson, 2005). DNA methylation is regulated by a family of DNMTs: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Bestor, 2000).

Dnmt1 preferentially methylates hemimethylated DNA. Additionally, Dnmt1 also can repair DNA methylation (Mortusewicz et al., 2005).For this reason, Dnmt1 is called the maintenance Dnmt because it maintains the original pattern of DNA methylation in a cell lineage. Dnmt3a and Dnmt3b are highly similar in structure and function. Unlike Dnmt1, both Dnmt3a and Dnmt3b, when overexpressed, are capable of methylating both native and synthetic DNA with no preference for hemimethylated (M et al., 1999). For this reason, Dnmt3a and Dnmt3b are referred to as de novo Dnmt because they can introduce methylation into naked DNA. A final member of the Dnmt family is Dnmt3L, a protein that lacks the catalytic domain present in other Dnmt enzymes. Dnmt3L is mainly expressed in early development and is restricted to the germ cells and thymus in adulthood (Aapola et al., 2000).

DNA methylation effects on transcriptional regulation differ depending on the location of the CpG site (intragenic vs promoter region vs enhancer). Most of the genome does not contain CpG sites. However, clusters of CpG sites, termed ‘CpG islands’, occur and commonly span promoters of housekeeping genes. These promoter CpG islands typically remain unmethylated, resulting in active gene expression (Stirzaker et al., 2014). Methylated CpG island promoters are associated with gene repression. DNA methylation frequently occurs in repeated genetic sequences and helps to suppress the expression and mobility of transposable elements and retroviral elements such as long interspersed nuclear element 1 (LINE-1) (Schulz et al., 2006).

DNA hydroxymethylation is another DNA modification in which the hydrogen atom at the C5 position is replaced by a hydroxymethyl (Guibert & Weber, 2013). This recent type of DNA modification gained interest due to its biological and epigenetic roles in cellular systems, including differentiation, development, and pluripotency (Richa & Sinha, 2014). 5-hydroxymethylcytosine (5-hmC) is formed by oxidation of 5mC by the Ten-Eleven Translocation (TET) family of proteins, including TET1, TET2, and TET3 are considered as “6th base” (Santiago et al., 2014). It is an intermediate step in active DNA demethylation reaction and essential for chromatin factors (Klug et al., 2013).

DNA methylation can coordinately regulate the chromatin state through the interaction of DNMTs with other modifications and components of the machinery that mediates these marks (Jin et al., 2011).

#### *2.3.3.1. DNA Methylation and Histone Modifications*

Covalent modification of DNA and histone proteins, the core components of chromatin, provides a heritable mechanism for regulating gene expression. Histone tails undergo a variety of covalent modifications, including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation, regulating key cellular processes such as gene transcription, DNA replication, and DNA repair. During mammalian development and cancer, DNA methylation and specific histone modifications appear to influence each other in deposition reciprocally: histone methylation may direct DNA methylation patterns, and DNA methylation may serve as a template for the establishment of specific histone modifications after DNA replication (Cedar & Bergman, 2009) (Figure 7).

Dnmt3L binds to H3 histone tails and recruits Dnmt3a and Dnmt3b to methylate DNA (Ooi et al, 2007). However, the presence of the active histone modification H3K4 trimethylation (H3K4me3) impairs the binding of Dnmt3a, Dnmt3b, and Dnmt3L to H3 histone tails and prevents methylation (Ooi et al, 2007; Zhang et al, 2010). CpG islands contain particularly high levels of H3K4me3 (Mikkelsen et al, 2007).

Methyl-binding proteins serve as the strongest link between DNA methylation and histone modification. Both the MBDs and the UHRF proteins interact with methylated DNA and histones to enhance gene repression (Nan et al, 1998; Ng et al, 1999; Sarraf and Stancheva, 2004; Citterio et al, 2004; Karagianni et al, 2008).

Methylation of histone tails occurs mainly at lysine (K) and arginine (R) residues, most commonly observed as mono-, di-, or trimethylation of the lysine residues on H3 and H4 histone tails. Methylation of H3K36 and H3K4, for instance, act as activating histone modifications, while H3K27 methylation has a role in repressing the gene expression (Bernstein et al., 2005). Similarly, H3K27ac and H3K9ac modifications are associated with active transcription and are predominantly associated with promoter and enhancer regions (Murray, n.d.; Nishida et al., 2006) (Table 1).

**Table 1. Histone modifications with their function and location**

| Histone modifications | Function | Location |
|---|---|---|
| H3K4me3 | Activation | Promoters, bivalent domains |
| H3K36me3 | Activation | Gene body |
| H3K9ac | Activation | Enhancers, promoters |
| H3K27ac | Activation | Enhancers, promoters |
| H3K27me3 | Repression | Promoters in gene-rich regions, bivalent domains |

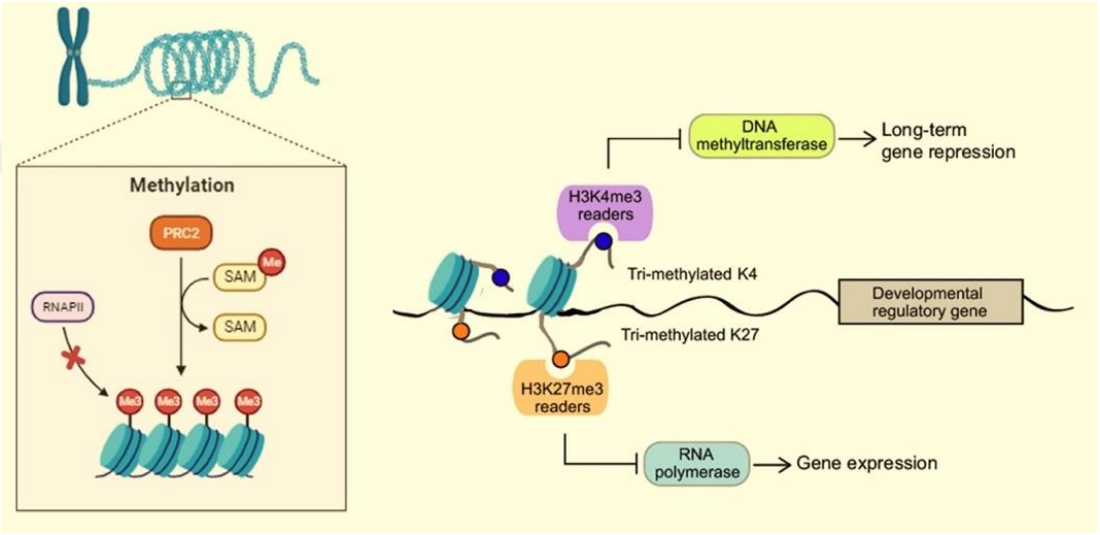


**Figure 7. The poised chromatin state**. Summary of the opposing effects of H3K4me3 and H3K27me3 and their proposed roles at poised promoters in the mammalian germ line (Adapted from Lesch & Page, 2014).

# 3. MATERIALS AND METHODS

## 3.1. Type of Study

This study pursues an analytical approach with in-vitro supported experiments.

## 3.2. Time and Place of Study

This study was conducted at Izmir International Biomedicine and Genome Center between March 2021 and December 2023.

## 3.3. Materials of Study

### *3.3.1. Cell Lines & Cell Culture Reagents*

**Table 2. Cell lines**

| Cell line | Medium |
|---|---|
| NMuMG<br>MEF | DMEM, high Glucose; 10%FBS;<br>1% Penicillin/Streptomycin;<br>1% nonessential amino acids;<br>1% sodium pyruvate |

**Table 3. Cell culture reagents**

| Materials | Cat. No | Vendor |
|---|---|---|
| DMEM, high glucose | 41965039 | Gibco |
| Trypsin-EDTA (0.25%), phenol red | 25-200-056 | Gibco |
| DPBS, no calcium, no magnesium | 14190094 | Gibco |
| Fetal Bovine Serum, qualified, Brazil | 10270106 | Gibco |
| MEM Non-Essential Amino Acids Solution (100X) | 11140035 | Gibco |
| Sodium pyruvate 100 mM | p04-43100 | PAN-Biotech |
| Penicillin-Streptomycin (10,000 U/mL) | 15140122 | Gibco |
| Recombinant Human TGFβ3 | 100-36E-10UG | Peprotech |
| Insulin solution human | I9278-5ML | Sigma Aldrich |
| FuGENE® HD Transfection Reagent | E2311 | Promega |
| Lipofectamine™ RNAiMAX Transfection Reagent | 13778150 | Invitrogen |
| DMSO | A3672,0100 | Applichem |

### *3.3.2. cDNA and qPCR Reagents*

**Table 4. cDNA and qPCR Reagents**

| Materials | Cat. No | Vendor |
|---|---|---|
| AMPIGENE® qPCR Probe Mix No-ROX | ENZ-NUC107-1000 | ENZO |
| MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL | 4346906 | Applied Biosystems |
| MicroAmp™ Optical Adhesive Film | 4360954 | Applied Biosystems |
| Universal Probe Library probes | | Roche |
| RealQ Plus 2x Master Mix | APQ-A323406 | Ampliqon |

### *3.3.3. Plasmid List*

**Table 5. Plasmids used in the study.**

| Plasmid Name |
|---|
| pcDNA 3.1c |
| CDS-Grhl3 |
| Elf3 |
| Cebpα |
| pCMV sport6 |
| pCMV sport6-Grhl3 |
| pCAGs2 -Klf4 |
| pEGFP.N2 |

### *3.3.4. siRNA Sequence Information*

**Table 6. siRNA Sequence**

| siRNA | Sequence | Vendor |
|---|---|---|
| Grhl3 | GGAAAGAAGUUCUACCACAtt | Thermo Fisher |
| Elf3 | TTGAACCAACTTGTTCGATAA | Qiagen |
| Cebpα | CGGCCGCTGGTGATCAAACAA | Qiagen |

### *3.3.5. Immunofluorescence Labelling Reagents*

**Table 7. Immunofluorescence Labelling Reagents**

| Materials | Cat. No | Vendor |
|---|---|---|
| Formaldehyde | F8775 | Sigma |
| Triton X-100 | T8787 | Sigma |
| E-cadherin antibody | 610181 | BD |
| DAPI | D1306 | Invitrogen |
| Alexa Fluor 488 Phalloidin | A12379 | Invitrogen |
| Prolong Diamond Antifade Mountant solution | P36962 | Thermo Fisher Scientific |

### *3.3.6. ChIP Reagents*

**Table 8. ChIP Reagents**

| Materials | Cat. No | Vendor |
|---|---|---|
| Formaldehyde | F8775 | Sigma |
| Glycine | CE.453807 | Carlo Erba |
| UltraPure Salmon Sperm DNA Solution | 15632011 | Thermo Scientific |
| Bovine Serum Albumin | D0500-4090 | Cegrogen |
| Proteinase K | P8107S | NEB |
| RNaseA | 740505.50 | MN |
| Grhl3 antibody | NBP2-16746 | Novus Biologicals |
| H3K4me3 antibody | 61379 | Active Motif |
| H3K27me3 antibody | 39155 | Active Motif |
| H3K9ac antibody | 61251 | Active Motif |
| H3K27ac antibody | 39685 | Active Motif |
| Rabbit control IgG | 2729S | Cell Signaling |
| Chelex | 142-1253 | Biorad |
| SDS | 817034100 | Merck |
| EDTA | 60-00-4 | Sigma |
| Trizma base | T1503-1KG | Sigma |
| Protease Inhibitor Cocktail | 04693116001 | Roche |
| NaCl | M106404.100 | Merck |
| Triton X-100 | T8787 | Sigma |
| NP-40 | A1694.0500 | Applichem |
| Sodium Deoxycholate | 30970 | Sigma |
| LiCl | L9650 | Sigma |
| Protein A-Sepharose® CL-4B | GE17-0780-01 | Cytiva |

### *3.3.7. Methylated DNA Immunoprecipitation (MeDIP) Reagents*

**Table 9. meDIP Reagents**

| Materials | Cat. No | Vendor |
|---|---|---|
| Sodium phosphate | 342483 | Sigma |
| NaCl | M106404.100 | Merck |
| Triton X-100 | T8787 | Sigma |
| Chelex | 142-1253 | Biorad |
| SDS | A7249,0500 | Applichem |
| EDTA | E5134-500G | Sigma Aldrich |
| UltraPure™ Salmon Sperm DNA Solution | 15632011 | Thermo Scientific |
| BSA | D0500-4090 | Cegrogen |
| Proteinase K | P8107S | NEB |
| RNaseA | 740505.50 | MN |
| 5-Methylcytosine (5-mC) antibody (mAb) | 61480 | Active motif |
| Rabbit control IgG | 2729S | Cell Signaling |
| Trizma base | T1503-1KG | Sigma |
| Protease Inhibitor Cocktail | 04693116001 | Roche |
| Protein A-Sepharose® CL-4B | GE17-0780-01 | Cytiva |

### *3.3.8. Other Chemicals, Consumables, and Commercial Kits*

**Table 10. Other Chemicals, Consumables, and Commercial kits**

| Materials | Cat. No | Vendor |
|---|---|---|
| NucleoSpin RNA kit | 740955.50 | Macherey-Nagel |
| DNA Clean & Concentrator™-5 | D4014 | Zymo Research |
| Zymoclean Gel DNA Recovery Kit | D4007 | Zymo Research |
| Quick-DNA™ Miniprep Kit | D3025 | Zymo Research |
| GeneRuler 1 kb DNA Ladder | SM0313 | Thermo Scientific |
| GeneRuler 100 bp DNA Ladder | SM0321 | Thermo Scientific |
| Agarose | A9536-500G | Sigma |
| Absolute Ethanol | 9.200.262.500 | Isolab |
| Ultrapure DNAse/RNAse free $dH_2O$ | 10977035 | Thermo Scientific |
| Safe View | G108 | ABM |
| Boric acid | 10043-35-3 | Merck |
| EDTA | E5134-500G | Sigma |
| Recombinant Human TGFβ3 | 100-36E-10UG | PEPROTECH |

### *3.3.9. Hardware & Machines*

**Table 11. Hardware and machines used in this study**

| Machine | Vendor |
|---|---|
| pH Meter | HANNA Instruments |
| Toploader Balance 820 g x 0.01g | Sartorius |
| SimpliAmp™ Thermal Cycler | Applied Biosystems |
| Owl™ EasyCast™ B1A Mini Gel Electrophoresis | ThermoFisher Scientific |
| Microwave oven | Beko |
| mySPIN™ 6 Mini Benchtop Centrifuge | ThermoFisher Scientific |
| Vortex Mixer | ThermoFisher Scientific |
| Hotplate Stirrer Thermolyne | ThermoFisher Scientific |
| Waterbath | Nuve |
| Centrifuge 5810R | Eppendorf |
| Centrifuge MicroCL 17R | ThermoFisher Scientific |
| ABI 7500 Fast Thermal Cycler | ThermoFisher Scientific |
| NanoDrop 2000 | ThermoFisher Scientific |
| Gel Doc XR+ System with Image Lab Software | Bio-Rad |
| CKX41 Inverted Microscope | Olympus |
| BX61 Fully Motorized Fluorescence Microscope | Olympus |
| Confocal Microscopy | Zeiss LSM880 |
| S220 focused-ultrasonicator | Covaris |
| Bioruptor Pico Sonication device | Diagenode |

### *3.3.10. Chemical Recipes*

#### *3.3.10.1. Electrophoresis Solutions*

**10X TBE (1L)**

108 g Tris
55 g Boric acid
40 ml 0.5 M pH:8 EDTA

**1% Agarose Gel**

0.5 g Agar
50 mL 0.5X TBE

### *3.3.10.2. ChIP Solutions*

**ChIP Lysis Buffer (50 mL)**

5 mL 10% SDS (1% Final Conc.)
1 mL 500 mM EDTA (10 mM Final Conc.)
2.5 mL 1M Tris (pH 8.0) (50 mM Final Conc.)
+ Protease Inhibitor Cocktail

**ChIP Dilution Solution (250 mL)**

250 µl 10% SDS (0.01% Final Conc.)
27.5 mL 10% Triton X-100 (1.1% Final Conc.)
600 µl 500 mM EDTA (1.2 mM Final Conc.)
4.175 mL Tris (pH 8.0) (16.7 mM Final Conc.)
8.35 mL 5M NaCl (167 mM Final Conc.)
+ Protease Inhibitor Cocktail

**Wash Buffer 1 (250 mL)**

2.5 mL 10% SDS (0.1 % Final Conc.)
25 mL 10% Triton X-100 (1% Final Conc.)
1 mL 500 mM EDTA (2 mM Final Conc.)
5 mL 1M Tris (pH 8.0) (20 mM Final Conc.)
7.5 mL 5M NaCl (150 mM Final Conc.)

**Wash Buffer 2 (250 mL)**

2.5 mL 10% SDS (0.1 % Final Conc.)
25 mL 10% Triton X-100 (1% Final Conc.)
1 mL 500 mM EDTA (2 mM Final Conc.)
5 mL 1M Tris (pH 8.0) (20 mM Final Conc.)
25 mL 5M NaCl (500 mM Final Conc.)

