

T.C.
DOKUZ EYLÜL UNIVERSITY
İZMİR INTERNATIONAL BIOMEDICINE AND GENOME INSTITUTE

**IDENTIFICATION OF TRANSCRIPTIONAL
NETWORKS CONTROLLING THE
MESENCHYMAL-EPITHELIAL TRANSITION**

BURCU ŞENGEZ

DEPARTMENT OF MOLECULAR BIOLOGY AND
GENETICS

DOCTOR OF PHILOSOPHY THESIS

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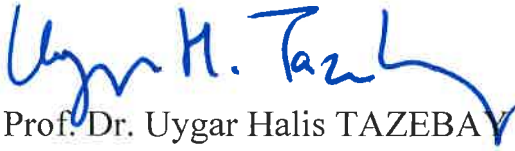
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LIST OF ABBREVIATIONS

EMT- Epithelial-Mesenchymal Transition

MET- Mesenchymal-Epithelial Transition

E-cad- E-cadherin

Elf3- E74-Like Factor 3 (Ets Domain Transcription Factor, Epithelial-Specific)

Grhl3- Grainyhead Like Transcription Factor 3

Ovol2- Ovo Like Zinc Finger 2

Ahr- Aryl Hydrocarbon Receptor

Nfya- Nuclear Transcription Factor Y Subunit Alpha

NMuMG- Normal Murine Mammary Gland

RNAi- RNA interference

TGFβ- Transforming Growth Factor Beta

EGF- Epidermal Growth Factor

FGF- Fibroblast Growth Factor

Snail- Snail Family Zinc Finger 1

Slug- Snai2, Snail Family Zinc Finger 2

Zeb1- Zinc Finger E-Box Binding Homeobox 1

Zeb2- Zinc Finger E-Box Binding Homeobox 2

Ca²⁺- Calcium Ion

Cdh1- Cadherin 1

miRNA- microRNA

TAM- Tumor Associated Macrophages

N-cad- N-cadherin

ZO-1- Zonula occludens-1, Tight junction protein-1

PI3K- Phosphoinositide 3-kinase, Phosphatidylinositol 3-kinase

MAPK- Mitogen-activated Protein Kinase

RNA- Ribonucleic Acid

iPSC- Induced Pluripotent Stem Cell

Klf4- Kruppel Like Factor 4

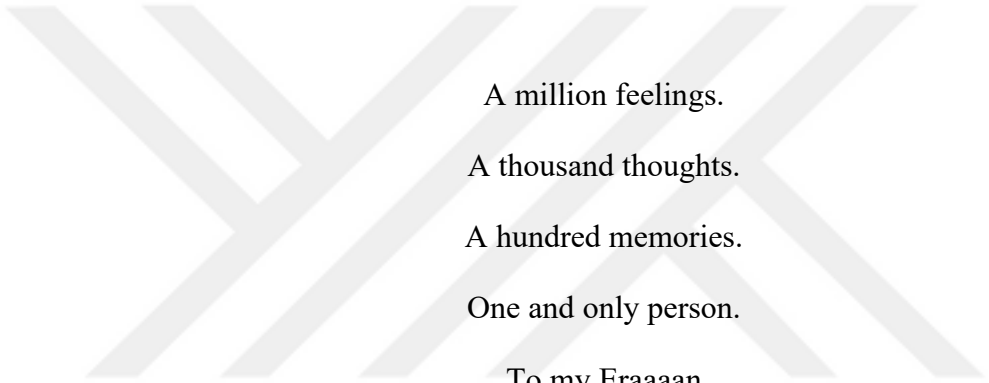
Nanog- Homeobox Transcription Factor Nanog

Hnf4a- Hepatocyte Nuclear Factor 4 Alpha

Oct4- Octamer-Binding Transcription Factor 4, POU Domain Class 5, Transcription Factor 1 (POU5F1)

Sox2- SRY-Box Transcription Factor 2
HoxB4- Homeobox B4
c-myc- Myc Proto-Oncogene Protein
Grh- Grainy Head
Elf-1- E74 Like ETS Transcription Factor 1
Lmo-4- LIM Domain Only 4
Pten- Phosphatase And Tensin Homolog
ESRP1- Epithelial Splicing Regulatory Protein 1
HCC- Hepatocellular Carcinoma
PolII- RNA polymerase II (RNAP II)
ATCC- American Type Culture Collection
GEO: Gene Expression Omnibus Database
DMEM- Dulbecco's Modified Eagle Medium
FBS- Fetal Bovine Serum
MEM NEAA- MEM Non-Essential Amino Acids Solution
BSA- Bovine Serum Albumin
HCl- Hydrochloric acid
PBS- Phosphate-Buffered Saline
PT: Post treatment
siRNA- Small interfering RNA
mRNA- Messenger RNA
cDNA- Complementary DNA
qPCR- Quantitative Polymerase Chain Reaction
Gapdh- Glyceraldehyde-3-Phosphate Dehydrogenase
ChIP- Chromatin immunoprecipitation
RnaseA- Ribonuclease A
SA- β -GAL- Senescence-associated beta-galactosidase
Dox- Doxorubicin
RT-PCR- Reverse Transcription Polymerase Chain Reaction
Mg- Magnesium
MgCl₂- Magnesium Chloride
UCSC- University of California, Santa Cruz
ChIP-Seq- Chromatin Immunoprecipitation Sequencing
SRA: Sequence Read Archive

BWA: Burrows-Wheeler Aligner
SAM: Sequence Alignment Map
BAM: Binary Alignment Map
Casz1- Castor Zinc Finger 1
Zfp750- Zinc Finger Protein 750
RegNetwork: Regulatory Network Repository
ChiBE: Chisio BioPAX Editor
Sp1-Proximal specificity protein 1
UTR- Untranslated Region
Hey2- Hes Related Family BHLH Transcription Factor With YRPW Motif 2
Atf3- Activating Transcription Factor 3
FOXA1- Forkhead Box A1
IRF6- Interferon Regulatory Factor 6
RIPK4- Receptor Interacting Serine/Threonine Kinase 4
Aop- Anterior Open
Eip74EF- Ecdysone-induced Protein 74EF
Ehf- ETS Homologous Factor
Elf5- E74 Like ETS Transcription Factor 5
Shg- DE-Cad
CO₂- Carbon dioxide
°C- Celsius
W- Watt
h- Hour
s: Second
ng- Nano gram
mL- Milliliter
nM- Nanomolar
M- Molarity
g- Gram
μl- Microliter
μg- Micro gram
μm- Micro molar
Sep- September
Feb- February



A million feelings.

A thousand thoughts.

A hundred memories.

One and only person.

To my Eraaaan

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IDENTIFICATION OF TRANSCRIPTIONAL NETWORKS CONTROLLING THE MESENCHYMAL-EPITHELIAL TRANSITION

Burcu Şengez, Izmir International Biomedicine and Genome Institute, Dokuz Eylül
University, 35340. Izmir, Turkey.

ABSTRACT

Dynamic fluctuations through cellular plasticity and intermolecular changes within the gene regulatory networks are utilized by conserved and complex mechanisms in which cells switch between different phenotypes without altering the genotype. Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) cellular programs, in which E-cadherin (E-cad) is the hallmark, endow with phenotypic plasticity. While much attention is being paid to EMT regulation, little attention is paid to the mechanisms underlying MET regulation.

This thesis explores the dynamic MET process from a distinct viewpoint from its counterparts in the literature. It is based on identifying a basic transcriptional network that controls cells' epithelial stability, which might play a crucial role in initiating the MET process. We carried out a comprehensive approach consisting of data mining, *in silico* analyses, and *in vitro* experimental studies to determine the possible network members and verify their relationships.

Analysis of gene expression profiles from NMuMG cells and the other datasets obtained from the literature, followed by loss of function experiments, revealed that transcription factors such as *Elf3*, *Grhl3*, *Ovol2*, *Ahr*, and *Nfya* are regulators of the MET process. Some transcriptional nodes were identified where *Grhl3* and *Elf3* are in the center of the network. It is also revealed that *Grhl3* and *Ovol2* transcriptional factors are most likely to collaborate in development processes and transcriptional regulation of important biological processes such as EMT.

In conclusion, our findings present novel insights into the regulation of MET and reveal some transcription factors such as *Elf3* as an indispensable guardian of the epithelial state within a transcriptional network.

Keywords: MET, Transcriptional Networks, EMT, *Elf3*, *Grhl3*, *Ovol2*

MEZENKİMAL-EPİTELYAL DÖNÜŞÜMÜNÜ (MET) KONTROL EDEN TRANSKRİPSİYONEL AĞLARIN BELİRLENMESİ

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ÖZET

Gen düzenleyici ağlar kapsamındaki moleküller arası değişimler ve hücresel plastisite arasındaki dinamik geçişler hücrelerin genotiplerini değiştirmeden farklı fenotipler arasında geçiş yaptığı korunmuş ve kompleks mekanizmalar tarafından kullanılırlar. E-kaderin (E-cad)'in ayırt edici özellik olduğu epitelyal-mezenkimal dönüşüm (EMT) ve mezenkimal-epitelyal dönüşüm (MET) hücresel programları fenotipik plastisite ile donatılmıştır. Literatürde EMT'in düzenlenmesi konusunda birçok çalışma bulunurken, MET'in düzenlenmesinin altında yatan mekanizmalara değinen yayınlara hala daha az rastlanmaktadır.

Bu tez çalışması, dinamik MET sürecini literatürdeki benzerlerinden farklı bir bakış açısıyla araştırmaktadır; çünkü MET sürecinin başlatılmasında önemli rol oynayan ve hücrelerin epitel durumdaki stabilitesini kontrol eden temel bir transkripsiyonel ağ varlığını belirlemeyi hedeflemiştir. Bu amaçla literatürden ve biyoinformatik veritabanlarından yararlanılarak olası ağ üyelerinin belirlenmesi ve birbirleri ile ilişkilerinin doğrulanması amacıyla in-vitro deneyler gerçekleştirilmiştir.

Sonuç olarak, MET sürecini kontrol eden *Elf3*, *Grhl3*, *Ovol2*, *Ahr* ve *Nfya* gibi transkripsiyon faktörleri saptanmıştır. *Grhl3* ve *Elf3*'ün ağ merkezinde bulunduğu bazı transkripsiyonel ağ odakları tanımlanmıştır. *Grhl3* ve *Ovol2* transkripsiyonel faktörlerinin gelişim süreci dışında EMT gibi önemli biyolojik süreçlerin transkripsiyonel düzenlenmesi gibi konularda ortak çalışma potansiyelinde oldukları gösterilmiştir.

Sonuç olarak, bulgularımızın MET sürecinin düzenlenmesi hakkında yeni bilgiler sunması ve epitel durumun düzenleyicisi olarak *Elf3* gibi bazı transkripsiyon faktörlerini bir transkripsiyonel ağ kapsamında ortaya çıkartması oldukça önemlidir.

Anahtar sözcükler: MET, Transkripsiyonel Ağlar, EMT, *Elf3*, *Grhl3*, *Ovol2*

1. INTRODUCTION AND AIM

1.1 Aim of the Study

Main aim: To define the transcription factors controlling the mesenchymal-epithelial transition and to identify the potential transcriptional networks governing their function.

Specific aims:

1. To determine the expression profiles of NMuMG cells using in-silico and in-vitro analyses to identify transcription factors enriched in the epithelial state.
2. To define the transcriptional relationships between the target genes using the RNAi mediated gene silencing.
3. To analyze possible protein-DNA interactions to define the role(s) and function(s) of selected genes within a network.

1.2 Hypothesis of the Study

EMT is induced by many different signals such as TGF β , EGF, and FGF but shares the activation of transcription factors like *Snail*, *Slug*, *Zeb1/2* called 'EMT-core regulators'. These transcription factors act as repressors of the Ca²⁺-dependent adhesion molecule E-cadherin, which is the hallmark of EMT-MET events. The expression of E-cadherin must be successfully downregulated for EMT and must be rapidly restored for proper MET. The activation of E-cad during MET can be partially explained by the downregulation of EMT inducers and the release of the repressors from its promoter, suggesting the presence of transcriptional activators, which could also be the regulators of MET. Based on the transcriptional relationships between some of the known *Cdh1* regulators, we hypothesized that such factors could be members of a more extensive transcriptional network controlling the stability of cells in the epithelial state, which could also be involved in the regulation of MET.

2. GENERAL INFORMATION

2.1 Cellular Plasticity

The embryogenic developmental process requires the programming of cellular differentiation systems, which are intimately linked with dynamic cell interactions (Keller 2005, Iacono, Benevento et al. 2017, Newman 2020). Gene regulatory networks regulate

phenotypic changes in response to environmental heterogeneity through epigenetic variations that lead to phenotypic changes (Thomas, Popovic et al. 2014, Jia, Jolly et al. 2017). Consequently, the dynamism of changes between molecules (such as genes, proteins, microRNAs) within the scope of these gene regulatory networks determines the plasticity between the stable and intermediate cell states (Gupta, Pastushenko et al. 2019, Teeuwssen and Fodde 2019).

It is well established that the epigenome can be altered through environmental fluctuations to control which genes are expressed in a given context through cellular plasticity programs, in which cells switch between different phenotypes without altering their genotypes (Duncan, Gluckman et al. 2014, Tian and Marsit 2018). Dynamic fluctuations through cellular plasticity and gene expression patterns are utilized by conserved and complex mechanisms (Gibson 2008, Varga and Greten 2017, Tiwari, Manzar et al. 2020). One of the most well-known processes determining cellular morphology and dynamic cell interactions is cell adhesion, which is tightly regulated by the E-cad molecule, which is also considered as a functional unit of adhesion (van Roy and Berx 2008, Erami, Timpson et al. 2015). Dynamic changes and plasticity needed for several biological processes require Cdh1 to be regulated in a controlled manner (Kroepil, Fluegen et al. 2012, Bedi, Mishra et al. 2014). In biological processes such as EMT and MET, the transitions between epithelial and mesenchymal states are usually associated with switching the expression states of E-cad among changes in other molecular signatures. The oscillations during the course of EMT-MET are utilized to stabilize cellular homeostasis (Bedi, Mishra et al. 2014, Macara, Guyer et al. 2014, Jolly, Tripathi et al. 2016).

Cellular plasticity also plays a crucial role in the vast majority of biological events, including primary tumorigenesis, metastasis, and drug resistance, which endows cancer cells with metastatic capacity (da Silva-Diz, Lorenzo-Sanz et al. 2018, Fanelli, Naccarato et al. 2020, Shen and Clairambault 2020). Cellular plasticity through tumorigenesis is highly associated with transition programs such as EMT and MET (Chaffer, San Juan et al. 2016, Bakir, Chiarella et al. 2020). Several publications suggested that cancer dissemination and metastatic colonization are promoted by many cancer cells such as breast cancer cells, tumor-associated macrophages (TAM), hepatic progenitor cells via induction of EMT (Giannelli, Koudelkova et al. 2016, Ye, Brabletz et al. 2017, Lin, Xu et al. 2019). Cell-specific lineage transitions between epithelial and mesenchymal states through the changes in cell' plasticity have direct links to

various cancer types enabling heterogeneity at the cellular level (Chen, You et al. 2017). In addition to cancer-initiating cells, there are also non-cancerous cells present in the tumor microenvironment, including fibroblasts and immune cells (Gajewski, Schreiber et al. 2013, De Jaeghere, Denys et al. 2019). Crosstalk between cancer cells with immune microenvironment exhibiting cellular plasticity in the course of EMT-MET is crucial for tumor initiation and immune surveillance. EMT inducing signaling pathways such as TGF- β and Wnt have been implicated in leading epithelial-mesenchymal plasticity by driving changes in the expression of EMT inducers including *Zeb1/2*, *Snail*, eventually providing cancer dissemination and immune evasion properties (Jing, Han et al. 2011, Jia, Li et al. 2019, Romeo, Caserta et al. 2019) (Figure 1).