**Wash Buffer 3 (250 mL)**

0.5 mL 500 mM EDTA (1 mM Final Conc.)
2.5 mL 1M Tris (pH 8.0) (10 mM Final Conc.)
25 mL 10% NP-40 (1% Final Conc.))
2.5 g sodium deoxycholate (1% Final Conc.)
7.8125 mL 8M LiCl (0.25M Final Conc.)

**Wash Buffer 4 (250 mL)**

2.5 mL 1M Tris (pH 8.0) (10 mM Final Conc.)
0.5 mL 500 mM EDTA (1 mM Final Conc.)

**10% Chelex (50 mL)**

5 g Chelex
Bring volume to 50 ml with sterile dd$H_2O$

#### *3.3.10.3. DIP Solutions*

**10× IP buffer**

100 mM Na-Phosphate, pH 7.0
1.4 M NaCl
0.5% Triton X-100

### 3.4. Variables of Study

Within the scope of the research, the independent variables are the NMuMG cell line model used in EMT and MET transitions and the MEF primary cell used in MET induction. The vectors and siRNAs with which the cells are transfected are the dependent variables. Promoter methylation levels in NMuMG cells represent the dependent variable in EMT-MET conversion. MET induced following Grhl3 overexpression in MEF cells is the dependent variable, with alteration of promoter methylation levels and subsequent binding of the Grhl3 transcription factor to the relevant DNA sequence.

### 3.5. Tools For Data Collection

The Graphpad Prism 9 program and Microsoft Excel were used to analyze qPCR results. Image J and Zen 3.1 programs were used to analyze IF stainings.

### 3.6. Study Plan and Calendar

| | |
|---|---|
| Oct-Dec 2021 | Read processing |
| January- April 2022 | Examination of gene expression levels of EMT and MET processes in NMuMG cells. Overexpression of Grhl3 in MEF cells |
| May-July 2022 | Examination of gene expression levels of MET process in MEF cells |
| September-December 2022 | Induction of MET in MEF cells using different transcription factors |
| January-April 2023 | Examining the effect of silencing transcription factors on MET. |
| May-August 2023 | Optimization and validation of MeDIP in MEF cells |
| September - November 2023 | Optimization and validation of ChIP assay in MEF cells overexpressing Grhl3 |
| December 2023 | Writing |

## 3.7. Data Evaluation

### *3.7.1. Cell Culture Maintaining and Handling*

Cell culture techniques were performed in a Class II Biological Safety Cabinet. The cells in the cryovials taken from the liquid nitrogen tank were thawed in a 37°C water bath. After being suspended by adding 10 ml of medium, they were centrifuged at 1200 rpm (300g) for 6 minutes to remove DMSO. After the supernatant was aspirated, it was resuspended with culture medium. The cell suspension was transferred to the cell culture dish using a sterile pipette. MEF cells were grown at 37°C in a humidified environment containing 5% $CO_2$. NMuMG cells were grown at 37 °C in a humidified environment containing 10% $CO_2$. The next day, the medium was changed to eliminate the effect of DMSO on the cells and to remove cells that died during the freezing process. Once the cells reached 80–90% confluency, the growth medium was aspirated, and the cells were washed with 10 mL of room temperature DPBS to remove any remaining growth medium. DPBS was then aspirated, and 1 mL of 0.25% Trypsin-EDTA at room temperature was added to detach the cells from the surface. The dishes were incubated at 37°C for 3-5 minutes. After the cells were detached from the cell plate surface, the medium was added and centrifuged to collect them as a cell/medium mixture. After the supernatant was aspirated, the cells were resuspended with fresh growth medium. A cell count of $4.0x10^5$ - $5.0x10^5$ was seeded in a Corning® 100 mm culture dish.

After collection and centrifugation, the cells are thawed in a freezing medium containing 90% FBS and 10% DMSO to freeze the cells. The cell suspension is then immediately transferred to cryovials. Cryovials were placed in a propanol-based freezing device and transferred to liquid nitrogen to ensure long-term storage conditions.

### *3.7.2. Induction of EMT and MET in NMuMG Cells*

Normal Murine Mammary Gland Cell line was provided from ATCC (ATCC® CRL-1636). NMuMG (Normal Murine Mammary Gland) cell line, which is a normal mouse mammary gland epithelial cell line, was chosen as the positive control of MET. Cells were grown in high glucose DMEM containing 10% FBS, 100U/ml penicillin/0.1mg/ml streptomycin, 1% non-essential amino acid mixture, and 10 µg/ml insulin added in incubators at 37°C, 10% $CO_2$ conditions. For EMT induction, TGFβ3 was added to the cell culture medium for 72 hours, with a final concentration of 5 ng/ml. TGFβ3 was dissolved in a buffer containing pure water with a final concentration of 7.5% BSA and 1.2 M HCl and stored at -20°C. The process of returning to epithelial form (MET) was induced by washing away TGFβ3. For this, cells treated with TGFβ3 for 72 hours were washed 3 times with PBS, and a fresh medium without TGFβ3 was added. The return of the cells to their old epithelial form was monitored morphologically for 72 hours.

### *3.7.3. Induction of MET in MEF cells via Plasmid Transfection*

MEF cells isolated from C57BL/6J E13.5 embryos were provided from Dimitrov Lab. The harvested cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (10,000 U/mL), 1x MEM NEAA, and 1x Sodium pyruvate. MET was induced in mouse embryonic fibroblasts (MEFs) using Grhl3 and other epithelial transcription factors, which have been identified as one of the transcription factors that can initiate the MET program when overexpressed (Alotaibi Lab).

In this context, Fugene transfection reagent was used to overexpress various epithelial genes. Before use, FuGENE® HD Transfection reagent was kept at room temperature and then mixed by inverting. First, plasmid mixtures containing 1µg DNA in 1.5ml Eppendorf tubes were prepared. Pre-warmed basic DMEM, 100 µl for each well to be transfected, was added to a sterile Eppendorf tube. For a 3:1 FuGENE® HD Transfection Reagent:DNA ratio, 3 µl of FuGENE® HD Transfection Reagent per well was added directly to the medium and mixed immediately. After incubating for 5 minutes at room temperature, the prepared plasmids were mixed with the mixture containing FuGENE® HD Transfection Reagent and incubated for 15 minutes at room temperature. Immediately after the incubation, the reagent/DNA mixture was added to the cells. After 48 hours, the medium was aspirated, and a fresh growth medium was added. After 72 hours, RNA was isolated from the transfected cells using the RNA isolation kit (Macharey-Nagel NucleoSpin® RNA).

MEF cells transfected with plasmids expressing various transcription factors were harvested after 72 h to determine expression changes of epithelial and mesenchymal genes and confirmed by qPCR.

### *3.7.4. RNA Knockdown*

The relevant siRNAs and Lipofectamine RNAiMAX were diluted separately in 250 µl DMEM and incubated for 5 minutes at room temperature. DMEMs containing siRNAs and Lipofectamine RNAiMAX reagent were combined, resulting in 500 µl transfection mix. It was incubated for 20 minutes at room temperature. The amount of siRNA was calculated to be 50 nM per well, and the final volume was completed to 2 mL by adding 500 µl of reaction mixture to the relevant well. Cells transfected with relevant siRNAs were collected 72 h later.

### *3.7.5. RNA isolation*

Total RNA was isolated from three technical replicates. The media of the cells incubated for 72 hours were aspirated and washed twice with 1X PBS. For total RNA isolation, Nucleospin RNA extraction Kit (Macherey Nagel, Germany) was used. First, lysis buffer was added to each well according to the steps in the kit protocol. 70% ethanol was added to the resulting cell lysate

and mixed by vortexing. Then, it was transferred to the NucleoSpin® RNA Column (light blue ring) provided by the kit. All remaining cell components (cell debris, protein, gDNA) were eliminated. There is only a biochemical basis that allows RNA to bind to the silica column. To elute the RNA from the column, 35 µl RNAse-free water was used to rehydrate it.

To assess the RNA concentration and purity of each sample, the absorbance of each sample was measured at 260/280 and 260/230 nm by UV spectroscopy (Nanodrop 2000), respectively. The 260/280 ratio of RNA was around 2.0, and the 260/230 ratio was in the range of 2.0-2.2.

### *3.7.6. cDNA Synthesis*

The Maxima® First Strand cDNA Synthesis Kit is a convenient system optimized for cDNA synthesis in two-step real-time quantitative RT-PCR (RT-qPCR) applications. Total RNA (1 µg) was converted to cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA USA). The main components of the synthesis include an RNA template, maxima enzyme mix containing maxima reverse transcriptase and RiboLock RNase Inhibitor, 5X reaction mix containing reaction buffer, dNTPs, oligo (dT)18 and random hexamer primers, RNase-free water. The kit components were dissolved, mixed, and centrifuged to prepare a master mix containing all components for the RT reaction except template RNA.

Added into a sterile, RNase-free tube on ice in the indicated order:

- 5X Reaction Mix 4 µL
- Maxima Enzyme Mix 2 µL
- Template RNA 1 µg
- Water, nuclease-free up to 20 µL

Once all these components were mixed gently and centrifuged, the Thermal Cycler device was set to appropriate conditions.

- 25ºC: 10 minutes
- 50ºC: 30 minutes
- 85ºC: 5 minutes

After the synthesis reaction was done, cDNAs could be used directly for qPCR or stored at -20°C) for up to a month or at -80°C) for extended periods of time.

### *3.7.7. qPCR validation of differential expressed genes*

The cDNAs used for qPCR were diluted 1/10 before use and kept at 4°C. AMPIGENE® qPCR Probe Mix No-ROX was used for the qPCR experiment according to the manufacturer's instructions. Briefly, each well of the 96-well qPCR plate included 6.25 µl of (2X) AMPIGENE® qPCR Probe Mix, 2.5 µl of

nuclease-free water, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 0.25 µl probe, and 2.5 µl cDNA template for each target gene reaction. After plating the reaction mixtures, the plate was sealed and centrifuged at 1200 rpm for 1 min. Quantitative real-time PCR was performed using the AMPIGENE® qPCR Probe Mix and Universal Probe Library probes (Roche, Basel, Switzerland), and amplification was carried out in the ABI 7500 Fast qPCR System (Thermo Scientific, Waltham, MA USA) with primers and probes at 95°C for 15 min, 50 cycles of 95°C for 15 s and 60°C for 1 min. Additionally, the reaction conditions for SYBR Green are as follows.

- Holding Stage: 95°C, 10 minutes
- Cycling Stage: 95°C, 15 seconds
- 60°C: 1 minute
- Melt Curve Stage: 95°C, 15 seconds
- 60°C: 1 minute
- 95°C: 30 seconds
- 60°C: 15 seconds

Obtained triplicate Ct values were averaged and normalized with respective housekeeping Ddx17 Ct values. For analysis, $2^{-\Delta\Delta Ct}$ method was used.

### *3.7.8. Immunofluorescence Staining*

The cells in which transfection experiments were performed were seeded on the cover for immunofluorescence staining. Cells were grown before they were fully confluent. Coverslips were washed 3 times with PBS after incubation and incubated in freshly prepared 4% paraformaldehyde in PBS for 10 minutes at room temperature. Coverslips were washed with PBS for 5 minutes and incubated in 0.5% Triton X-100 in PBS for 10 minutes at room temperature. Coverslips containing permeabilization buffer were washed by incubating in PBS for 5 min. Cells were incubated with the primary antibody anti-E-cadherin (BD) diluted in 50 µl of 5% BSA (1:200) for 1 h at room temperature in the dark. Then, the slides were washed again 3 times with PBS. Cells were incubated with Alexa Fluor™ goat anti-mouse IgG (Invitrogen, USA) diluted in 5% BSA (1:500) and secondary antibody 488 Phalloidin (Invitrogen, USA) diluted in 5% BSA (1:200) for 1 hour in the dark. To visualize the nuclei, 1:1000 DAPI (Invitrogen, USA) was added. The slides were mounted to the lams using Prolong Diamond Antifade Mountant Solution (Thermo Fisher Scientific, USA). Visualization was performed with an Olympus BX61 Fluorescence Microscope.

### *3.7.9. Metylated DNA Immunoprecipitation (MeDIP) During MET in MEF cells*

Following Grhl3 overexpression in MEF cells, DNA isolation from qPCR-validated cells was performed using the Zymo Research kit. The obtained DNA (1 µg) was processed using the Bioruptor Pico Sonication device (B01060010),

30/90; It was sonicated for 10/10 cycles. Unsonicated and sonicated gDNA were run on a 1% agarose gel to determine the fragment size obtained as a result of the sonication process. After the fragment size range of the sonicated DNA sample was confirmed on the agarose gel, it was denatured in boiling water at 95°C for 10 minutes and immediately placed on ice to prevent rebinding. The denatured DNAs, kept on ice for 5 minutes, were transferred to 1.5 µl microcentrifuge tubes and diluted by adding 900 µl of 1× IP buffer. IgG and 5mC antibodies were used for each sonicated sample obtained from control MEF cells and Grhl3 overexpressing cells. The tubes were incubated on the rotator device at 4°C, 14 rpm for 16 hours.40 µl Protein A sepharose beads were added to each and incubated on the rotator for 2 hours. After this stage, the immune complexes collected with beads were washed 3 times with IP buffer and once with TE Buffer. 10% Chelex was added and incubated at 95°C Heat Block, then Proteinase K was added and incubated at 55°C Heat Block. To denature Proteinase K, it was kept in the 95°C Heat Block again and centrifuged at 2000 rpm / 1 min. The supernatant was separated and carefully transferred to the purification column, and the DNA was purified by centrifugation. The enrichment rates of the obtained methylated DNAs were examined using the qPCR method (Figure 8). Following the DIP method, the promoters of genes that play a critical role in EMT and MET were evaluated in terms of methylation profiles.

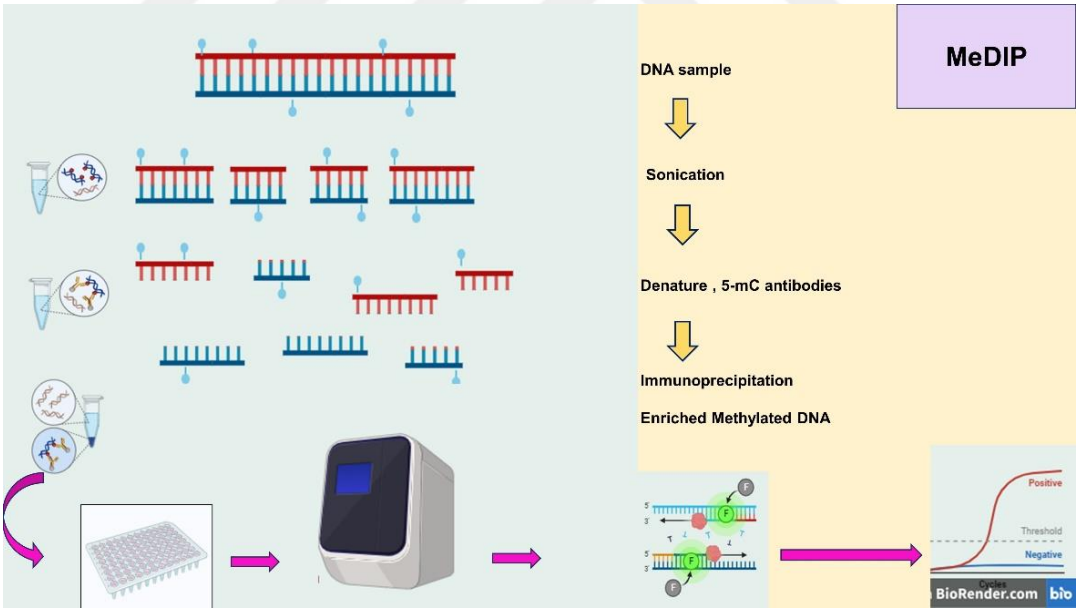


**Figure 8. Flow chart of DNA immunoprecipitation.**

### *3.7.10. Chromatin Immunoprecipitation (ChIP) During MET in MEF Cells*

Transfected MEF cells were grown and treated in 10 cm plates. 37% formaldehyde was added to a final concentration of 1% and fixed for 10 minutes at room temperature. 1.25M glycine was added to the cells and incubated for 10 minutes at room temperature. After washing 3 times with PBS,

it was scraped with growth medium. The pellets obtained after centrifugation at 3000 g for 5 minutes were lysed in 1 mL cell lysis buffer. 50 µl of unsonicated DNA from the cell lysate was taken into a separate tube and labeled as control. In order to obtain fragments between 200-1000 bp in size, they were sonicated using a S220 ultrasonicator (Covaris, Woburn, MA, USA) sonication device (60sec pulse/60sec rest) x 30 min (Peak Power: 140 W; Duty factor: 15; Cycles/ burst: 200; water temperature: 4°C). Samples labeled as unsonicated and obtained immediately after sonication were loaded onto a 1.5% agarose gel for interpretation of chromatin fragmentation. After verification in the gel, it was diluted 1:10 with ChIP dilution solution and incubated with Rabbit IgG, Grhl3, H3K4me3, H3K27me3, H3K9ac, and H3K27ac antibodies at 4°C overnight at 12 rpm on a rotator. The prepared Protein A (50% slurry) was added to the samples and incubated with antibodies at 12 rpm at 4 °C for 2 hours. It was then centrifuged at 3000 g and centrifuged after each washing solution. To reverse the crosslinking of the DNA and proteins, 10% Chelex was added (10 minutes at 95°C). Then, these samples were incubated with Proteinase K (30 minutes at 55°C) and RNase A (15 minutes at 37°C). Then, the obtained samples were passed through the column. Quantitative real-time PCR was performed using the AMPIGENE® qPCR Probe Mix No-ROX and Universal Probe Library probes (Roche, Basel, Switzerland), and amplification was carried out in the ABI 7500 Fast qPCR System (Thermo Scientific, Waltham, MA USA) with primers and probes at 95°C for 15 min, 50 cycles of 95°C for 15 s and 60°C for 1 min. Data were normalized to the input and presented as a percentage of input.