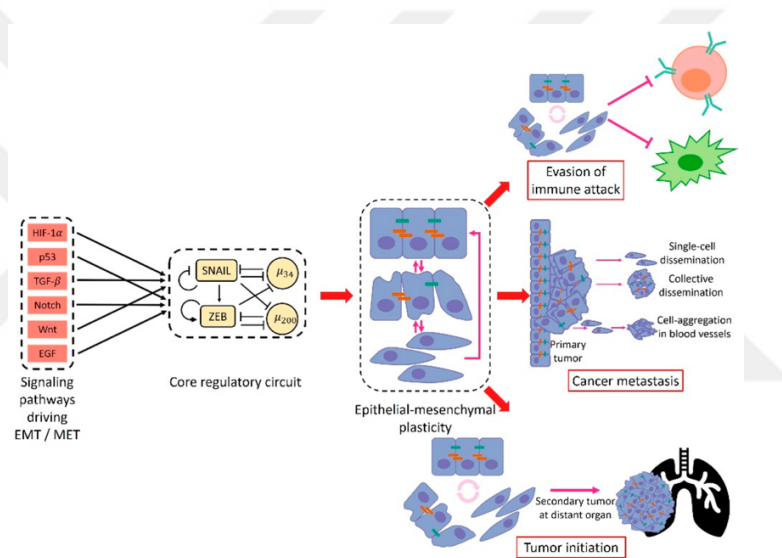


Figure 1. A proposed model of epithelial–mesenchymal plasticity. EMT program can be activated via signaling pathways such as TGF- β , Wnt and EGF which also drive the activation of core regulatory network including transcription factors SNAIL and ZEB. Changes of the epithelial repertoire allow cells for transitions between epithelial and mesenchymal states through cellular plasticity which is also able to enhance metastasis and immune evasion.

EMT program can be activated via signaling pathways such as TGF- β , Wnt, and EGF, which also drive the activation of core regulatory network, including transcription factors SNAIL and ZEB. Changes in the epithelial repertoire allow cells to undergo transitions between epithelial and mesenchymal states through cellular plasticity, enhancing metastasis and immune evasion.

Since cellular plasticity and EMT-MET programs share similar partners in the induction and the regulation of the processes, improving our understanding of mechanisms underlying

cancer cell plasticity and tumor heterogeneity, which represents a significant challenge in terms of developing more efficient therapeutic strategies, is fundamental to better management of metastatic diseases (Santamaria, Moreno-Bueno et al. 2017, Voon, Huang et al. 2017).

2.2 EMT-MET

EMT and MET are highly coordinated cellular programs that lead to changes in the epithelial architecture (Lamouille, Xu et al. 2014, Tan, Olsson et al. 2015). While epithelial cells are tightly attached cells with apical-basal polarity, mesenchymal cells, on the contrary, are non-polarized cells that can move individually due to their poor intercellular connections (Kalluri and Weinberg 2009, Nelson 2009). Unlike epithelial cells, mesenchymal cells are unspecialized cells, which are more prone to cell migration (Shu and Pei 2014). Cytoskeletal rearrangements and modulation of the expression of many different genes, including cell adhesion molecules, are the hallmarks of EMT and MET events (Thiery, Acloque et al. 2009, Brabletz 2012, Lamouille, Xu et al. 2014). While EMT and MET programs are indispensable duo, they are dissimilar in many ways, particularly in the function of the regulatory partners during the course of the processes (He, Qiu et al. 2018). Although it is well established that they are fundamental processes during embryogenesis, wound healing, and tumorigenesis (Barriere, Fici et al. 2015); many points are still not understood and described.

2.2.1 Epithelial-Mesenchymal Transition (EMT)

During embryonic development, cell states change dynamically by modulating cellular plasticity (Martinez Arias, Nichols et al. 2013, Nieto, Huang et al. 2016). Epithelial cells show remarkable plasticity during embryonic morphogenesis processes such as mesoderm formation and neural crest development, and they transform into mesenchymal cells following the EMT process (Acloque, Adams et al. 2009, Tsai and Yang 2013). Epithelial-mesenchymal transition (EMT) is a reversible molecular program that defines the transformation of cells into more invasive and non-polarized mesenchymal cells by losing their polarized morphology (Diepenbruck and Christofori 2016). EMT plays an important role in several biological processes, such as embryogenesis and cancer development (Thiery, Acloque et al. 2009, Roche 2018). During EMT, cell interactions are remodeled, and transcriptional machinery is altered to define the cell's fate (Chen, Han et al. 2012). It is known that the expression of about 4000 genes (almost 10% of the human genome) is changed during EMT. This global changes in the gene expression levels and cytoskeletal rearrangements during EMT depend on the association

of some signaling molecules, mediators, and regulators. They can be divided into three: EMT effectors, EMT core regulators, and EMT inducers (Tsai and Yang 2013).

EMT is a process in which cellular polarity, adherent morphology, and epithelial gene expressions are lost by suppressing the expression of cell adhesion molecules, also known as EMT effector molecules (Tsai and Yang 2013, Lamouille, Xu et al. 2014). They are cellular proteins such as E-cad, fibronectin, and N-cadherin (N-cad) that function through cell migration and invasion (Lamouille, Xu et al. 2014). An essential step of EMT is the downregulation of E-cad (Lombaerts, van Wezel et al. 2006, Wells, Yates et al. 2008), a Ca^{2+} dependent adhesion molecule. In contrast, its expression is rapidly restored during MET. Induction of EMT is integrated by many different signals but shares the activation of intracellular EMT-inducers like *Snail*, *Slug*, *Twist*, *Zeb1/2*, and others, which are at the core of a transcriptional regulatory network controlling the EMT program (Fazilaty, Rago et al. 2019, Lavin and Tiwari 2020). These transcription factors orchestrating the EMT process are considered as EMT core regulators (Tsai and Yang 2013). In agreement with a required rapid downregulation of E-cad, all of these transcription factors are, in fact, repressors of E-cad expression. They all bind to several evolutionary conserved E-boxes present in the proximal promoter of *Cdh1* (Bolos, Peinado et al. 2003, Canel, Serrels et al. 2013, Serrano-Gomez, Maziveyi et al. 2016). The *Cdh1* promoter alone, including all known E-box elements, was shown to be insufficient for conferring strict cell-type specificity. On the other hand, intron 2 of E-cad has emerged as an essential regulator of *Cdh1* expression (Stemmler, Hecht et al. 2005). They can also repress the activation of cellular junction proteins, including *claudins* and *ZO-1* (Lamouille, Xu et al. 2014). Although EMT inducing signaling pathways are context and cell-dependent (Aiello and Kang 2019, Cook and Vanderhyden 2020), almost all critical signaling pathways such as TGF β , Wnt, and Notch are associated with the induction of the EMT program (Xu, Lamouille et al. 2009, Zhang, Tian et al. 2016). TGF β signaling pathway is defined as the main signaling pathway of EMT, as it can provide direct activation of central EMT regulators such as *Snail*, *Slug*, *Twist*, and *Zeb1/2* (Wendt, Allington et al. 2009, Xu, Lamouille et al. 2009). TGF β has a critical role in important cellular functions such as proliferation, differentiation, and apoptosis in adult and embryonic cells (Ramesh, Wildey et al. 2009, Lafontaine, Chaudhry et al. 2011, Zhang, Alexander et al. 2017). The crosstalk between TGF β and Smad signaling to induce EMT are closely linked to other signaling pathways such as PI3K and MAPK (Zhang 2009, Chapnick,

Warner et al. 2011). During the EMT process, TGF β increases cell motility, invasion, and facilitates metastasis (Massague 2008).

EMT is also categorized into three types based on their origin (Figure 2): The developmental (type I) EMT is required for mesoderm formation, neural crest delamination, establishment of the heart valve, palatogenesis, and myogenesis. After tissue damage, EMT becomes activated during fibrosis and wound healing (type II). Since EMT is not only a developmental event, it is also aberrantly activated during tumorigenesis when cancer cells start to disseminate, invade and form metastases (type III) (Kalluri and Weinberg 2009, Thiery, Acloque et al. 2009, Sisto, Lisi et al. 2018).

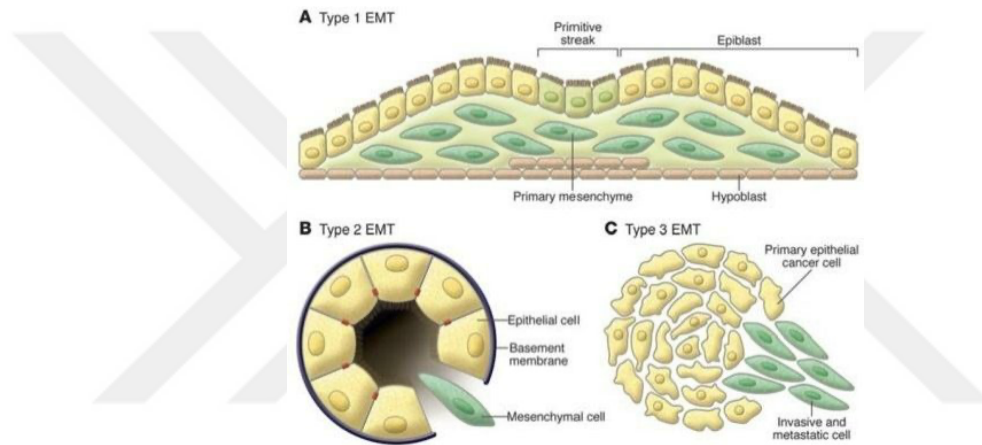


Figure 2. Three types of EMT. Type 1 EMT (A), Type 2 EMT (B) and Type 3 EMT (C). Type 1 EMT is associated with developmental processes, whereas Type2 and Type 3 EMTs are associated mainly with fibrosis and metastasis, respectively.

In summary, the EMT program entails tightly regulated signaling events which are also controlled by many different levels through epigenetic modifications, transcriptional and post-transcriptional/translational regulation, alternative splicing, and non-coding RNAs (Serrano-Gomez, Maziveyi et al. 2016, Pradella, Naro et al. 2017, Gugnoni and Ciarrocchi 2019, Peixoto, Etcheverry et al. 2019).

2.2.2 Mesenchymal-Epithelial Transition (MET)

Most cellular processes are achieved by establishing a balance between EMT and MET programs (Roca, Hernandez et al. 2013). Although classical definitions define the MET process as the reverse process of EMT (Kalluri and Weinberg 2009), recent studies and the data we have obtained emphasize the complex and dynamic nature of the MET process (Li, Zheng et al. 2011, Yao, Dai et al. 2011, Alotaibi, Basilicata et al. 2015, Kim, Xing et al. 2017). The

importance of MET has been lately discovered. Even though MET is recently considered a vital reprogramming event (Li, Liang et al. 2010), the mechanism underlying MET regulation is still debatable.

The expression of E-cadherin suppressed during the EMT process is rapidly restored during MET. The increase in the expression levels of epithelial genes is accompanied by a decrease in mesenchymal genes' expression. In this way, cells regain their apicobasal polarity (Yao, Dai et al. 2011). Reactivation of E-cad expression is critical for MET. Different studies have shown that the decrease in the expression of the central EMT regulators and their release from the *Cdh1* promoter region are not sufficient for the rapid activation of the *Cdh1* locus during the MET process (Stemmler, Hecht et al. 2003, Stemmler, Hecht et al. 2005, Alotaibi, Basilicata et al. 2015). The immediate activation of the *Cdh1* locus during MET can only in part be explained by the release and downregulation of these repressors. The *Cdh1* promoter alone, including all known E-box elements, was shown to be insufficient for conferring strict cell-type specificity. On the other hand, intron 2 of E-cad has emerged as an essential regulator of *Cdh1* expression (Stemmler, Hecht et al. 2005).

Epithelial signature defining cellular homeostasis represents the most relevant hallmark of the MET specific transcriptional network. It encompasses altering the cellular identity when exposed to different signals along EMT-MET processes (Lamouille, Xu et al. 2014, Macara, Guyer et al. 2014). Similar to the EMT process, the MET event can be manifested by many signaling pathways, transcription factors, and epigenetic modifiers at different levels in cells, and these parameters may vary depending on physiological and pathological conditions (Lamouille, Subramanyam et al. 2013). MET is recently considered one of the first key events during the reprogramming of mouse fibroblasts into iPSCs (Li, Liang et al. 2010, Polo and Hochedlinger 2010, Esteban, Bao et al. 2012). It is described that the activation of the endogenous loci of the core pluripotency network is achieved by the MET process while creating the iPSCs. This is partly achieved by the exogenous *Klf4* binding to specific regions in the promoter and activating E-cad expression.

Interestingly, E-fate was found to support the initiation of MET and improve reprogramming efficiency (Redmer, Diecke et al. 2011). It was also determined that E-cad is required to keep embryonic stem cells in a pluripotent state (Redmer, Diecke et al. 2011), and the reduction of E-cad expression during induced pluripotent stem cell generation interferes

with the initiation of MET and reduces the potential of somatic cell programming (Bedzhov, Alotaibi et al. 2013). Besides managing morphogenetic events in embryogenesis, MET also plays a critical role in metastasis and colonization of tumor cells in distant regions (Banyard and Bielenberg 2015, Bakir, Chiarella et al. 2020). Association of pluripotency network and EMT-MET events are required to establish metastatic foci (Figure 3) (Voutsadakis 2015). Previous studies also discovered that some types of cancer cells, including sarcoma and melanoma, exhibit a pluripotent-like state through MET-mediated reprogramming (Zhang, Cruz et al. 2013, Takaishi, Tarutani et al. 2016).

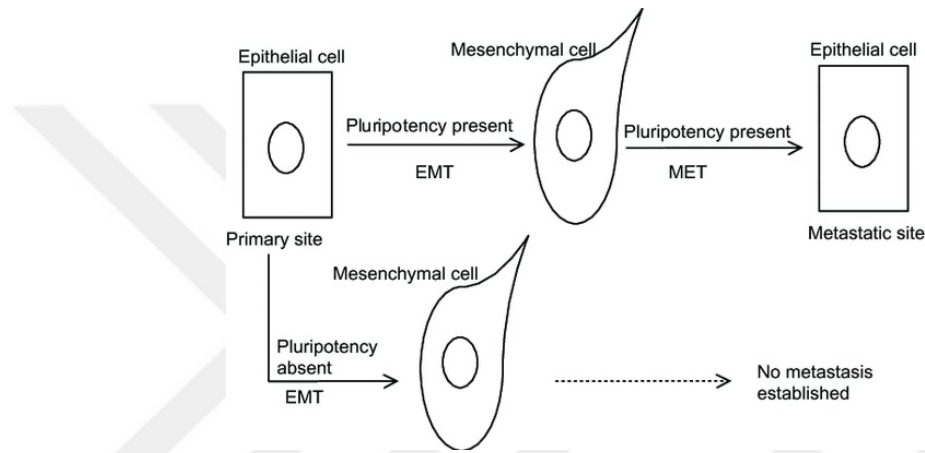


Figure 3. An overview of the association of the pluripotency network and EMT-MET processes. An activated pluripotency network is required for cells undergoing EMT to establish the metastatic cascade.