### 3.8. Statistical Analysis

Statistical significance was determined by performing the Student's t-test using the normalized values of each test sample compared to the normalized value of the control, using a 95% confidence interval; p-values less than 0.05 were considered significant. Data are presented as the mean of at least three independent experiments, done in triplicates. Error bars represent standard error of the mean.

### 3.9. Limitation of the Study

No limitations are to be reported.

### 3.10. Ethics Committee Approval

This study was approved by the IBG local ethics committee with ethics protocol number 2022-011.

# 4. RESULTS

## 4.1. Induction of MET in MEF Cells

### *4.1.1. Overexpression of Grhl3 in MEF Cells*

Fibroblast cells, a heterogeneous cell population of mesenchymal origin that plays important roles in wound repair and healing, are located in various locations and have different appearances and activities and can be considered multipotent progenitor sources. Embryos are a rich source of fibroblast content. Primary mouse embryonic fibroblasts (MEFs) have many advantages compared to other cell types, making them stand out as a model system. It is easy to establish and maintain compared to other primary explant cultures. They are isolated in the early stages of embryonic development (E13.5–E14.5) and have a rapid growth rate. It is a powerful model for testing the physiological consequences of gene function. MEFs are efficiently transfected using plasmid DNAs. EMT and MET are critical biological processes involved in physiological and pathological events. Considering the previously reported regulation of MET by Grhl3 (Alotaibi et al., 2015), we wanted to investigate the impact of introducing Grhl3 in MEFs. MEFs were isolated from E13.5 mouse embryos. We first optimized the transfection conditions using a GFP expressing plasmid. Using FuGENE transfection reagent we were able to successfully and reproducibly transfect MEFs with high efficiency (Figure 9).

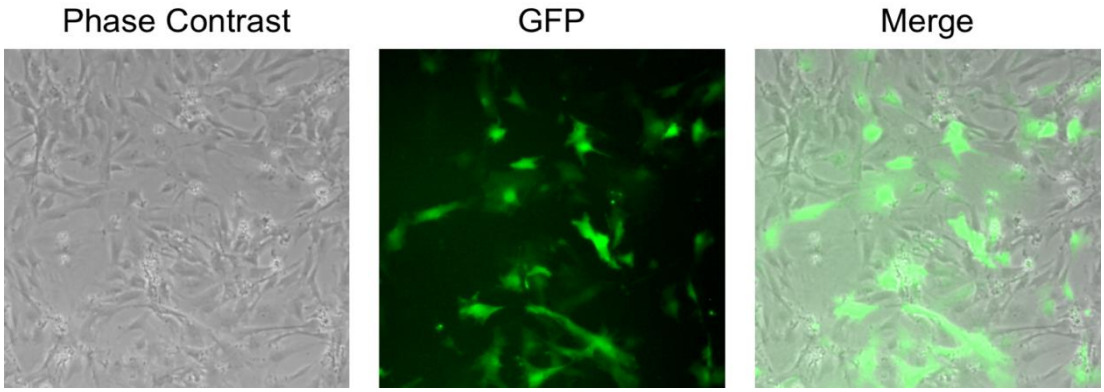


**Figure 9. Transfecting MEF cells with GFP plasmid.**

To study the effects of Grhl3 on MEFs, cells were transfected with a plasmid expressing Grhl3 (CDS) and were examined for their ability to induce MET. 72 hours after transfection, cells were harvested to prepare RNA. Grhl3 and Cdh1 mRNA levels were investigated by qPCR. When Grhl3 and Cdh1 mRNA levels obtained from control cells and Grhl3 overexpressing cells were compared, a significant increase in the transcript level was detected (Figure 10).

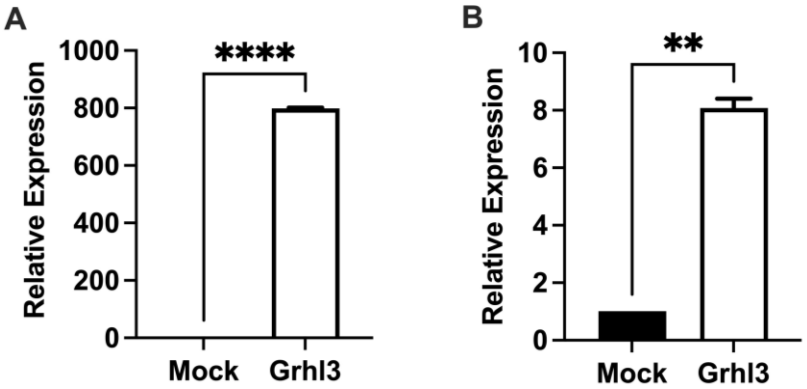


**Figure 10. Analyzing the change of transcript levels of Grhl3 and Cdh1.** (A) Grhl3 and (B) Cdh1 genes after transfecting MEF cells with CDS-Grhl3 plasmid by qPCR.

### *4.1.2. Confirmation of Grhl3 Overexpression*

We observed a very strong expression of Grhl3 after transfection, we questioned whether this was a by-product or contaminated RNA with residual DNA (plasmid), which might result in such high fold change. For this we performed a control experiment using cDNA prepared without the addition of reverse transcriptase (RT) and performed the qPCR as comparing this control cDNA with the regular cDNA prepared using the regular methods. We concluded that there was no DNA contamination, the qPCR amplicons were also loaded on a 2% agarose gel for confirmation (Figure 11).

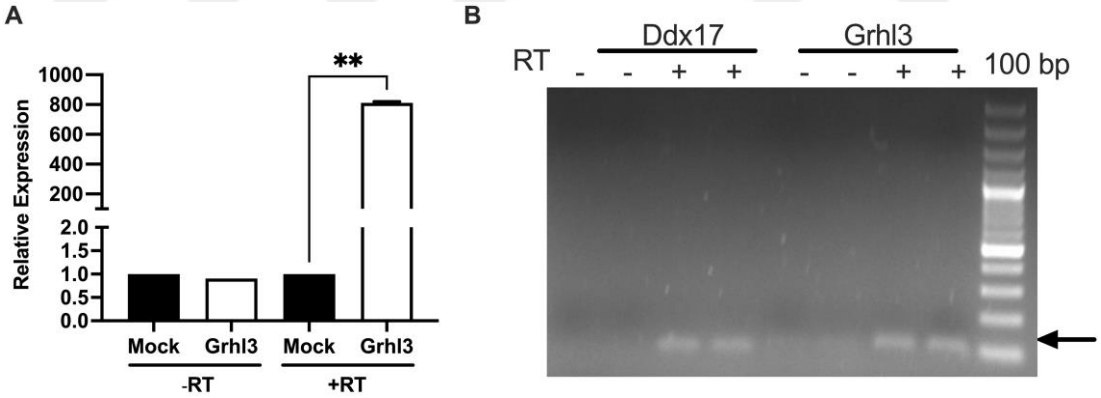


**Figure 11. Evaluation of the effect of Grhl3 overexpressed in MEF cells.** Demonstration of whether the increase in transcript level depends on the presence of plasmid. A) Comparison of Grhl3 levels in RT (-) and RT (+) samples. B) Gel image of PCR products.

We then transfected MEFs with another plasmid (pCMV-Sport6-Grhl3) in which Grhl3 is expressed with UTRs as opposed to the previous plasmid containing CDS only transcript and both plasmids were under the control of CMV promoter. Therefore, after transfecting MEF cells with these two plasmids, it was determined that the Grhl3 transcript level was higher in the cell transfected with the CDS-Grhl3 plasmid (Figure 12 A). On the other hand,

Cdh1 level for both plasmids were similar (Figure 12 B). 3'UTR region regulates mRNA localization, stability, and translation. This suggested that Grhl3 might be regulated post transcriptionally through its 3'UTR. We searched for putative miRNAs that could bind to the Grhl3 3'UTR and compiled a list of these miRNAs (Table 13), which could be further investigated. Since CDS-Grhl3 does not have a 3'UTR region, its half-life in the cell is longer. It is unlikely to be targeted by the miRNAs listed in Table 13 (appendix II). The gene expression level of epithelial markers is lower in MEF cells. Therefore, the cell's capacity to express these genes remains at a certain level, even if there is induction. Since both plasmids had a negligible cytotoxic effect on the induction of MET, CDS-Grhl3, which leads to higher Grhl3 transcript levels, was used in all subsequent experiments.

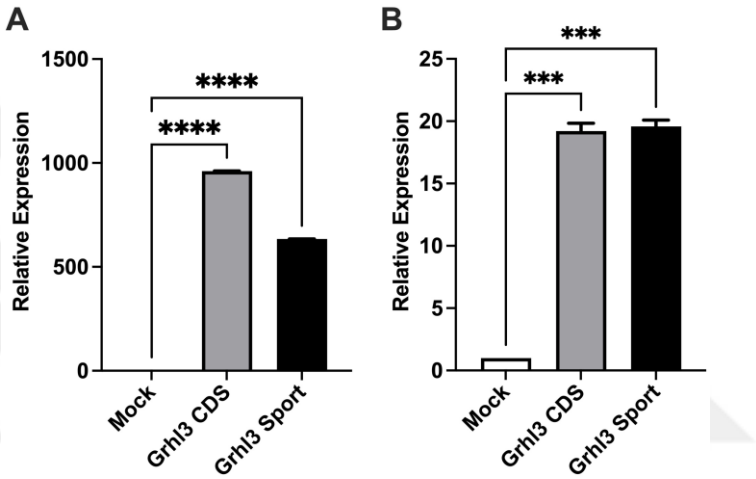


**Figure 12. Evaluation of the expression levels of Grhl3 using different expression vectors in MEF cells.** Grhl3 (A) and Cdh1 (B) transcript levels following overexpression using CDS-Grhl3 and pCMV Sport6-Grhl3 plasmids.

After establishing a successful expression of Grhl3 and Cdh1 in transfected MEFs as an indication of potentially initiating MET, we wanted to examine the expression of E-cadherin and its localization in transfected cells. The confocal microscope images show results of immunofluorescence staining following Grhl3 overexpression in MEF cells where E-cadherin protein was localized in the cytoplasm instead of the membrane (Figure 13A). The concentration of E-cadherin protein in MEF cells is relatively low compared to NMuMG cells, which exhibit epithelial properties (Figure 13B). Overexpression of the Grhl3 transcription factor enabled the induction of MET by playing a role in the regulation of the Cdh1 gene, which plays a role in the synthesis of the E-cadherin protein, which plays a vital role in the formation of the epithelial character. However, it can be said that the epithelial features observed in NMuMG cells are not fully formed in MEF cells because the E-cadherin protein, which mediates cell-cell adhesion, is not localized in the region where it will perform its function.

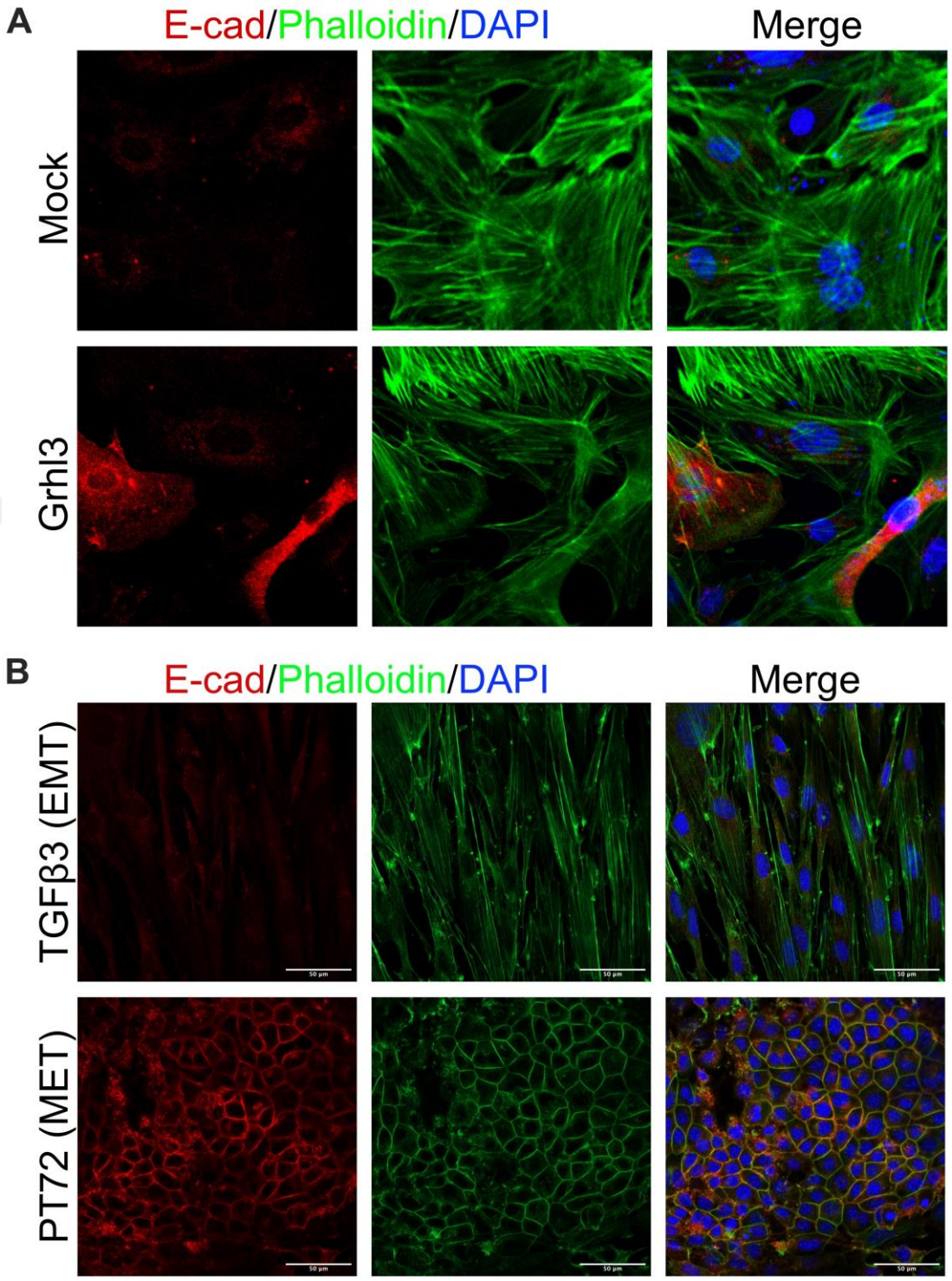


**Figure 13. Immunofluorescence staining of E-cadherin in MEF and NMuMG cells.** A) E-cadherin/Phalloidin/DAPI staining in control MEF cells and following overexpression of Grhl3. B) E-cadherin / Phalloidin /DAPI staining following EMT and MET processes induction in NMuMG cell.

The delay of E-cadherin to localize to the plasma membrane might be related to how E-cadherin is transported to the membrane. For This we

examined the expression levels of proteins know to be involved in E-cadherin trafficking and recycling. We have noticed that while some were upregulated, others were downregulated. Snx1 and Snx5 have been reported to prevent lysosomal degradation and promote the recycling of E-cadherin (Bryant et al., 2007). Snx1, Pals1, Tuberin, Rab11a, and Exoc9 levels increased following Grhl3 overexpression in MEF cells, on the other hand, Pak1, CblI1, and Pip5k were downregulated (Figure 14).

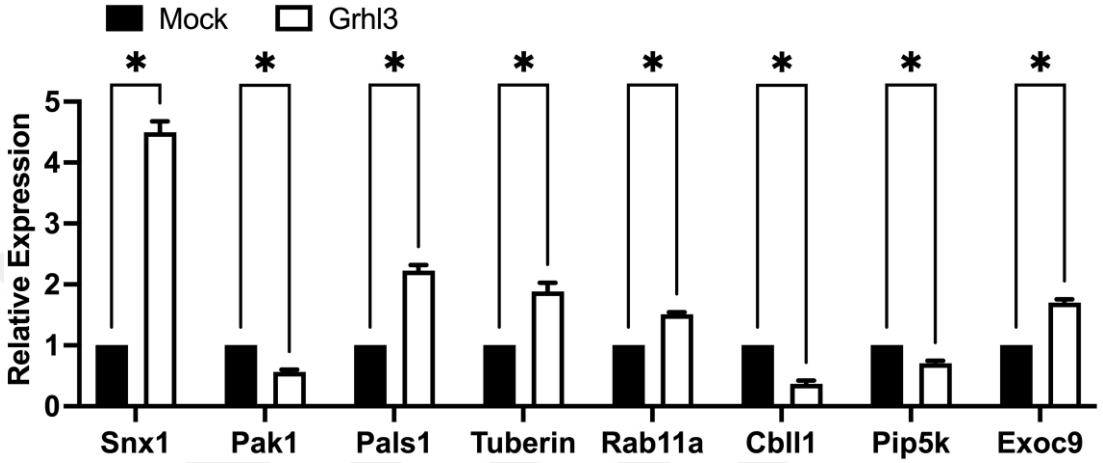


**Figure 14. Determination of mRNA expression levels of relevant genes following Grhl3 overexpression in MEF cells.** mRNA expression levels of Snx1, Pak1, Pals1, Tuberin, Rab11a, Cbll1, Pip5k, Exoc9 proteins, which are known to play a role in E-cadherin trafficking and recycling, were validated by qPCR.