In sum, exploring the dynamic nature of the MET event, together with its counterparts, is worth examining in detail to better understand how MET is regulated during physiological and pathological conditions. Moreover, this will also provide better knowledge on how MET contributes to key biological events such as chromatin regulation, epigenetics, pluripotency, cellular homeostasis, and metastasis.

2.3 Transcriptional Networks

Transcriptional networks have been described before (Blais and Dynlacht 2005); examples include, but are not limited to, the transcriptional network of EMT (Fazilaty, Rago et al. 2019), the core pluripotency network of embryonic stem cells (Kim, Chu et al. 2008, Zwaka 2012), and the transcriptional network of blood stem cells (Gottgens 2015). There are different kinds (or motifs) of transcriptional regulatory networks. These are sets of transcription factors that control the initiation and maintenance of specific transcriptional loops or chains to augment

or stabilize gene expression. Examples of the network motifs include auto-regulatory motif, feed-forward loop, single input module, and multi-input module (Figure 4) (Zhang, Xuan et al. 2008). For instance, in autoregulatory loops, a transcription factor regulates its own expression, such as the *Nanog* loop in the core pluripotency network (Boyer, Lee et al. 2005). In feed-forward loops, transcription factor X will regulate both Gene Y and Gene Z, where Gene Y encodes a transcription factor that also regulates Gene Z. An example of such regulatory loop comes from our previous study describing the initiation of MET, in which *Grhl3* regulates the expression of both *Cdh1* and *Hnf4a*; *Hnf4a* is then recruited to the *Cdh1* chromatin to boost up the *Grhl3*-dependent regulation (Alotaibi, Basilicata et al. 2015). There is not always a straightforward loop between the genes. In a multi-input model, transcription factors regulate the expression of their regulators in a positive feedback loop; a very well known example is the closed circuit of *Oct4/Sox2* in embryonic stem cells regulatory network (Rizzino 2009). In a regulatory chain, three or more transcription factors are ordered in a series; the chain ends when the transcription factor has no target or is autoregulatory. An example of such regulatory chains comes from the hematopoietic stem cells, where β -catenin regulates *HoxB4*, which, in turn, activates the expression of *c-myc* (Forrester and Jackson 2012). Common to most transcriptional network studies is the methodology used; most share similar sets of experimental and knowledge-based approaches, combining data mining, gene expression profiling, and gene silencing, all integrated in a powerful computational analysis to decipher transcriptional nodes and network motifs correctly.

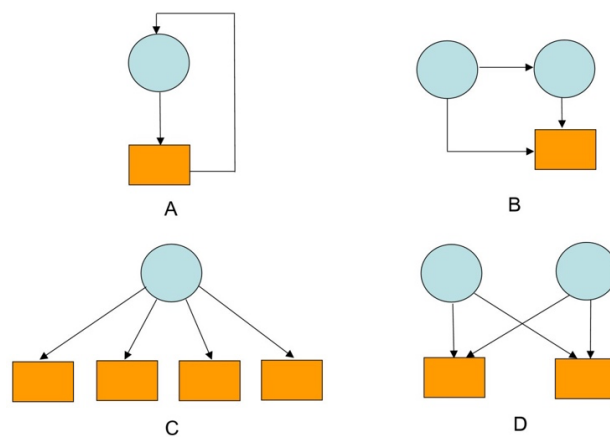


Figure 4. Transcriptional network motifs: Auto-regulatory motif (A), feed-forward loop (B), single-input module (C), and multi-input module (D). Transcription factors are represented as blue circles and genes as orange boxes. Arrowheads indicate the activation.

2.4. MET Core Regulators

While much attention is being paid to EMT regulation in recent publications, little attention is paid to the mechanisms underlying MET regulation since only EMT-inducing factors are thought to be responsible for MET regulation. On the contrary, recent studies identified *Grhl2/3*, *Hnf4a*, and *Ovol1/2*, which are also involved in the dynamic transcriptional trajectories during EMT-MET (Roca, Hernandez et al. 2013, Werner, Frey et al. 2013, Alotaibi, Basilicata et al. 2015).

2.4.1 Grainyhead-Like Proteins

Grainyhead (Grh) protein (previously known as *Elf-1* or *NTF-1*) was originally identified in *Drosophila*, expressed predominantly in the surface ectoderm (Bray, Burke et al. 1989). It plays a critical role in the mechanism of cuticle formation, maintenance of wing and hair polarity, and epithelial differentiation in *Drosophila* (Ren, Zhu et al. 2005). Grainyhead-mutated embryos cannot hatch, have loose cuticles, vascular and interrupted skeleton, and irregular tracheal tube (Gustavsson, Copp et al. 2008). It is known that *Grh* also interacts with Lmo-4 (LIM-only protein 4) protein, which is essential in epigenetic morphogenesis (Moussian and Uv 2005).

Vertebrate orthologs are called Grainyhead-like genes and have three known members: *GRHL1*, *GRHL2*, and *GRHL3*. These highly conserved transcription factors are expressed in many ectodermal and endothermal cell types during embryonic development and adult skin due to their contributions to epidermal barrier formation. Their expressions are tissue-specific and may vary during developmental processes and pathological conditions (Boglev, Wilanowski et al. 2011, Mlacki, Kikulska et al. 2015).

GRHL proteins are considered as a conserved controller of epidermal integrity by altering the chromatin infrastructure (Jacobs, Atkins et al. 2018, Sundararajan, Pang et al. 2020). The most common function of Grh and GRHL family members is the epidermal differentiation event. Besides, they also share common features during most developmental and pathological events such as epigenetic regulation, chromatin remodeling, and differentiation (Sundararajan, Pang et al. 2020) (Figure 5). Among them, *GRHL2* is defined as a gatekeeper of the epithelial traits in many cancers by controlling the expression of many genes, including E-cad and claudins (Chung, Tan et al. 2016). *Grhl3* (grainyhead-like protein 3), previously named *GET1*, *MGR*, *LBP-32*, *SOM*, and *BOM*, is a critical factor responsible for epidermal differentiation,

barrier formation, and homeostasis of the surface ectoderm in many species (Klein, Lin et al. 2017, Kimura-Yoshida, Mochida et al. 2018). It controls epidermal traits and wound-repair via altering the expression of *Transglutaminase-1* and *RhoGEF19* (Ting, Caddy et al. 2005, Caddy, Wilanowski et al. 2010). Although the mammalian *Grhl3* protein does not bind directly to *Lmo-4* as its *Drosophila* ortholog, it has been found that it is involved in a multiple protein complex together with *Lmo-4*, and this association is particularly required for neural tube closure and epidermal barrier formation (Hislop, Caddy et al. 2008). *Grhl3* knock-out mice have spina bifida, a disease that occurs as a developmental defect of the neural tube (De Castro, Hirst et al. 2018). Non-*Grhl3* mice cannot form a suitable skin barrier and die of dehydration as soon as they are born (Ting, Caddy et al. 2005, Goldie, Cottle et al. 2018).

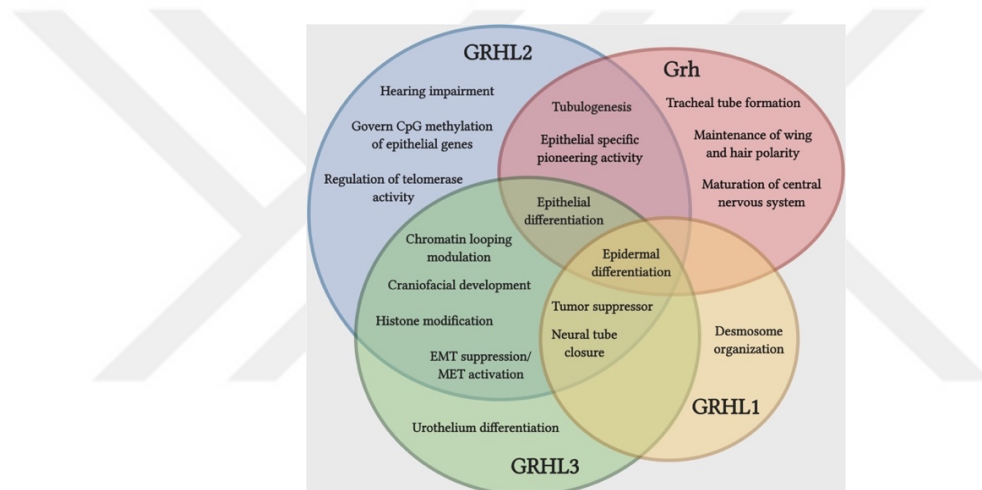


Figure 5. Common functions of Grh and GRHL family members. Grh and GRHL family members share common features such as chromatin remodeling, epigenetic regulation, and epidermal differentiation, which is the most common among developmental and pathological conditions.

Studies conducted in recent years also emphasize the tumor-promoting functions of GRHL members, mostly through EMT-MET processes. GRHL factors are known to be associated with cancer types such as skin squamous cell carcinoma, breast cancer, gastric cancer, hepatocellular carcinoma, colorectal cancer, prostate and cervical cancer (Mlacki, Kikulska et al. 2015, Frisch, Farris et al. 2017, Kotarba, Taracha-Wisniewska et al. 2020). It was demonstrated that *GRHL2* alters the expression of EMT inducer *ZEB1* and *miR-200* through self-activation and indirect downregulation of its repressor in many cancer types, including ovarian and sarcoma (Chung, Tan et al. 2016, Somarelli, Shetler et al. 2016). Besides,

GRHL2 is shown to drive MET-like phenotypical changes in MDA-MB-231 cells and sarcomas and identified as a MET inducer in breast cancer (Werner, Frey et al. 2013).

GRHL3 has been thought to play an essential role in the critical tumor suppressor pathways in squamous cell carcinomas through the loss of *Pten* expression (Darido, Georgy et al. 2011). Recent studies revealed that *Grhl3* could function both as an activator and a repressor and may have different roles in regulating the migration and invasion of epithelial cells (Klein, Lin et al. 2017). For instance, *Grhl3* has been implicated as a key player in the induction of epithelial signature by the downregulation of E-cad expression (Alotaibi, Basilicata et al. 2015, Zhao, Guo et al. 2016).

2.4.2 *Ovol Proteins*

OVOLs are zinc finger transcription factors; vertebrate homologs of *Drosophila* OVO functions as a key regulator of embryogenesis and developmental processes (Saxena, Srikrishnan et al. 2020). They have been thus identified as master regulators of EMT-MET since altering the expression of EMT core regulators (Li and Yang 2014, Jia, Jolly et al. 2015). They can either act as an inhibitor of EMT or promoter of MET (Roca, Hernandez et al. 2013, Saxena, Srikrishnan et al. 2020) (Figure 6).

Evidences have shown that OVOL family members have vital roles in driving the changes in cell fates and considered as the gatekeepers of epithelial traits (Li and Yang 2014, Renaud, Chakraborty et al. 2015). Together with GRHL members, they act as phenotypic stability factors (Roca, Hernandez et al. 2013, Jolly, Tripathi et al. 2016). Although it has been shown that these master regulators act in transcriptional and splicing regulatory programs and modulate the stability of cell traits in human cancer cells, there is still limited knowledge about the role of the OVOL members in the regulation of MET. It has been recently described that *OVOL1* and *OVOL2* are crucial regulators of MET in many cancer types, including prostate and breast cancer cells (Roca, Hernandez et al. 2013). Expression of either *OVOL1* or *OVOL2* plays a crucial role in the repression of the EMT core regulator *ZEB1* and *SNAIL* (Bai, Kusama et al. 2018, Liu, Wu et al. 2018, Murata, Ito et al. 2020). Furthermore, they have been implicated in reprogramming fibroblasts to epithelial cells (Watanabe, Liu et al. 2019, Saxena, Srikrishnan et al. 2020).

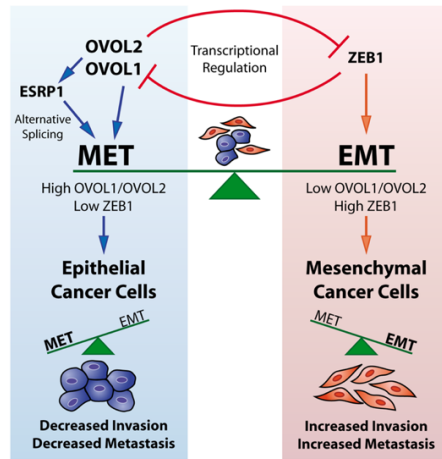


Figure 6. The proposed mechanism of OVOL proteins in the regulation of EMT-MET. EMT-MET programs are controlled by the balance between the expression of OVOL1/2 and ZEB1. OVOL1/2 transcription factors also regulate the expression of splicing regulator ESRP1, which contributes to MET.

Consequently, it is reasonable to conclude that the temporal dynamics of the phenotypic transitions between epithelial and mesenchymal states are tightly regulated by *miR200/ZEB1/OVOL/GRHL* circuit. Such association enables cells to gain stemness and plasticity through various biological events, including embryogenesis, cancerogenesis, and cellular reprogramming.

2.4.3 *Hnf4α*

Hepatic differentiation factor *Hnf4α* belongs to the transcriptional nuclear receptor superfamily abundantly expressed in a tissue-specific manner in the liver, kidney, and intestine (Li, Ning et al. 2000, Babeu and Boudreau 2014). It is essential for maintaining cellular homeostasis, inflammation, and regulating epithelial phenotype, lipid, and glucose metabolism. The balance between EMT core regulators and *Hnf4α* influence EMT-MET events (Cattin, Le Beyec et al. 2009, Hatziapostolou, Polyarchou et al. 2011, Moore, Khurana et al. 2016).

Hnf4α has been associated with MET initiation and cause MET-like phenotypic changes via modulating epithelial signature and repression of the mesenchymal program (Kanazawa, Ichii et al. 2011). Moreover, it is associated with EMT core regulator *Snail* in HCC cells by regulating miR-29a and miR-29b expression (Cicchini, de Nonno et al. 2015, Dubois, Staels et al. 2020). It functions in HCC cells' metastability and orchestrates the balance between epithelial and mesenchymal signatures (Yao, Peng et al. 2013). *Grhl3/Hnf4α* circuit also operates in stabilizing the epithelial signature by the transcriptional repression of the

mesenchymal program. Our team also revealed that *Grhl3* contributes to the regulation of *Hnf4α* during MET in NMuMG cells (Alotaibi, Basilicata et al. 2015) (Figure 7).

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

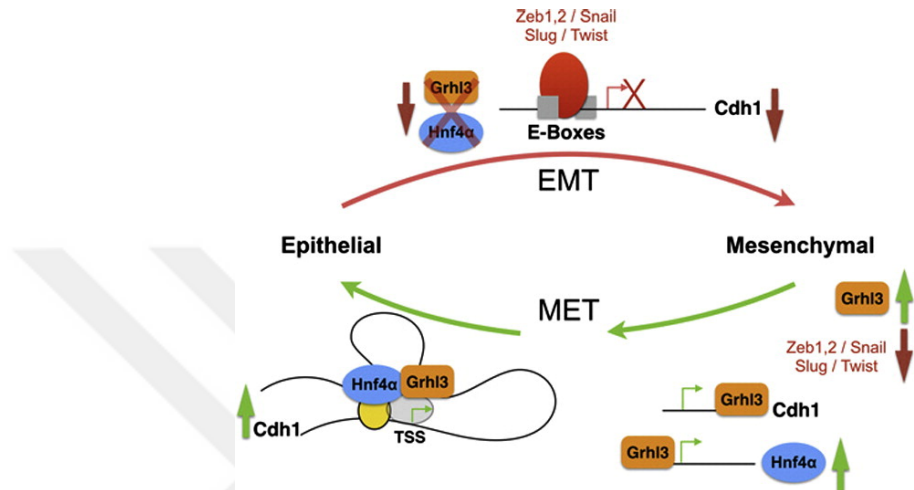


Figure 7. Proposed model of Grhl3/Hnf4α circuit during MET. During EMT, the expression of E-cad activators is downregulated and *Cdh1* locus is inactivated by binding of EMT core regulators such as Zeb1/2 and Twist. Upon MET induction, Grhl3 binds to specific sites at *Cdh1* and *Hnf4α* which leads to recruitment of Grhl3/Hnf4α to intronic enhancers and interaction with PolII (grey) and p300 (yellow) which is defined as enhancer cooperativity.