Previous reports demonstrated Klf4 as a regulator of Cdh1 expression (Koopmansch et al., 2013) and that Klf4 is able to initiate MET in MEFs, Klf4 induced MET in MEFs is believed to be the first step towards the reprogramming of MEFs into iPSCs (R. Li et al., 2010). When we examined the data we also noticed that the expression of E-cadherin in Klf4 transfected MEFs was cytoplasmic similar to the Grhl3 transfected MEFs described here. This led us to conclude that at least a similar initiation of MET is possible.

### *4.1.3. Analysis of mRNA Expression of Epithelial and Mesenchymal Genes in Response to Grhl3 Overexpression*

Since we have established an increase of Cdh1 mRNA and protein levels we wanted examine more epithelial and mesenchymal markers to understand the extent of Grhl3 induced MET. Following overexpression of the Grhl3 in MEF cells using the CDS-Grhl3, the cells were collected at the 72nd hour to prepare RNA. Expression levels of epithelial and mesenchymal genes were evaluated using quantitative polymerase chain reaction. It was determined that the mRNA levels of epithelial markers such as Elf3, Klf4, Cebpα, Ovol2, Ehf, Crif1, and Hnf4α were increased compared to the control (Figure 15).

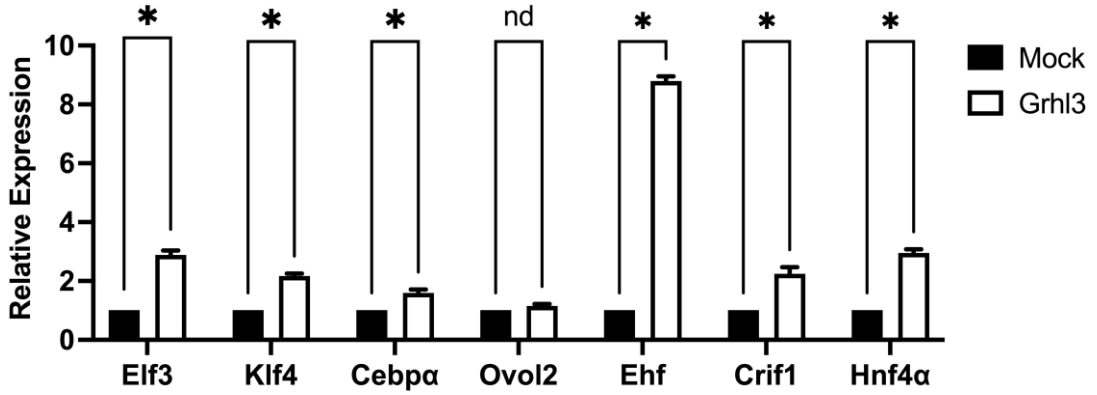


**Figure 15. The effect of Grhl3 transfection on mRNA expression levels of epithelial genes.** Determination of mRNA expression levels of Elf3, Klf4, Cebpα, Ovol2, Ehf, Crif1, and Hnf4α epithelial genes, respectively, according to qPCR validation performed after Grhl3 overexpression in MEF cells.

The levels of mesenchymal genes such as Cdh2, Zeb1, Zeb2, Snai1, Snai2, Vim, Fn1 showed a decrease (Figure 16). Taken together, we concluded that Grhl3 transfected MEFs had initiated MET.

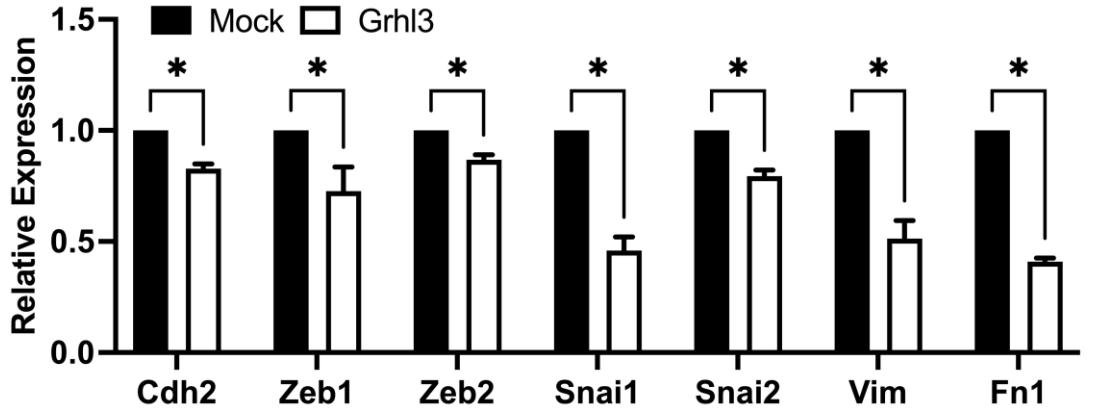


**Figure 16. The effect of Grhl3 transfection on mRNA expression levels of mesenchymal genes.** Determination of mRNA expression levels of Cdh2, Zeb1, Zeb2, Snai1, Snai2, Vim and Fn1 mesenchymal genes, respectively, according to qPCR validation performed after Grhl3 overexpression in MEF cells.

### *4.1.4. Effect of Overexpressed Transcription Factors on Inducing MET*

Induction of MET in MEF cells following transfection with the CDS-Grhl3 plasmid was determined by the increase in Cdh1 mRNA, one of the critical epithelial markers. For this reason, the ability of transcription factors such as Elf3, Klf4, and Cebpα to trigger and maintain MET was compared with Grhl3 (Figure 17). After overexpression of each transcription factor, the changes they caused in the mRNA levels of Cdh1, Elf3, Klf4, Cebpα, and Grhl3, respectively, were determined by qPCR. Accordingly, the increase in the mRNA transcript of the relevant transcription factor after each overexpression compared to the

control shows that the transfection is effective. In addition, it was determined that the Grhl3 transcription factor had a more significant effect on the Cdh1 gene expression level than other transcription factors (Figure 17A).

Additionally, when Grhl3 is overexpressed, Elf3 transcript level increases. In cells where Cebpα and Elf3 are overexpressed, the Grhl3 transcript level increases. Cebpα and Elf3 regulate Grhl3, while Grhl3 appears to play a role in regulating Elf3.

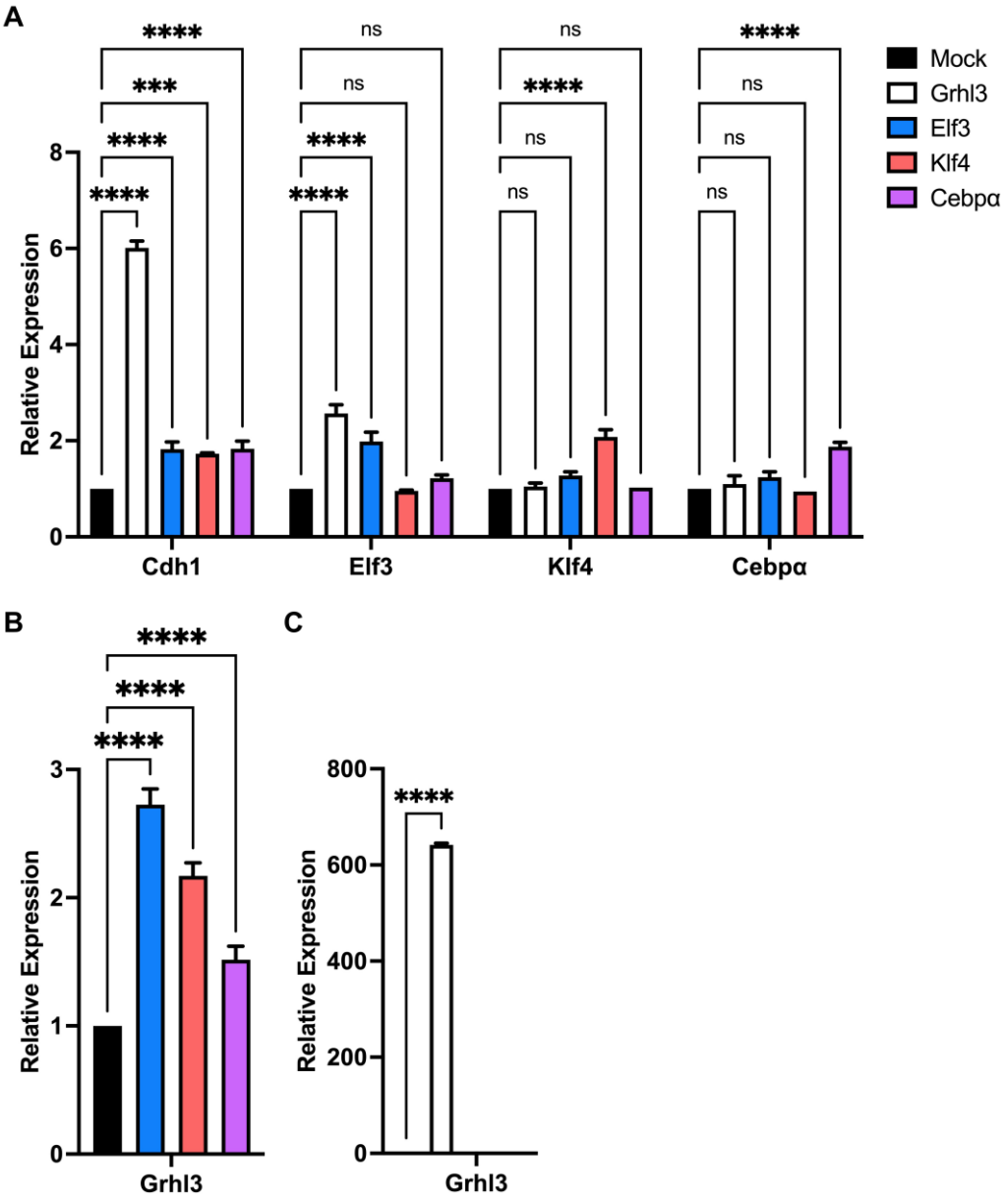


**Figure 17. Comparison of the effects of overexpressed epithelial genes on each other at the expression levels**. (A) Determination of the effects of overexpression of Grhl3, Elf3, Klf4, Cebpα on the transcript levels of Cdh1, Elf3, Klf4, Cebpα and (B) Grhl3 genes, respectively.

We then wanted to study the cooperativity of different transcription factors on the initiation of MET. The combination of transcription factors was evaluated for their ability to induce MET. MEF cells were transfected with transcription factors such as Klf4, Elf3, and Cebpα during the first transfection and re-transfected using the same transcription factors or Grhl3 (Figure 18). Cells were harvested 72 h after the second transfection to prepare RNA. The presence of Grhl3 in the second transfection resulted in a robust increase of Cdh1 levels as compared to the introduction of the same transcription factor in the second transfection. It was concluded that the presence of Grhl3 was more effective than the presence of other transcription factors.

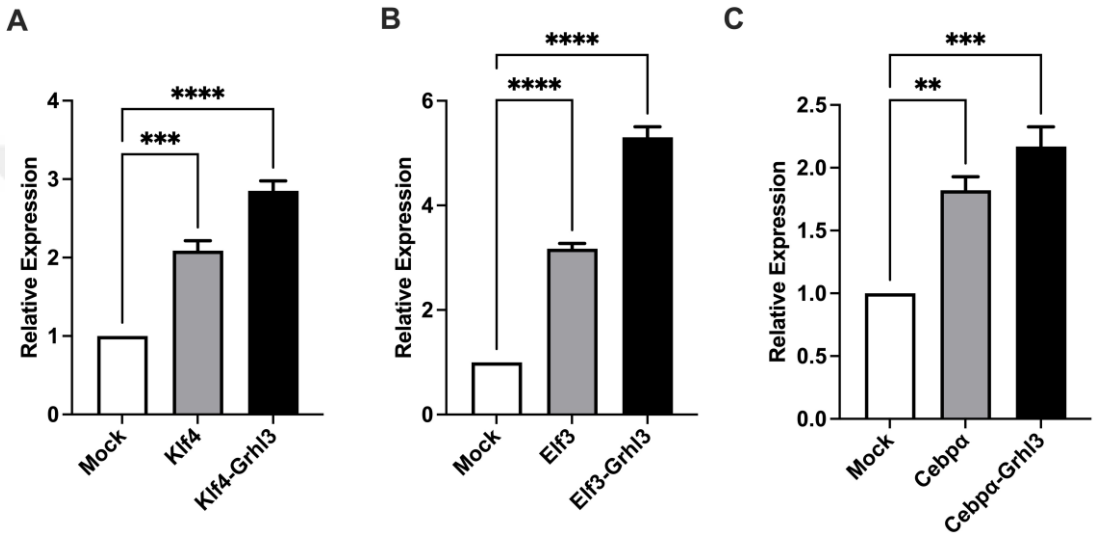


**Figure 18. Effect of Grhl3 on Cdh1 expression during the induced MET by Klf4, Elf3, and Cebpα.** Klf4, Elf3, and Cebpα were used for double transfection in the first group of MEF cells. In the other group, Grhl3 was used for the second transfection after Klf4, Elf3, and Cebpα factors. Comparison of the effects of two transfection treatments on Cdh1 mRNA levels utilized for (A) Klf4, (B) Elf3 and (C) Cebpα .

## 4.2. Transcription Factors Effective in Maintaining Induced MET

### *4.2.1. Effect of Silenced Transcription Factors on Maintaining Induced MET in MEF*

After inducing MET in MEF cells by Grhl3, gene expression was silenced with Cebpα, Elf3, and Grhl3 by the RNAi method. The decrease in transcription level after silencing each transcription factor is successful. In Grhl3 knockdown cells, the level of Cdh1 is lower compared to the other two transcription factors. The decrease in Elf3 transcript level in Grhl3 knockdown cells supports that Grhl3 regulates Elf3. Likewise, Grhl3 transcript level was severely reduced in Cebpα knockdown and Elf3 knockdown cells (Figure 19). These results confirm that there is a reciprocal regulation between these transcription factors.

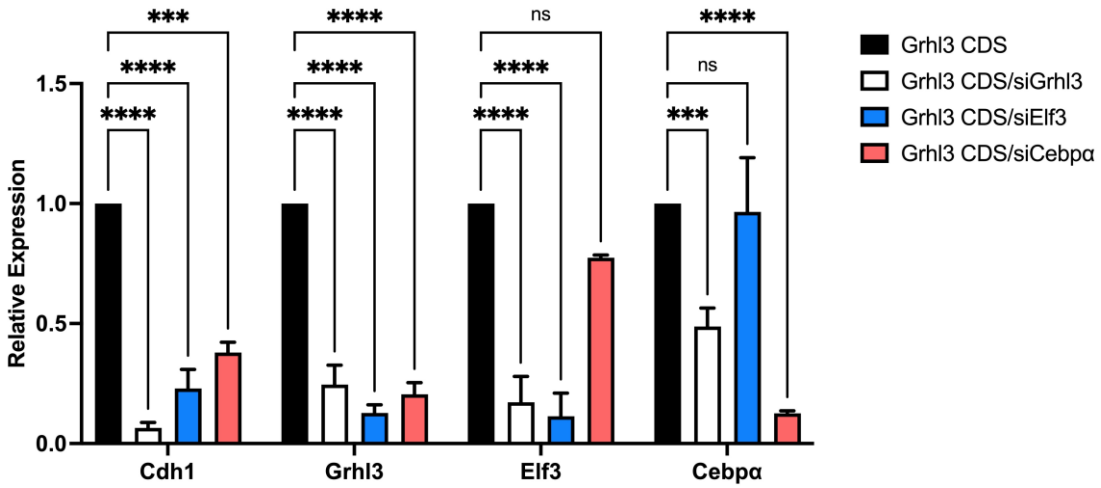


**Figure 19. Crosstalk between MET-TFs.** Analysis of Cdh1, Grhl3, Elf3, and Cebpα mRNA levels to determine knockdown efficiency following MET by Grhl3 overexpression and silencing by Grhl3, Elf3 and Cebpα.

As a result of silencing Grhl3 following the overexpression of Elf3 and Cebpα (Figure 20), the mRNA expression levels of Cdh1 and Grhl3 gene expressions decreased. Following the overexpression of Elf3 and Cebpα, an increase in the mRNA levels of both Cdh1 and Grhl3 was observed. Elf3 and Cebpα appear to affect the indicated epithelial genes. Overexpression of these two transcription factors caused an increase in both their own mRNA levels and each other's mRNA levels. Subsequently, the decrease in Elf3 and Cebpα mRNA levels after Grhl3 silencing indicates that these transcription factors might be under the control of Grhl3. These factors are effective in regulating each other, Grhl3, and Cdh1 transcription. The results obtained confirm that there is a reciprocal regulation between these transcription factors.