3. MATERIALS AND METHODS

3.1 Type of the Study

It is an experimental study supported by in-silico experiments.

3.2 Time and Place of the Study

This study was conducted at İzmir International Biomedicine and Genome Center between March 2017-October 2020.

3.3 Population and Samples of the Study

All the research experiments were performed using commercial cell lines and in-silico methods. No human biological material was used in the experiments.

3.4 Research Material

The Normal Murine Mammary Gland cell line (NMuMG) used in the experiments were commercially obtained from ATCC (ATCC® CRL-1636) in 2017. It is a very well-known and frequently used EMT-MET model (Miettinen, Ebner et al. 1994) since the significant gene signature changes are reflected in observable morphological features. These cells undergo an epithelial-mesenchymal transition upon treatment with TGF β . They lose E-cad expression, relocate cortical actin to stress fibers, and upregulate the expressions of mesenchymal genes such as N-cadherin and Vimentin. Once TGF β is removed from the culture medium, the cells revert to the epithelial state through MET in 72h (Alotaibi, Basilicata et al. 2015) (Figure 8).

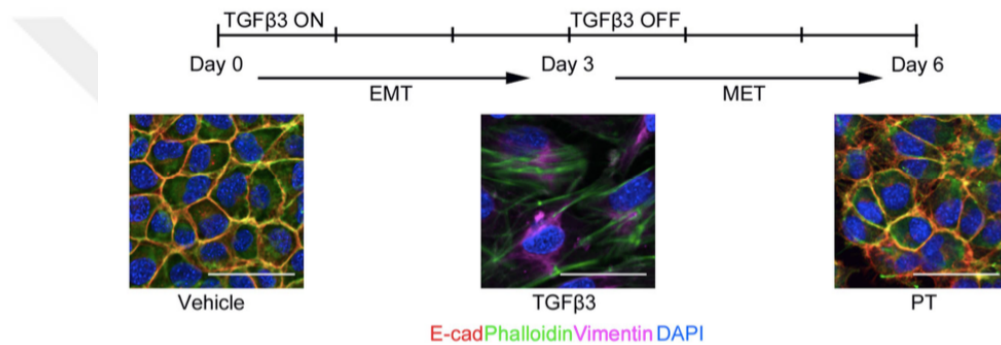


Figure 8. EMT-MET cellular model. Representation of the morphological changes of NMuMG cells with and withdrawal of TGF β 3 (PT: Post-treatment). Epithelial origin cells undergo EMT upon treatment with TGF β in 72h. They lose E-cad expression and gain mesenchymal features. By TGF β withdrawal, the cells revert to the epithelial state through MET in 72h.

3.5 Variables of the Study

The EMT-MET cellular model NMuMG is an independent variable. The siRNAs used in silencing experiments and binding sites of transcription factors identified and GEO datasets consist of array and sequence-based data of publicly available curated gene expression profiles are dependent variables.

3.6 Tools for Data Collection

3.6.1 Materials and Methods

To construct a transcriptional regulatory network, an integrated approach including data mining, high throughput expression profiling, gene silencing, and validation by DNA-protein interaction techniques were used.

3.6.1.1 Microarray Data Analysis and Correlation Study

Raw data (CEL files) of four publicly available gene expression datasets based on the GPL6246 platform (GSE130548, GSE77551, GSE55711, and GSE55072) were downloaded from the GEO database. Accession numbers of the datasets and samples included in the analysis are shown in Table 1. We applied several criteria for transcription factor selection:

1. Our primary target will be the transcription factors present in the epithelial state and absent from the mesenchymal state. The second group will include transcription factors enriched in the epithelial state compared to the mesenchymal.
2. All data sets should contain both epithelial and mesenchymal samples.
3. All data sets should contain at least two samples in each group.
4. Factors with expression patterns similar to key epithelial markers such as E-cadherin will be given higher priority.

Thus, samples with distinct epithelial and mesenchymal states were selected for further analysis.

Table 1. NCBI GEO accession numbers and phenotypes of samples used in correlation studies.

Study GEO Accession	Phenotype	Sample
GSE130548	Epithelial	GSM3742048.CEL
GSE130548	Epithelial	GSM3742049.CEL
GSE130548	Epithelial	GSM3742050.CEL
GSE130548	Mesenchymal	GSM3742051.CEL
GSE130548	Mesenchymal	GSM3742052.CEL
GSE130548	Mesenchymal	GSM3742053.CEL
GSE55072	Epithelial	GSM1329077.CEL
GSE55072	Epithelial	GSM1329078.CEL
GSE55072	Mesenchymal	GSM1329081.CEL
GSE55072	Mesenchymal	GSM1329082.CEL
GSE55711	Epithelial	GSM1342162.CEL
GSE55711	Epithelial	GSM1342163.CEL
GSE55711	Mesenchymal	GSM1342164.CEL
GSE55711	Mesenchymal	GSM1342165.CEL

GSE77551	Epithelial	GSM2054383.CEL
GSE77551	Epithelial	GSM2054384.CEL
GSE77551	Epithelial	GSM2054385.CEL
GSE77551	Mesenchymal	GSM2054377.CEL
GSE77551	Mesenchymal	GSM2054378.CEL
GSE77551	Mesenchymal	GSM2054379.CEL

We simultaneously performed a time-course experiment in our laboratory using NMuMG cells. Obtained and verified duplicate RNA samples in specified time points (details are given in the ‘Results’ section) were subjected to the microarray analysis. Hybridization was performed using the platform "GeneChip Mouse Gene 2.0 ST Array" (Affymetrix, Inc.) in Ayka Medikal, Istanbul.

Data were RMA normalized using the Bioconductor package oligo. Normalized data were merged and transformed into z-scores using the scale function in R. The z-score transformation was performed both on the sample and on the gene level. Merged z-scores profile was used to evaluate spearman correlation between *Cdh1* and all other probe sets present in the expression profile. Differential expression analysis between epithelial and mesenchymal samples was performed using the Limma package to retrieve log fold changes and p-values. Gene to probe set annotation was performed using the annotate package and mogene10sttranscriptcluster.db annotation library. A list of mouse transcription factors was retrieved from Animal TFDB3.0. *Cdh1* correlation and differential expression profile of transcription factors were extracted from the performed analysis. The Ggplot2 package was used to draw the plots.

3.6.1.2 Cell Culture and Induction of EMT-MET

NMuMG cells were cultured in DMEM (11965092, Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, 10500064, Gibco), 1% penicillin-streptomycin (10,000 U/mL, 15140122, Gibco), 1X MEM NEAA (1140035, Gibco), and 10 µg/mL insulin (19278-5 mL, Sigma, Munich, Germany). NMuMG cells were grown at 37 °C in a humid environment with 10% CO₂. Confluent cells were split 1:10 in each two days.

For EMT induction, NMuMG cells were maintained in complete medium supplemented with 5 ng/mL TGFβ3 (100-36E, PeproTech, London, UK) for 72 h (labeled as TGFβ3 or β3). TGFβ3 was dissolved in a buffer containing purified water to a final concentration of 7.5%

BSA and 1.2 M HCl and stored at -20 °C for further experiments. The medium was removed, cells were washed twice with PBS, and fresh medium supplemented with TGFβ3 was added at 48 h. The induction of MET was initiated by TGFβ3 withdrawal and washing the plates three times with PBS and incubation for an additional 72 h in fresh complete medium (labeled PT as in Post-treatment). NMuMG cells used as control were labeled as Vehicle.