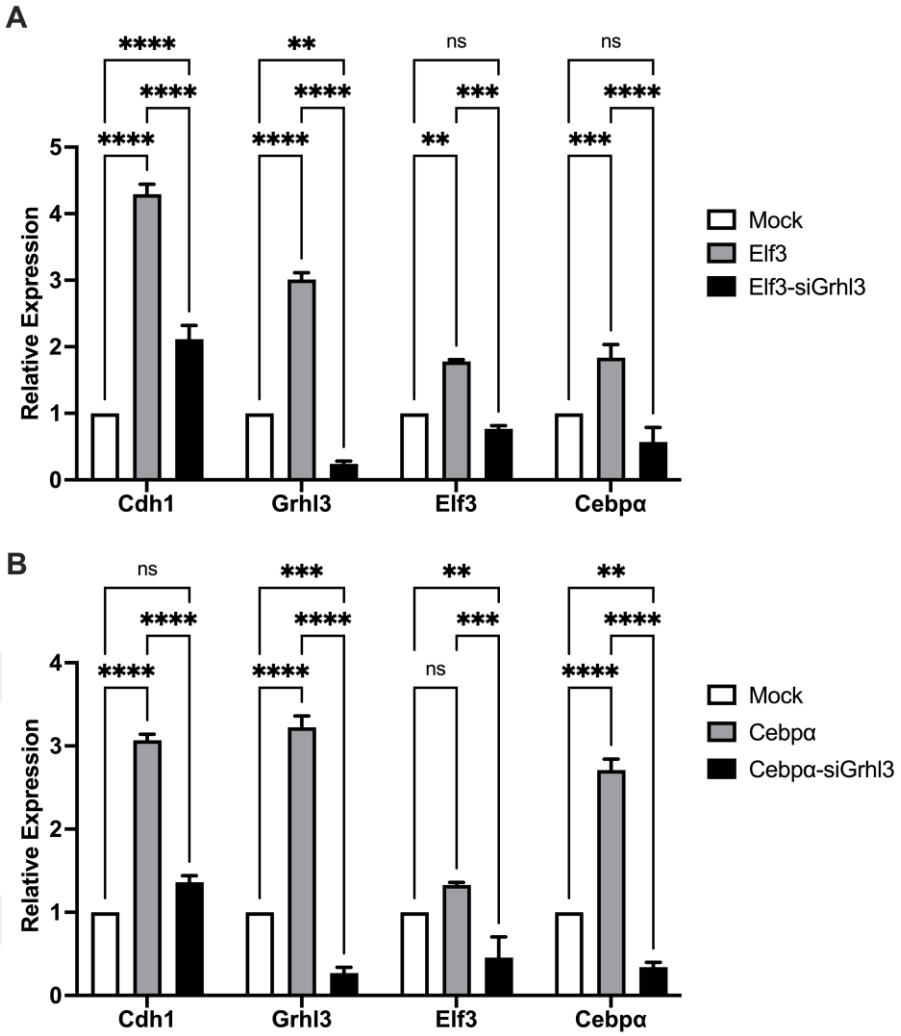


**Figure 20. Crosstalk between MET-TFs.** Analysis of Cdh1, Grhl3, Elf3, and Cebpα mRNA levels to determine the knockdown efficiency of Grhl3 silencing following induction of MET by overexpression of (A) Elf3 and (B) Cebpα.

### *4.2.2. Effect of Silenced Transcription Factor on Maintaining Induced MET in NMuMG*

Normal Murine Mammary Gland (NMuMG) cell line is a well-known cell model frequently used in EMT-MET studies. These cells, originally in epithelial phenotype, switch to a mesenchymal phenotype when treated with TGFβ3. In this process, while cells lose their E-cadherin expression on the membrane, their cortical Actin is replaced by stress fibers, and the expression of mesenchymal markers such as N-cadherin and vimentin increases. When TGFβ3 is removed from the culture medium, the cells return to the epithelial state after the MET process, although not 100%. Although there is an established procedure for evaluating these processes, the timing of these processes is not yet fully known. However, these dramatic changes in gene expression are reflected in morphological features that can be easily observed, making NMuMG cells a good cell line model for EMT-MET studies.

As a result of Grhl3 silencing using 50 nM siRNA while initiating the MET process in NMuMG cells, control cells completed the MET process, while Grhl3-silenced cells could not enter MET, as observed in BF microscope images (Figure 21A). When we look at the Vehicle, which is the untreated state of the NMuMG cell, the EMT that was exposed to TGFβ3 treatment, and the cells that entered the MET process 72 hours after the removal of TGFβ3, it is clear that the ability to undergo EMT/MET, proliferation rate, and apoptosis level are dynamically preserved in the regulation of plasticity. After Grhl3 silencing, this balance is disrupted in cells.

All samples (Veh, EMT, MET, Grhl3 silencing in MET) were collected and validated by qPCR to assess the effectiveness of silencing for both Cdh1 and Grhl3 (Figure 21B). According to the results, Grhl3 is an important transcription factor in maintaining epithelial characteristics in NMuMG cells (Figure 21C).

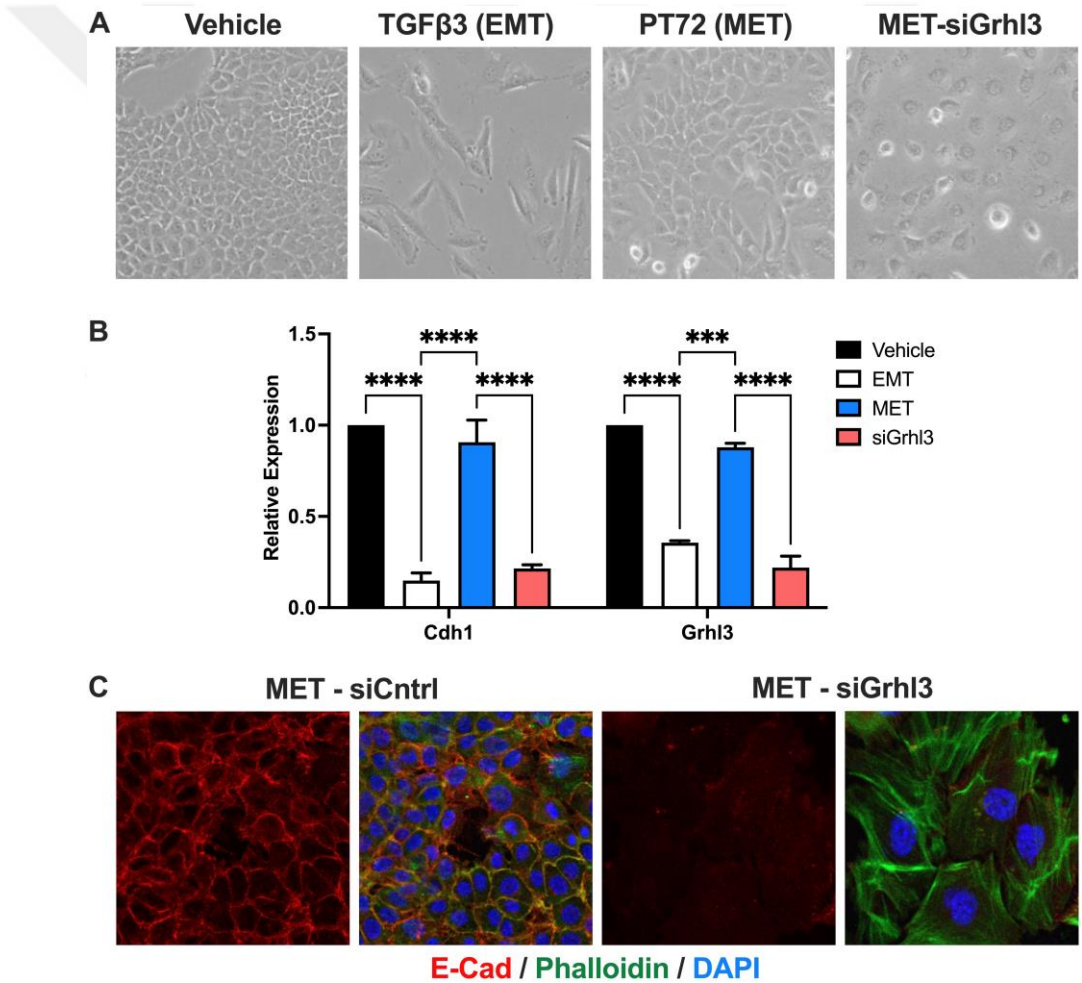


**Figure 21. Grhl3 silencing in NMuMG cells results in MET failure.** A) Bright field (BF) microscope images obtained Veh, EMT, MET and after silencing Grhl3 during MET. B) Determination of Cdh1 and Grhl3 transcript levels for collected samples by qPCR. C) Confocal microscope images of E-cad/phalloidin/DAPI staining obtained following silencing of Grhl3.

### 4.3. Methylation Profiles of Promoters During MET

In this part we examine the epigenetics of Grhl3 induced MET by studying DNA methylation states of relevant promoters using methylated DNA immunoprecipitation (MeDIP). Genomic DNA was isolated after overexpressing Grhl3, followed by validating Grhl3 and Cdh1 mRNA levels by qPCR. One µg of genomic DNA was used for each immunoprecipitation reaction. It was sonicated for 20 cycles using the Bioruptor device. In order to determine whether sonication was effective, 100 and 50 ng of the unsonicated and sonicated samples, respectively, were loaded onto the agarose gel and confirmed the successful sonication to the desired fragment size range of the between 200-500 bp (Figure 22). Sonicated genomic DNA samples were subjected to immunoprecipitation.

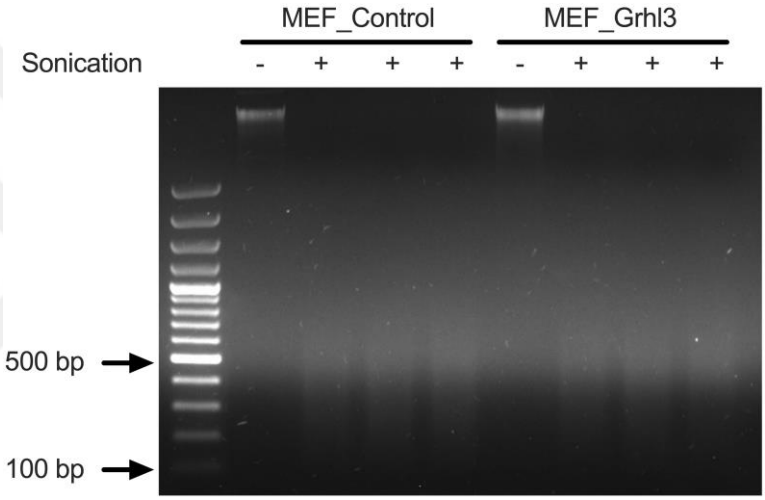


**Figure 22. Agarose gel image of sonicated DNA prepared for MeDIP.** Genomic DNAs were isolated from control MEF cells and MEF cells harvested following overexpression of Grhl3. The DNAs obtained as a result of the isolation were sonicated and then loaded onto agarose gel to determine the range of fragmentation.

Following immunoprecipitation, qPCR was performed to determine the enrichment using antibodies to determine the methylation levels of promoters of epithelial and mesenchymal genes.It is expected that MET begins in Grhl3 transfected MEF cells, and therefore, the methylation levels in the promoters of epithelial genes will decrease, and the methylation levels in the promoters of mesenchymal genes will increase. After MeDIP following Grhl3 overexpression, qPCR was performed to determine the methylation change in the promoters of epithelial genes such as Cdh1, Grhl3, Ovol2, and Hnf4α (Figure 23). These results determined a decrease in the methylation levels of the Cdh1, Grhl3, and Hnf4α promoters. Methylation in the Ovol2 promoter did not change significantly. This is in agreement with the results shown in Figure 15, where Grhl3 transfection did not cause a change in the gene expression level of Ovol2.

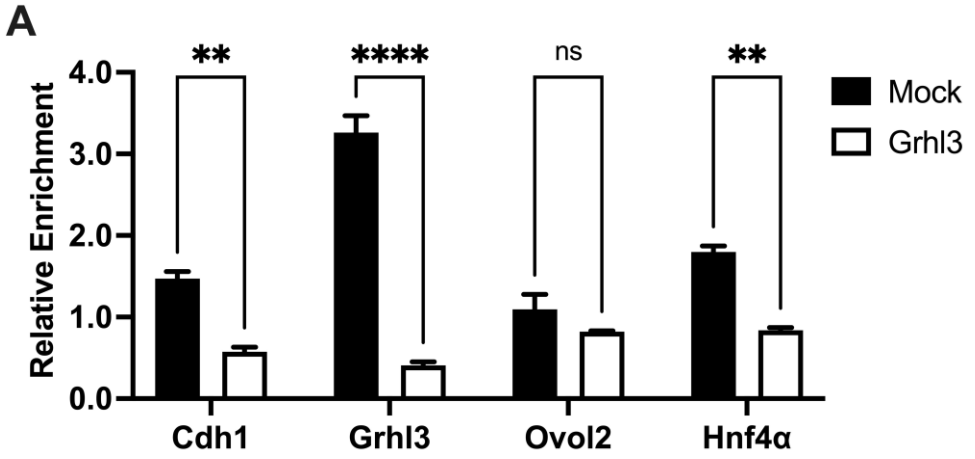


**Figure 23. DNA methylation of epithelial promoters in response to Grhl3 transfection.** Following stimulation of MET with Grhl3, enrichment analysis of the methylation profiles of the promoters of the indicated epithelial genes, Cdh1, Grhl3, Ovol2, and Hnf4α, respectively, was confirmed by qPCR.

An increase in DNA methylation of promoters of mesenchymal genes is expected after Grhl3 overexpression in MEF cells. As seen in Figure 24, the increase in DNA methylation occurring in the promoters of mesenchymal genes such as Zeb1 and Snai1 is statistically very significant.

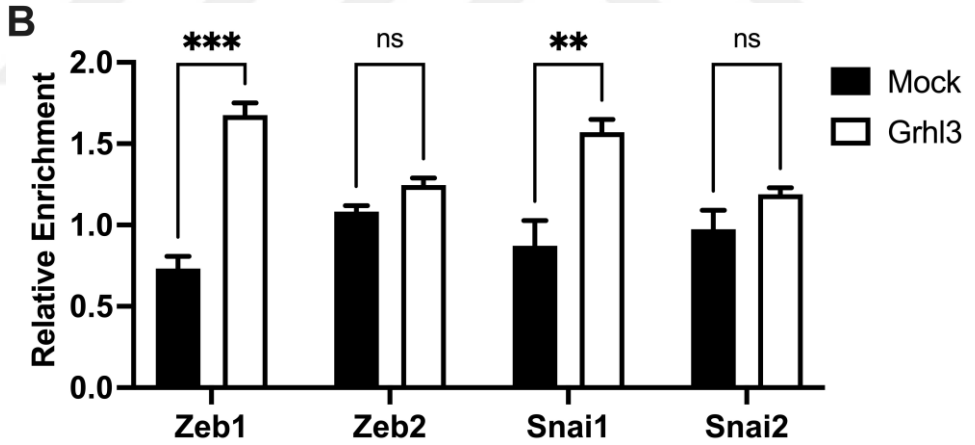


**Figure 24. DNA methylation of mesenchymal promoters in response to Grhl3 transfection.** Following induction of MET by Grhl3, enrichment analysis of methylation profiles of the promoters of mesenchymal genes Zeb1, Zeb2, Snai1 and Snai2 was confirmed by qPCR.

### 4.4. Chromatin Immunoprecipitation During MET in MEF Cells

We then wanted to associate the expression levels of epithelial and mesenchymal genes and their DNA methylation states with changes in histone marks known to represent active or repressed promoters using ChIP assays. First, the samples obtained after overexpression of Grhl3 in MEF cells were sonicated on the Covaris S220 device for 15 cycles, 60 seconds on, 60

seconds off, Peak Power 140 W, duty factor 15, cycle/burst 200, and the whole process was carried out at 4 degrees. The range of chromatin sonication was then evaluated by gel electrophoresis and determined to be between 0.5-1.0 kb (Figure 25).

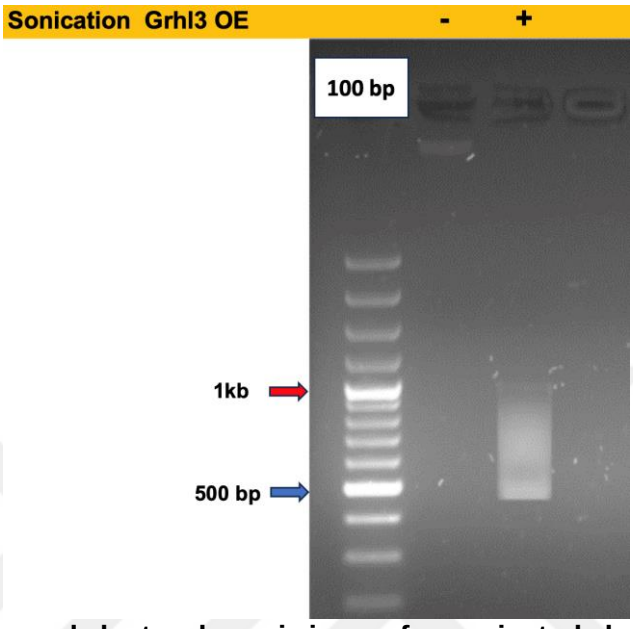


**Figure 25. Agarose gel electrophoresis image for sonicated chromatin.** MET was induced by overexpression of Grhl3 in MEF cells. After 72 hours, the cells were cross-linked, and the fixation process was stopped in the presence of glycine. After sonication, the resulting DNA was isolated and loaded onto the gel to evaluate the sonication quality.

To examine the distribution of histone marks in selected promoter regions, we performed ChIP using H3K4me3, H3K27me3, H3K9ac, and H3K27ac antibodies. In this context, firstly, the distribution of histone marks located in the Grhl3_52 and Grhl3_108 promoter regions was validated by qPCR (Figure 26). Considering that the opposing effects of H3K4me3 and H3K27me3 affect the organization of chromatin in the euchromatin and heterochromatin direction, protein complexes interacting with H3K4me3 inhibit the activity of DNA methyltransferases, thus increasing DNA methylation and prevents associated chromatin condensation and long-term gene repression. Protein complexes interacting with H3K27me3 prevent active transcription, thereby maintaining somatic gene silencing in the germline. According to Figure 26, High levels of H3K4me3, H3K9ac, and H3k27ac at both Grhl3 promoters indicate continued active transcription. Additionally, the detection of a decrease in the H3K27me3 histone methylation mark in both Grhl3_52 and Grhl3_108 promoter regions shows that the Grhl3 transfection process is effective.

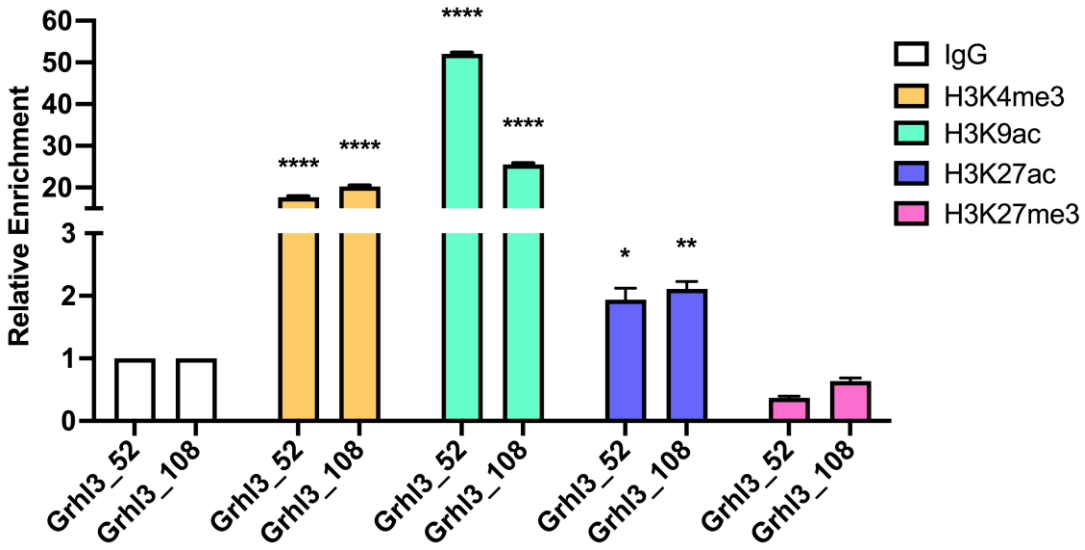


**Figure 26. Distribution of selected histone marks in the promoter regions of the Grhl3 transcription factor.** Examination of histone marks such as H3K4me3, H3K27me3, H3K9ac, and H3K27ac with overexpression of Grhl3 in MEF cells. After 72 hours, cells were cross-linked, and the fixation process was stopped in the presence of glycine. Immunoprecipitation procedures were then performed. DNA enriched as a result of ChIP, enrichment of histone marks in Grhl3_52 and Grhl3_108 promoters was determined by qPCR method compared to the control antibody.

Following this result, the presence of the Grhl3 transcription factor in the Cdh1, Elf3, Cebpα, Uhrf1, Dnmt1, Dnmt3b, and Zeb1 promoters and the change of H3K4me3, H3K27me3, H3K9ac and H3K27ac histone marks in these regions were examined. Selected genomic regions of the CDH1 gene s (Figure 27) are transcriptionally active, as expected, considering both the enrichment of Grhl3 and the enrichment of histone marks involved in active transcription. These results are in agreement with the known regulatory role Grhl3 on Cdh1 expression during MET (Alotaibi et al., 2015).

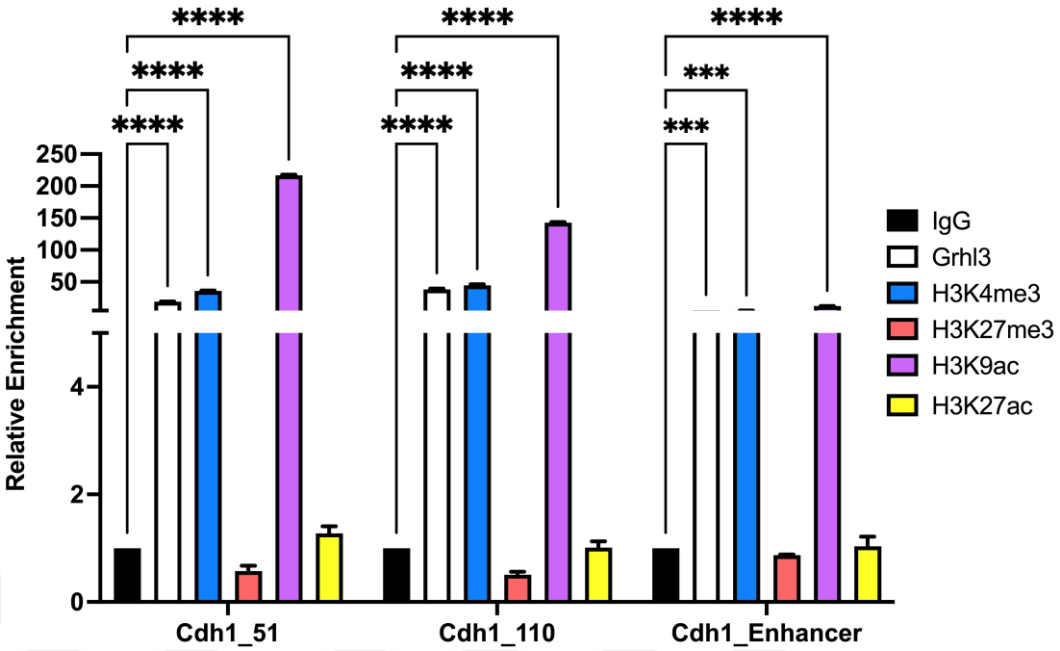


**Figure 27. Determination of the presence of the Grhl3 transcription factor in the Cdh1 promoter and enhancer regions after Grhl3 overexpression and the change of histone marks in these regions.** After 72 hours, cells were cross-linked, and fixation was stopped in the presence of glycine. Then, immunoprecipitation procedures were performed. DNA was enriched as a result of ChIP. The enrichment of the Grhl3 transcription factor and histone marks such as H3K4me3, H3K27me3, H3K9ac, and H3K27ac in the Cdh1_51, Cdh1_110 promoter regions and the selected enhancer genomic region, respectively, compared to the control antibody, was determined by the qPCR method.

The presence of Grhl3 has been detected in the promoter regions of epithelial genes such as Elf3 and Cebpα (Figure 28). It can be said that Grhl3 plays a role in the establishment of epithelial characteristics by binding to the promoter regions of determined epithelial genes. Although details regarding all genes involved in this context are not available, the potential of Grhl3 to direct MET should be evaluated. When the MeDIP results performed on MEF cells were examined, it was determined that the promoter regions of the Zeb1, Zeb2, Snai1, and Snai2 genes were methylated. It was observed that the methylation of the Zeb1 promoter increased more than other mesenchymal genes. According to the results obtained from the subsequent ChIP, it was determined that Grhl3 bound to the Zeb1 promoter (Figure 28). It has been determined that with the increase of H3K27me3, one of the histone marks in this region, the active transcription of this gene is suppressed, and therefore, methylation shifts from the Zeb1/Dnmt1 regulatory axis, which is in an advantageous position for mesenchymal genes. Therefore, it can be said that the MEF cell can no longer maintain its mesenchymal character.

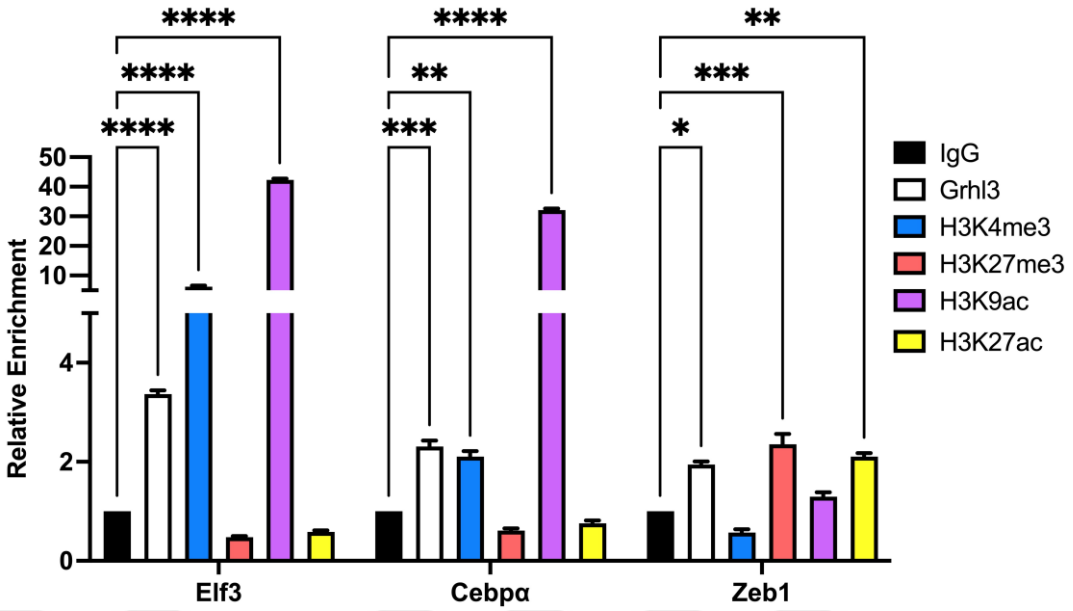


**Figure 28. Determination of the presence of the Grhl3 transcription factor in the Elf3, Cebpα, and Zeb1 promoter regions after Grhl3 overexpression and the change of histone marks in these regions.** After 72 hours, cells were cross-linked, and fixation was stopped in the presence of glycine. Then, immunoprecipitation procedures were performed. DNA was enriched as a result of ChIP. The enrichment of the Grhl3 transcription factor and histone marks such as H3K4me3, H3K27me3, H3K9ac, and H3K27ac in the Elf3, Cebpα, and Zeb1 genomic regions, respectively, compared to the control antibody, was determined by the qPCR method**.**

Following the induction of MET using Grhl3 in MEF cells, H3K4me3 is enriched on the promoter regions of genes such as Dnmt1, Uhrf1, and Dnmt3b (Figure 29). Therefore, it is inevitable to conclude that the transcription of these proteins must be active for the methylation of mesenchymal genes to occur and that the binding of Grhl3 to these regions results from the interaction caused directly or in another way. This may also suggest that Grhl3 could be involved in the transcriptional regulation of these DNA methyltransferases. This novel function of Grhl3 will increase our understanding of the capacity of Grhl3 to initiated MET.

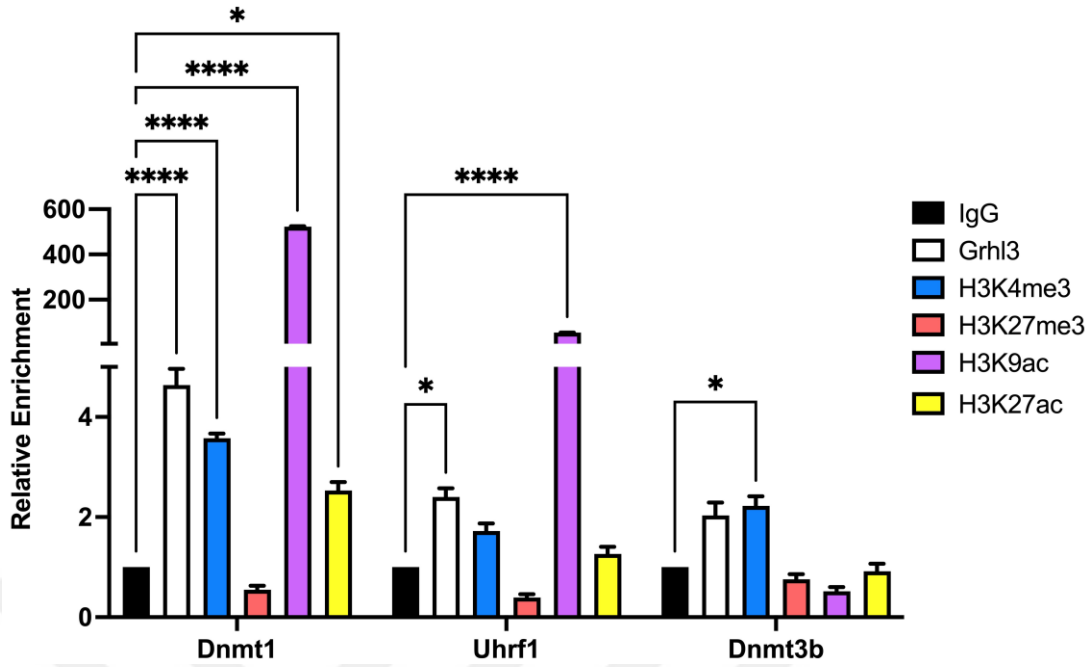


**Figure 29. Determination of the presence of the Grhl3 transcription factor in the Dnmt1, Uhrf1 and Dnmt3b promoter regions after Grhl3 overexpression and the change of histone marks in these regions.** After 72 hours, cells were cross-linked, and fixation was stopped in the presence of glycine. Then, immunoprecipitation procedures were performed. DNA was enriched as a result of ChIP. The enrichment of the Grhl3 transcription factor and histone marks such as H3K4me3, H3K27me3, H3K9ac, and H3K27ac in the Dnmt1, Uhrf1, and Dnmt3b genomic regions, respectively, compared to the control antibody, was determined by the qPCR method.

# 5. DISCUSSION

The ability of a gene to be expressed and function under certain conditions depends on many conditions. Formation of chromatin infrastructure in the presence of both histone modifications and histone variants, transcription factors responsible for activating or repressing a gene regulate each other and the relevant gene through physical interaction, regulations carried out by miRNA, non-coding RNA, alternative splicing, post-translational modifications needed for the active conformation of the protein, the packaging and transportation process of the newly synthesized product and finally, as a result of all these processes, cell metabolism is reprogrammed.

We focus on epithelial-mesenchymal transformation (EMT) and mesenchymal-epithelial transformation (MET) processes, which play a role in regulating the plasticity of the cell, among the many biochemical reactions that take place inside the cell. EMT and MET stand out as mechanisms that play a critical role in many biological processes, such as morphogenesis, organogenesis, wound healing, and migration of tumor cells and their ability to gain metastasis capacity. Although MET has been accepted as the opposite process of EMT according to the understanding that has been established for many years, research conducted at Alotaibi Lab indicates that the MET process is not only a process that works in reverse of EMT but also a dynamic fundamental program regulated by a unique transcription mechanism. Since EMT is the first step during metastasis, it has led to an intensification of research on cell plasticity rearranged in this direction. This has resulted in a large gap between the understanding of the two processes.

E-cadherin (E-cad), encoded by the gene Cdh1, is a member of the cadherin superfamily. E-cad is a $Ca^{+2}$-dependent cell-cell adhesion glycoprotein, which shares about 70% similarity with N-cadherin (N-cad) (Bedzhov et al., 2013). Cadherins anchor the cytoskeleton through interactions with p120-catenin, alpha-catenin, and beta catenins to form adherens junctions (Stemmler, 2008). E-cad and N-cad are expressed in a mutually exclusive manner; E-cad is restricted to epithelial cells, while N-cad is expressed in mesenchymal cells.

Although reduction of E-cadherin and repression of EMT inducers like Zeb1/2 and Snai1/2 are the hallmarks of EMT (Adhikary et al. 2014; Kang and Massague 2004), it is not limited to EMT inducers. Several transcription factors (TFs) also work as molecular switches to retain cellular plasticity, which is regulated by epigenetic mechanisms (Skrypek et al. 2017; Choi et al. 2017; Lee and Kong 2016), which indicates that MET is not simply the reverse process of EMT and is a fundamental program as well. It involves a series of dynamic and tightly regulated events by either transcriptional or posttranscriptional mechanisms, in the center of which is the initiation and maintenance of Cdh1 expression and the presence of E-cadherin at the

plasma membrane, which are essential steps for proper entry to MET (Sengez et al. 2019).

Normal mouse mammary gland cells (Normal Murine Mammary Gland / NMuMG) were used to examine EMT and MET processes. NMuMG cells are a cell line frequently used in our laboratory in previous studies and are accepted in the literature as an EMT/MET model. NMuMG cells have two main characteristics: the first is that the NMuMG cell is not a cancer/tumor cell. NMuMG cells contain a typical number of chromosomes and have no known mutations. Secondly, these cells respond to TGFβ3 and undergo EMT more quickly than other EMT cell models and can undergo MET after TGFβ3 is removed from the environment. Especially if the cells do not undergo a good EMT, even a few remaining epithelial cells can quickly mask the changes in MET by proliferating if MET is triggered. For this reason, although it is a cell line that has been used in our laboratory for a long time, we analyze whether EMT and MET transitions are healthy in every experiment we conduct.

According to the data obtained within the scope of the projects completed as a result of the completion of the data in the literature and the hypothesis established, Elf3, Grhl3, Ovol2, Hnf4α, and Cebpα were identified as some of the regulatory factors controlling the MET process. Their role in the MET-specific transcriptional network has begun to be investigated.

Some enhancers in the intron-2 region, critical in regulating E-cadherin, were first identified. It has been determined that Grhl3 and Hnf4α transcription factors associated with these enhancer regions contribute to the regulation of E-cadherin expression in the MET process. Additionally, Grhl3 can regulate the expression of Hnf4α by binding to the Hnf4α promoter. Our results show that the MET program cannot be initiated in NMuMG cells with the Grhl3 gene silenced, and therefore, Grhl3 is an absolute factor for the initiation of MET (Alotaibi et al., 2015). However, for E-cadherin to be fully activated during MET, Hnf4α must be recruited to the Cdh1 enhancer. Hnf4α is responsible for creating the chromatin infrastructure necessary for Grhl3 to function. When the MET process is completed, Grhl3 dissociates from the Cdh1 locus, and only Hnf4α is needed for the steady-state expression of E-cadherin.

Grhl3 and Ovol2 transcriptional factors have been shown to play a joint role in the transcriptional regulation of important biological processes such as regulation of gene expression and developmental processes, EMT, apoptosis, programmed cell death, chromatin organization, and epigenetic regulation. In addition, the Elf3 transcription factor was identified as the regulator of Grhl3, which is critical for the initiation of MET and was published and introduced to the literature (Sengez et al., 2019). The close relationship of Elf3, Grhl3, Cebpα, and Hnf4α transcription factors with factors regulating the chromatin infrastructure suggests that the essential roles of these factors during MET can be explained by this mechanism. It is a known fact that many factors, such as transcriptional factors, chromatin regulatory factors, and adhesion molecules, are responsible for keeping the cell in balance in terms of phenotypic plasticity,

and the mechanisms in which they take part affect other mechanisms with a domino effect. The dynamism of the epigenome is provided by the relationship of transcription factors with chromatin and chromatin regulatory enzymes. By activating transcription factors, the chromatin infrastructure changes, and these factors bind to DNA regulatory regions. Enzymes that can change chromatin (chromatin modifying and remodeling enzymes) play an active role in this entire process (Brettingham-Moore et al., 2015).

In order to evaluate MET conversion in MEF cells, we used the NMuMG cell model as a positive control. This literature will help shorten the time taken to study MET in the NMuMG cell line. Considering that all these processes are dynamically regulated during or after embryo formation, the nature of the differentiating cell is regulated differently. The use of a cell line with embryonic character is vital in examining the retrospective stages of this process. Although induced pluripotent stem cells (iPSC) are not in the scope of this thesis, the cell adhesion molecule E-cadherin has important functions in pluripotency and reprogramming. When evaluated accordingly, the question arises if the process can be triggered in this direction. MEF cell line is a heterogeneous cell of mesenchymal origin that plays a critical role in wound repair and healing, is found in various locations, has different appearances and different activities, and can be considered as multipotent progenitor source. At the same time, they can be reprogrammed into cells that can perform different types of functions (such as neurons and adipocytes) by being treated under different conditions. The aim here is to evaluate the capacity of MEF cells to program MET with the help of the Grhl3 factor or other epithelial factors and their effectiveness in maintaining this epithelial character.

There is a significant increase in Grhl3 and Cdh1 mRNA levels after Grhl3 overexpression compared to control cells (Figure 10A and Figure 10B). According to images obtained by confocal microscopy, it was determined that the increase in Cdh1 mRNA level also caused an increase in E-cadherin protein in MEF cells compared to the control. (Figure 13A). In addition, following Grhl3 overexpression, the E-cadherin protein synthesized by the MEF cell appears to be localized in the cytoplasm compared to the NMuMG cell (Figure 13B). Considering these results, overexpression of Grhl3 may initiate the MET process in MEF cells. For MET to be sustainable, the E-cadherin protein must be localized on the plasma membrane. The absence of E-cadherin at the plasma membrane could be due to the premature synthesis of the responsible trafficking proteins, which may require additional time or the activation of other cues still absent in MEFs.

E-cadherin localization to the zonula adherens is fundamental for epithelial differentiation, but the mechanisms controlling localization are unclear. E-cadherin transport was examined in an in vivo model using Drosophila follicular epithelium. Three mechanisms mediating E-cadherin accumulation in zonula adhesions have been interpreted. Two membrane trafficking pathways deliver newly synthesized E-cadherin to the plasma membrane. It is Rab11 dependent and targets E-cadherin directly to the zonula

adhesions, while the other transports E-cadherin to the lateral membrane. Differential activation of these pathways could facilitate cell shape changes during morphogenesis, while their misregulation compromises cell adhesion and tissue architecture in differentiated epithelia (Woichansky et al., 2016). As stated in the literature, this is a delicate process. If we need to compare Klf4 in the literature and Grhl3 in the scope of this thesis, they can be summarized as follows.

Klf4, one of the exogenous reprogramming factors, activates the epithelial program. It is stated in the literature that fibroblasts turn off essential mesenchymal genes to overcome the EMT epigenetic barrier and create a stable ground condition "near pluripotency" in the absence of sufficient E-cadherin (R. Li et al., 2010). Although Klf4-induced increase in Cdh1 was detected, E-cad is still localized in the cytoplasm, as seen with Grhl3. Transport of E-cad protein to the membrane is essential for a complete MET process. The key desired step is: Does Grhl3 have the potential to direct E-cad to the membrane? If so, could it be associated with ER and Golgi-related factors to increase transport? Although E-cad transportation is not emphasized within the scope of this thesis, it should be emphasized that work still needs to be done for a complete MET. If this condition is met, Grhl3 may be a candidate as a factor that entirely directs the MET process.

Another question should be whether transfection of the plasmid in mammalian cells followed by transfer to subsequent next generation cells is a low-efficiency process. When evaluated in this context, there may be intense plasmid entry into specific cells, but this is not preferred for the energetic processes of both parental and the next generation. Therefore, the number of cells expressing the E-cadherin protein, which plays a role in establishing intercellular connections, may be low, or the cells receiving the plasmid may be localized further away than the distance required to form intercellular adhesive connections, thus limiting both paracrine signal transmission and physical interactions. Even in epithelial cells that actively express the E-cadherin protein, the membrane transport process is tightly controlled. Therefore, it is more likely that the time period from the induction of E-cadherin expression in MEF cells to the transport process extends over a long period of time.

Following Grhl3 transfection in MEF cells, mRNA levels of epithelial and mesenchymal gene markers were confirmed by qPCR. Among the epithelial genes, the increase in Grhl3 and Cdh1 is the highest. As expected, an increase in the gene levels of genes such as Elf3, Klf4, Cebpα, Ehf, Crif1, and Hnf4α, which are involved in maintaining epithelial properties, was detected. No significant changes were detected for Ovol2. A decrease was detected in the mRNA levels of Cdh2, Zeb1, Zeb2, Snai1, Snai2, Vim, and Fn1 genes, which play a role in the display of mesenchymal features. As a result, transcript levels of proteins such as Fn1, which connects with the extracellular matrix, and Vim, which plays a role in mechanosensing control, showed a significant decrease

after Grhl3 transfection, suggesting that a balance between the inside and outside of the cell is being re-established.

Grhl3 stands out as the strongest candidate for activating the Cdh1 locus following Grhl3, Elf3, Cebpα, and Klf4 transfection. It is Elf3 that increases the Grhl3 transcript level the most after Grhl3 overexpression. Likewise, the Elf3 transcript level is higher following Grhl3 overexpression compared to Elf3 overexpression. These two transcription factors potentially serve as regulators of each other.

After inducing MET by transfecting Grhl3 in MEF cells, these cells were individually silenced by Cebpα, Elf3, and Grhl3. In Grhl3 knockdown cells, the Cdh1 transcript level is lower compared to the other two transcription factors. The decrease in Elf3 transcript level in Grhl3 knockdown cells confirms that Grhl3 regulates Elf3. Likewise, the Grhl3 transcript level was severely reduced in Cebpα and Elf3 knockdown cells.

The increase in Elf3 and Cebpα mRNA levels after overexpression and decrease after silencing indicates that these transcription factors are regulated by Grhl3. When the changes in the mRNA levels of Cdh1 and Grhl3 are examined, the increase in transcript levels after overexpression of the relevant factors and the decrease in transcript levels after silencing are consistent with other data. These results confirm that a reciprocal regulation between transcription factors coordinates changes in Cdh1 mRNA level. In order to answer the question of whether the Grhl3 transcription factor is truly indispensable for MET, it was silenced in NMuMG cells during the initiation of the MET process. It has been determined that following this silencing, the homeostasis that the cell tries to maintain between differentiation, proliferation, and cell death is disrupted. The decrease in the adhesion ability of cells is associated with E-cadherin degradation. Based on the microscope images, it can be seen that the cytoplasm content of most of the cells is released. Grhl3 is a protective epithelial transcription factor involved in maintaining the cell's balance between differentiation, proliferation, and cell death in both NMuMG and induced MEF cells.

Currently, there are three primary methods to identify and quantify DNA methylation. Bisulfite conversion and sequencing, differential enzymatic cleavage of DNA, and affinity capture of methylated DNA (Laird, 2010). Restriction enzyme-based differential cleavage of methylated DNA is locus-specific. However, affinity-capture and bisulfite conversion followed by sequencing methods have been used for both gene-specific and genome-wide analysis (N. Li et al., 2010). The most commonly reported DNA affinity capture method is methylated DNA immunoprecipitation (MeDIP), which uses methyl DNA-specific antibodies or methyl capture using methyl-CpG binding domain (MBD) proteins. Each approach is sensitive but has limitations, like antibody cross-reactivity or methylcytosine density dependency (Nair et al., 2011). There are also other reagents to study DNA methylation. For example, CpG DNA methyltransferase is useful for CpG-methylated gene expression studies

in a cell culture system. Similarly, methylated DNA controls are useful for methylation-specific PCR after bisulfite conversion of DNA. Although each method has advantages and disadvantages, the MeDIP method was used on the grounds that it separates 5mC and 5hmC.

When methylation levels of epithelial promoters were examined after overexpression of Grhl3 in MEF cells, Cdh1, Grhl3, and Hnf4α, methylation levels were reduced as expected. As remembered from Figure 15, the increase in the transcript level of the Ovol2 gene increased the least compared to all other epithelial genes. Therefore, after MET induction of other epithelial genes, the DNA methylation levels of their promoters decrease more and the increase in transcription levels is greater compared to Ovol2, which is related to the fact that the two data support each other. We also expect an increase in the promoter regions of mesenchymal genes. As expected, the promoters of all Zeb1, Zeb2, Snai1, and Snai2 genes are methylated. This methylation increase is not much. Therefore, the decrease in the transcript level of these genes after Grhl3 overexpression will not be significant. This result confirms that the previous reduction in mesenchymal gene levels was insufficient. The fact that the increase represents both types of plasticity leads to the conclusion that the MEF cell population initiates but cannot fully complete MET after Grhl3 overexpression.

Although not included in the results, it was previously determined that when the EMT state is induced in the NMuMG cell, the methylation of the promoter regions of epithelial genes increases. When the MET state is induced, the methylation of the promoter regions of the epithelial genes decreases. In mesenchymal genes, the methylation change during these processes works in the opposite way. During EMT, the methylation of the promoters of these regions decreases. It increases during MET.

MeDIP, performed after Grhl3 overexpression, one of the factors that most affect the Cdh1 transcript level, was not performed after knockdown. The amount of DNA obtained following Grhl3, Elf3, and Cebpα silencing performed during MET in both MEF cells and NMuMG cells is less than the input required to initiate the MeDIP experiment. It is also costly as it requires large amounts of siRNA.

When the ChIP-qPCR results performed after overexpression of the Grhl3 transcription factor are interpreted, the Grhl3 transcription factor is localized in the promoter region of the Cdh1 gene. It was also detected in the enhancer region of the Cdh1 gene. In this case, a comment can be made as follows. Grhl3 is responsible for the regulation of the Cdh1 gene by being located in both the enhancer and promoter regions, and the reason for its prominence compared to other transcription factors is understood. Grhl3 was enriched on the promoter of Uhrf1, one factor that plays a role in the recruitment of Dnmt1 to hemimethylated DNA. In addition, since it is located in the promoters of Dnmt1 and Dnmt3b genes, it can be said that it plays a role in regulating the enzymes involved in the regulation of DNA methylation.

Additionally, the presence of Grhl3 in the Elf3 and Cebpα regions confirms the regulation between these factors. Grhl3 has been found to be enriched in the promoters of DNA methylation enzymes and, in particular, prominent transcription factors such as Elf3 Cebpα, and Zeb1. It outlines that Grhl3 shapes chromatin organization by mediating the change of DNA methylation profile and regulation of histone marks, and thus its role in regulating the transcription of epithelial and mesenchymal genes.

# 6. CONCLUSION

Cells faced with different signals throughout EMT-MET processes have the ability to change their cellular identity reversibly. Mesenchymal-epithelial transition (MET) is a key event involved in different biological processes such as developmental process, tissue regeneration, reprogramming into iPSC cells, cancer development, etc. As mentioned extensively in this thesis, similar to the EMT process, MET is regulated by many signaling pathways, transcription factors, and epigenetic factors at different levels in cells, and the cause-effect relationship formed by these factors may vary depending on physiological and pathological conditions. Although MET is thought to be triggered by the rapid decrease in the binding affinity of factors involved in epithelial-mesenchymal transition (EMT) regulation to the Cdh1 locus, several studies have determined that MET regulation is not limited to this.

The dynamism of the epigenome is provided by the relationship of transcription factors with chromatin and chromatin regulatory enzymes. Grhl3, apart from the regulation of gene expression and development processes, has been found to work in the transcriptional regulation of critical biological processes such as EMT, apoptosis, programmed cell death, chromatin organization and epigenetic regulation.

Factors such as Elf3, Cebpα, Klf4, and Grhl3, which are members of the MET transcriptional network, were determined to be critical factors for the initiation of MET in MEF cells. Grhl3 is one of the most prominent factors among these factors, as it has the powerful potential to regulate the Cdh1 gene, which encodes the E-cadherin protein.

It is Elf3 that increases the Grhl3 transcript level the most after Grhl3 overexpression. Similarly, the Elf3 transcript level is higher following Grhl3 overexpression compared to Elf3 overexpression. According to previously obtained information, Elf3 regulates this factor by associating with the promoter region of Grhl3, and Grhl3 regulates this factor by associating with a cis-regulatory element on the Cdh1 gene. It is thought to affect the localization of E-cadherin. When we look at the Cdh1 mRNA level in the Elf3-Grhl3 association, this data is confirmed.

In Grhl3 knockdown cells, the Cdh1 transcription level is lower compared to the other two transcription factors. The decrease in Elf3 transcript level in Grhl3 knockdown cells confirms that Grhl3 might be a regulator of Elf3. Grhl3 transcript level was severely reduced in Cebpα and Elf3 depleted cells. Both the fact that the relevant transcription factors cause an increase in each other's mRNA expression levels after their overexpression and the decrease in mRNA levels after silencing led to the conclusion that there is a mutual regulation between the transcription factors and that they affect the Cdh1 level.

Looking at the silencing of Elf3 and Grhl3 in the MEF cell, it is also seen in confocal images that the MET process was attenuated. Knocking down Grhl3 is more severe than knocking down Elf3 and Cebpα. There is a serious decrease in the number of proliferating cells. Considering the potential of the mentioned factors to regulate each other, the absence of one will affect the other, suggesting a potential regulatory network in play.

To answer the question of whether the Grhl3 transcription factor is truly indispensable for MET, it was also silenced in NMuMG cells during the initiation of the MET process. After silencing, the decrease in adhesion ability between cells is associated with the downregulation of E-cadherin. Grhl3 appears as a protective epithelial transcription factor that plays a role in maintaining the cell's balance between differentiation, proliferation, and cell death in both NMuMG and induced MEF cells, making a big difference in the sustainability of MET. Although the importance of DNA methylation in epithelial gene silencing during EMT has been well studied, the comprehensive picture of the DNA methylation pattern of MET is still lacking. Despite efforts to investigate the epigenetic mechanisms associated with EMT, relatively little attention has been paid to the involvement of epigenetic mechanisms in MET. By analyzing the DNA methylation profile in MEF cells on a small scale, new information was obtained to interpret the DNA methylation status of the cell and, therefore, a part of its complete mechanical picture under this heading. In this context, ChIP was performed, and it was determined that Grhl3 binds to the promoter region of both Elf3 and Cebpα transcription factors. H3K4me3, H3K9ac, and H3K27ac, which are active histone marks, increase in these promoter regions, and H3K27me3 decrease supports the induction of MET. Histone methylation plays a role in recruiting DNA methylation to particular genomic regions and in its exclusion from others. H3K4me3 is mutually exclusive with and is, therefore, a candidate for histone modification that blocks DNA methylation. Its presence in the Cdh1, Elf3, and Cebpα regions is like a protective layer for the epithelial character.

H3K4me3 is dense in the promoter regions of proteins such as Dnmt1, Uhrf1, and Dnmt3b. Therefore, it is inevitable to conclude that for methylation of mesenchymal genes to occur, the transcription of these proteins must be active, and Grhl3's binding to these regions is due to the interaction it causes either directly or through another way. Consequently, Grhl3 might be a Dnmt1/Uhrf1 complex regulator required to maintain global DNA methylation and/or hypomethylation.

EMT inducers such as Snai1 and Zeb1 are known to act during the EMT program by acting on the miR200 family, recruiting DNA methyltransferases (DNMTs) histone-modifying enzymes to the Cdh1 promoter. Following Grhl3 overexpression, the presence of Grhl3 was determined in the Zeb1 promoter, and an increase in the H3K27me3 mark showed that the activity of Zeb1 was silenced; therefore, DNA methylation shifted from the Zeb1/Dnmt1 axis and other mesenchymal genes were methylated and mesenchymal character could not be maintained any further. The interplay between these systems

maintains genome and epigenome integrities during several biological processes, including EMT and MET. Therefore, integrating gene regulation with epigenetic markers stands out in terms of obtaining new gains for the MET process.

Induced pluripotent stem cells (iPSC) have improved therapeutic and translational potential. Genomic integration of transcription factors into the relevant cell for their continued activity may cause the risk of introducing mutations into the genome of the target cell, resulting in tumorigenesis. Therefore, instead of retrotransposons being used to overcome the low reprogramming efficiency in iPSC, different vectors such as plasmids, adenoviruses, and transposon vectors have been used to prevent genomic insertion.

The activation of endogenous loci of the core pluripotency network is described as accomplished by the MET process when generating iPSCs. E-cadherin is the adherent junction protein required to maintain embryonic stem cells in a pluripotent state. A few regulators of Cdh1 have been reported in the literature (Alotaibi et al., 2015; Sengez et al., 2019). According to previous reports, Klf4 has been proposed as a potential MET-TF that can simultaneously repress many EMT-TFs and inhibit EMT through multiple parallel pathways. For example, E-cadherin expression is repressed by Zeb2/ SIP1 while it is induced by Klf4 (Koopmansch et al., 2013). Independent data from the literature indicate that these two transcription factors could bind close to each other in the proximal region of the Cdh1 gene promoter.

In the scope of this thesis, Grhl3 has the potential to be a more potent activator than Klf4, as assessed in terms of transcript enrichment upon binding to the Cdh1 promoter. Therefore, although more results are needed to evaluate iPSC-inducing ability, it stands out compared to its effect on Cdh1. It is comparable to the effect exerted by Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc). Due to the inadequacy in iPSC yield, Grhl3 can be recommended as a strong candidate.

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# 8. APPENDIX

## 8.1. Appendix I.

**Table 12. qPCR Primers and Probe Informations**

| Primer | Sequence | Probe |
|---|---|---|
| Cdh1 F | ATCCTCGCCCTGCTGATT | 18 |
| Cdh1 R | ACCACCGTTCTCCTCCGTA | 18 |
| Grhl3 F | AAGGAAGATGTCGAATGAACTTG | 100 |
| Grhl3 R | TCGTCCTCATTACTGTAGGGAAA | 100 |
| Elf3 F | ACCGAACCCTGACACACC | 46 |
| Elf3 R | AGCTGTACATGGCGTTGAAGT | 46 |
| Cebpα F | AAACAACGCAACGTGGAGA | 47 |
| Cebpα R | GCGGTCATTGTCACTGGTC | 47 |
| Klf4 F | CGGGAAGGGAGAAGACAC | 62 |
| Klf4 R | GAGTTCCTCACGCCAACG | 62 |
| Ovol2 F | GTGAGGATTGCGGCTACAC | 79 |
| Ovol2 R | TGGTCACTGTTCACATGCAG | 79 |
| Crif1 F | GCTTAGCGACCATGCAAG | 40 |
| Crif1 R | CACTCTGCGATACGCTGCT | 40 |
| Ehf F | TCATTGTCAAGACTGAACAAACC | 33 |
| Ehf R | GTCCAACAGATCTACTGTGCTACC | 33 |
| Hnf4α F | AGCCTAAATGACAGGTCCTCT | 73 |
| Hnf4α R | TTAATCAGCTGGCCCCATT | 73 |
| Zeb1 F | TGGAGTTCA AAGGTTGTCGTT | 109 |
| Zeb1 R | TTGCCACATCAACACTGGTC | 109 |

| | | |
|---|---|---|
| Zeb2 F | TTGCTCCAGGATGTGTGG | 64 |
| Zeb2 R | CACACACTTGTTTGTGTGCATATC | 64 |
| Snai1 F | CTTGCTCCACAAGCACCA | 71 |
| Snai1 R | GAGGATGGGGAGGTAGCAG | 71 |
| Snai2 F | ATCCTTGGGGCGTGT AAG T | 6 |
| Snai2 R | TGAACCACTGTCATCCTTGG | 6 |
| Cdh2 F | TCCCTGAGATACAGCGTC ACT | 17 |
| Cdh2 R | ATA ATGAAGATGCCCGTTGG | 17 |
| Vim F | ACGTTTCCA AGCCTG ACCT | 74 |
| Vim R | AGCCACGCTTTCATACTGCT | 74 |
| Fn1 F | AGGTGGACCCCGCTAAAC | 31 |
| Fn1 R | TGCCGCAACTACTGTGATTC | 31 |
| Snx1 F | GCCAACAAGCCTGACAAAC | 40 |
| Snx1 R | ACCCGAGATTCCCACTCTG | 40 |
| Exosc9 F | CAAGGTGCCCCTATAGTGCT | 106 |
| Exosc9 R | GGTCTGAGCTCTTATTTTCTTTGG | 106 |
| Pak1 F | ACGGTTCGAGAAGATTGGAC | 98 |
| Pak1 R | TGTGGCTACATCCATTGCAG | 98 |
| Pals1 F | GCATCTGCCGTTCCTCATAC | 33 |
| Pals1 R | TGCCGAGAAACAAAGTGGTA | 33 |
| Tuberin F | CGCAGCATCAGTGTATCTGAA | 84 |
| Tuberin R | GCGCTCGTAAGGGATGTCT | 84 |
| Cbll1 F | TGCCTCCACAGCAACATTAC | 10 |
| Cbll1 R | GCATTGGATGGCTTATTGGT | 10 |
| Rab11a F | AAGGCACAGATATGGGACACA | 51 |

| | | |
|---|---|---|
| Rab11a R | GCACCTACTGCTCCACGATAG | 51 |
| Pip5k1c F | GCTGGCTTCCAAGGACTG | 16 |
| Pip5k1c R | CTGGCCATGATGATTGTGTC | 16 |
| Ddx17 F | GTGGAGGGTTTGGAGCAAGA | 165 |
| Ddx17 R | GTA ACCGCTCCCCAGGATTAC | 165 |
| Cdh1 TSS_51 F | TGAGTTCCCAAGAACTTCTGC | 51 |
| Cdh1 TSS_51 R | GCCGAGCAAACACTG AGC | 51 |
| Cdh1 TSS_110 F | ACAGGGGTGGAGGAAGTTG | 110 |
| Cdh1 TSS_110 R | CTCCCACACCAGTGAGCAG | 110 |
| Cdh1 14100_F | CCCTTGGTTCTAAAGTTGGTTG | 107 |
| Cdh1 14100_R | GGAATGAGGAAACTGGTTTGAC | 107 |
| Dnmt1_F | TGGCAAGCAAACCAGAGTCA | 132 |
| Dnmt 1_R | TTGCCTGAAGAGGTGGCTC | 132 |
| Dnmt3b_F | GTCGAAAAGCCAATGCAGGG | 120 |
| Dnmt3b _R | TTCACAGGTCTGGATGAGCG | 120 |
| Uhrf_F | TCAGAAGTCTCGGGGTGGT | 14 |
| Uhrf_R | CTGCCGGCTATGCTCACTT | 14 |
| Grhl3 P_52 F | TCTAGTTCTCCCGTTCCTTCC | 52 |
| Grhl3 P_52 R | TTCTTAGCTGAGGGGGTGAG | 52 |
| Grhl3 P_108 F | GGAGAAAAGAGAGGGGACTCA | 108 |
| Grhl3 P_108 R | TCACCTTCTCTACCCGGAAA | 108 |
| Ovol2 P_F | GCAAACATTCATGCCTAAGGA | 27 |
| Ovol2 P_R | AACAAGCTCGCTACCTGTCC | 27 |
| HNF4α P2_F | ATGTCAGCTGGGTTGTCCAC | 66 |
| HNF4α P2_R | AACAGGACTGGCATTGGTTG | 66 |

| | | |
|---|---|---|
| Zeb1 P_F | CCTAGGAAGCCACGCTGTC | 45 |
| Zeb1 P_R | CTCAGGTGTGGTGGAGTAGC | 45 |
| Zeb2 P_F | CTGGATAAAGGGCAGGGAGG | 52 |
| Zeb2 P_R | TCGCCTGGGGATTGGAAAC | 52 |
| Snai1 P_F | GTTCCAGGAACCCCTAAGCG | 129 |
| Snai1 P_R | CCCGAGCTGCTGACCTTT | 129 |

## 8.2. Appendix II.

**Table 13. putative microRNas targeting the 3'UTR of Grhl3**

| Target Rank | Target Score | miRNA name | miRNA Sequence |
|---|---|---|---|
| 1 | 97 | mmu-miR-467a-3p | CAUAUACAUACACACACCUACA |
| 2 | 97 | mmu-miR-669b-3p | CAUAUACAUACACACAAACAUAU |
| 3 | 97 | mmu-miR-669f-3p | CAUAUACAUACACACACACGUAU |
| 4 | 96 | mmu-miR-203-3p | GUGAAAUGUUUAGGACCACUAG |
| 5 | 93 | mmu-miR-26b-5p | UUCAAGUAAUUCAGGAUAGGU |
| 6 | 93 | mmu-miR-381-3p | UAUACAAGGGCAAGCUCUCUGU |
| 7 | 93 | mmu-miR-26a-5p | UUCAAGUAAUCCAGGAUAGGCU |
| 8 | 92 | mmu-let-7a-1-3p | CUAUACAAUCUACUGUCUUUCC |
| 9 | 92 | mmu-let-7f-1-3p | CUAUACAAUCUAUUGCCUUCCC |
| 10 | 92 | mmu-miR-539-3p | CAUACAAGGAUAAUUUCUUUUU |
| 11 | 92 | mmu-miR-669h-3p | UAUGCAUAUACACACAUGCACA |
| 12 | 92 | mmu-miR-669k-3p | UAUGCAUAUACACGCAUGCAA |
| 13 | 92 | mmu-miR-98-3p | CUAUACAACUUACUACUUUCCU |
| 14 | 92 | mmu-let-7b-3p | CUAUACAACCUACUGCCUUCCC |
| 15 | 92 | mmu-let-7c-2-3p | CUAUACAAUCUACUGUCUUUCC |
| 16 | 88 | mmu-miR-126b-5p | AUUAUUACUCACGGUACGAGUU |
| 17 | 87 | mmu-miR-6981-3p | CAACAGCCUACUGUCUCCUCAG |
| 18 | 87 | mmu-miR-21a-3p | CAACAGCAGUCGAUGGGCUGUC |
| 19 | 87 | mmu-miR-448-3p | UUGCAUAUGUAGGAUGUCCCAU |
| 20 | 86 | mmu-miR-7037-5p | AAGGUGGCCACAGGAGAUCAUGGU |
| 21 | 86 | mmu-miR-511-3p | AAUGUGUAGCAAAAGACAGGAU |

| | | | |
|---|---|---|---|
| 22 | 85 | mmu-miR-7211-5p | UCUUUCCCUCUGCCACUCCACC |
| 23 | 84 | mmu-miR-12188-5p | UAGAAGAGCUUUCGCUGGCCCAGA |
| 24 | 84 | mmu-miR-6371 | UUCAAGUGGCUUGAAGCUCUUCU |
| 25 | 82 | mmu-miR-669d-3p | UAUACAUACACACCCAUAUAC |
| 26 | 82 | mmu-miR-466e-3p | UAUACAUACACGCACACAUAAGA |
| 27 | 82 | mmu-miR-297b-3p | UAUACAUACACACAUACCCAUA |
| 28 | 82 | mmu-miR-466d-3p | UAUACAUACACGCACACAUAG |
| 29 | 82 | mmu-miR-466a-3p | UAUACAUACACGCACACAUAAGA |
| 30 | 82 | mmu-miR-297c-3p | UAUACAUACACACAUACCCAUA |
| 31 | 82 | mmu-miR-7226-3p | UGACACAGCCAUUCUCUGAGCAG |
| 32 | 82 | mmu-miR-297a-3p | UAUACAUACACACAUACCCAUA |
| 33 | 82 | mmu-miR-6955-5p | AGGGCCUCAGGACUCAGGAGGUGA |
| 34 | 82 | mmu-miR-467g | UAUACAUACACACACAUAUAU |
| 35 | 80 | mmu-miR-496b | CAACAUGGCCAAUUCCUUUCAU |
| 36 | 80 | mmu-miR-669j | UGCAUAUACUCACAUGCAAACA |
| 37 | 80 | mmu-miR-6957-5p | CAGAAGAGAGACAGGAUCUAG |
| 38 | 80 | mmu-miR-218-1-3p | AAACAUGGUUCCGUCAAGCACC |
| 39 | 80 | mmu-miR-669i | UGCAUAUACACACAUGCAUAC |
| 40 | 76 | mmu-miR-466o-3p | UACAUACAUGCACACAUAAGAC |
| 41 | 76 | mmu-miR-466m-3p | UACAUACACACAUACACACGCA |
| 42 | 72 | mmu-miR-7650-3p | GUUUUGAUAUAUACAAGAAGGA |
| 43 | 70 | mmu-miR-379-3p | UAUGUAACAUGGUCCACUAACU |
| 44 | 70 | mmu-miR-411-3p | UAUGUAACACGGUCCACUAACC |
| 45 | 70 | mmu-miR-3066-5p | UUGGUUGCUGUAGAUUAAGUAG |
| 46 | 70 | mmu-miR-194-5p | UGUAACAGCAACUCCAUGUGGA |

| | | | |
|---|---|---|---|
| 47 | 69 | mmu-miR-1195 | UGAGUUCGAGGCCAGCCUGCUCA |
| 48 | 68 | mmu-miR-7210-3p | UUAUAUGUAAGUAACAAUGUGGCU |
| 49 | 66 | mmu-miR-493-5p | UUGUACAUGGUAGGCUUUC |

## 8.3. Appendix III

**İZMİR BİYOTIP VE GENOM MERKEZİ**
**GİRİŞİMSEL OLMAYAN ARAŞTIRMALAR ETİK KURULU (İBG-GOEK)**
**KARARI**

| | | | |
|---|---|---|---|
| **Toplantı Tarihi** | : 04/03/2022 | **Toplantı Günü** | : Cuma |
| **Toplantı Sayısı** | : 4 | **Toplantı Saati** | : 10:30 |

**Sayın Dr. Hani ALOTAİBİ,**

**2022-011 Protokol No'lu;** sorumlusu olduğunuz "Mezenkimal Epitelyal Dönüşümün Epigenetiği" başlıklı araştırmanın uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

| | |
|---|---|
| **Prof. Dr. H. Alper BAGRIYANIK**<br>**Başkan** | **Dr. Serap ERKEK ÖZHAN**<br>**Başkan Yardımcısı** |
| **Prof. Dr. Sedef AKGÜNGÖR**<br>**Üye** | **Prof. Dr. İnci ALACACIOĞLU**<br>**Üye** (Katılmadı) |
| **Prof. Dr. Gülgün OKTAY**<br>**Üye** | **Prof. Dr. Hilmi ORHAN**<br>**Üye** (Katılmadı) |
| **Prof. Dr. Şermin GENÇ**<br>**Üye** (Katılmadı) | **Doç. Dr. Canan Aslı YILDIRIM**<br>**Üye** |
| **Doç. Dr. Can KÜÇÜK**<br>**Üye** | **Dr. Öğr. Üyesi Yavuz OKTAY**<br>**Üye** |