3.6.1.3 siRNA Knockdown and mRNA Expression Analysis

siRNAs against *Elf3*, *Grhl3*, *Ovol2*, *Ahr*, and *Nfya* were purchased from Qiagen for siElf3_2, siGrhl3, siOvol2, siAhr, siNfya; Dharmacon for siElf3_10, siElf3_12, and; sequences are available in Table 2. Early passage NMuMG cells were transfected with 50 nM siRNA using the Lipofectamine RNAi Max (13778150, Invitrogen, Waltham, MA USA) as recommended by the manufacturer and collected 72 h later.

Table 2. Sequence information of siRNAs used.

siRNA	Sequences	Vendor
siElf3_2	TTGAACCAACTTGTTTCGATAA	Qiagen
siElf3_10	GGUAUUACUACAAACGGGA	Dharmacon
siElf3_12	GGUUGGAGAGAGAGUCGGAAU	Dharmacon
siCntrl	Silencer™ Negative Control No. 1 siRNA	Qiagen
siGrhl3_45	GGAAAGAAGUUCUACCACAtt	Ambion
siAhr_5	TGGCATTAAGATAAAGATAAA	Qiagen
siNfya_4	CAGAACTACTTAGCTCAGTA	Qiagen
siOvol2_2	TTCCTTGGAGTCCCACCTAAA	Qiagen

NMuMG cells were grown and treated in 6 well plates. Total RNA was isolated using the Nucleospin RNA II kit (740955.50, Macherey-Nagel, Düren, Germany) recommended by the manufacturer. Their concentrations were measured using the instrument ‘NanoDrop 2000’ (Thermo Scientific). Total RNA (1 µg) was converted to cDNA using the Maxima First Strand cDNA Synthesis Kit (K1642, Thermo Scientific, Waltham, MA USA). cDNA was then diluted 1:20 and used for qPCR analysis with the TaqMan™ Universal Master Mix II (4440040, Applied Biosystems, Waltham, MA USA) and Universal Probe Library probes (Roche, Basel, Switzerland). All qPCR reactions were performed using ‘Applied Biosystems Fast 7500’ instrument at 95°C for 15 min, 40-50 cycles of 95°C for 15s, and 60°C for 1 min. All qPCR

data were normalized to the *Gapdh* gene. Primers and probes were designed specifically for each gene via ‘Roche Life Sciences, Universal probe library’ website. Primers and probes used in the experiments are available in Table 3. Quantification was performed using the $\Delta\Delta C_t$ method.

Table 3. Primers and corresponding UPL probes used in qPCR experiments.

Primers	Forward	Reverse	Probe
Ahr	TGCACAAGGAGTGGACGA	AGGAAGCTGGTCTGGGGTAT	27
Cdh1	ATCCTCGCCCTGCTGATT	ACCACCGTTCTCCTCCGTA	18
Cdh2	TCCCTGAGATACAGCGTCACT	ATAATGAAGATGCCCCGTTGG	17
Cebpa	AAACAACGCAACGTGGAGA	GCGGTCATTGTCACTGGTC	67
Ehf	TCATTGTCAAGACTGAACAAACC	GTCCAACAGATCTACTGTGCTACC	33
Elf3	ACCGAACCCTGACACACCT	AGCTGTACATGGCGTTGAAGT	46
Exosc9	CAAGGTGCCCCCTATAGTGCT	GGTCTGAGCTCTTATTTTCTTTGG	106
Fn1	AGGTGGACCCCGCTAAAC	TGCCGCAACTACTGTGATTC	31
Gapdh	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG	9
Grhl3	AAGGAAGATGTCTGAATGAACTTG	TCGTCCTCATTACTGTAGGGAAA	100
Ovol2	GTGAGGATTGCGGCTACAC	TGGTCACTGTTACATGCAG	79
Snai1	CTTGCTCCACAAGCACCA	GAGGATGGGGAGGTAGCAG	71
Snai2	ATCCTTGGGGCGTGTAAGT	TGAACCACTGTGATCCTTGG	6
Zeb1	TGGAGTTCAAAGGTTGTCGTT	TTGCCACATCAACACTGGTC	109
Zeb2	TTGCTCCAGGATGTGTGG	CACACACTTGTTTGTGTGCATATC	64
Twist1	GCCCGTGGACAGAGATTC	CTATCAGAATGCAGAGGTGTG	74

3.6.1.4 Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described with minor modifications (Alotaibi, Yaman et al. 2010). NMuMG cells were grown and treated in 10 cm plates. Following 10 min cross-linking with formaldehyde (final concentration is 1%) and 10 min quenching with glycine (final concentration is 0.125 M), cells were washed twice with PBS, and cells were scraped off in 10 ml DMEM medium. Pellets obtained after 5 minutes of centrifugation at 3000 g were lysed in 1 ml cell lysis buffer. 5 μ l of unsonicated DNA from the cell lysate was taken into a separate

tube as control. Cell lysates were then sonicated in the S220 focused-ultrasonicator (Covaris, Woburn, MA, USA) for 30 min (60 s on/60 s off; Peak Power: 140 W; Duty factor: 15; Cycles/burst: 200; water temperature: 4 °C). Chromatin shearing was tested by agarose gel electrophoresis. The samples obtained in the desired fragment range were subjected to dilution with ChIP dilution solution (30 µg DNA in 1 ml ChIP dilution buffer). Immunoprecipitation was then performed at 4 °C for 16 h using an antibody against ELF3/ESE-1 (NBP1-30873, Novus Biologicals, Centennial, CO, USA), GRHL3 (NBP2-16746, Novus Biologicals, Centennial, CO, USA), and a rabbit control IgG (2729S, Cell Signaling, Danvers, MA, USA). 50 µl Protein A (50% slurry) pre-absorbed with salmon sperm DNA (and washed with dilution buffer) were added and incubated with tumbling at 4 °C for 2 hours. Immunoprecipitations were centrifuged for 2 minutes at 3000 g, and supernatants were carefully removed. Pellets were then washed with 4 different wash buffers (wash buffer 1-4) and centrifuged after each wash. Removed supernatant were then purified using 10% Chelex. To reverse the cross-linking of the DNA and proteins, samples were incubated 10 minutes at 95 °C and subjected to Proteinase K (30 min at 95 °C) and RNaseA incubation (15 min at 37 °C). Quantitative real-time PCR was performed using the TaqMan Universal master mix II and Universal Probe Library probes (Roche, Basel, Switzerland). Amplification was carried out in the ABI PRISM 7500 Fast qPCR System (Thermo Scientific, Waltham, MA USA) with primers and probes depicted in Table 4 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 percentage of input in Figure 21; normalized to control (IgG) and presented as fold enrichment in Figure 27.

Table 4. qPCR primers and UPL probes used in ChIP experiments.

Primers	Forward	Reverse	Probe
Grhl3_Prom_52	TCTAGTTCTCCCGTTCCTTCC	TTCTTAGCTGAGGGGGTGAG	52
Grhl3_Prom_108	GGAGAAAAGAGAGGGGACTCA	TCACCTTCTCTACCCGGA	108
Ovol2_Prom	GCAAACATTTCATGCCTAAGGA	AACAAGCTCGCTACCTGTCC	27
gr14100	CCCTTGGTTCTAAAGTTGGTTG	GGAATGAGGAACTGGTTTGAC	107

3.6.1.5 Immunofluorescence Labeling and Confocal Microscopy

NMuMG cells recovering from TGFβ3 were transfected with desired siRNAs, as described before. Vehicle and β3 treated cells were used as control. Cells were washed with

PBS and fixed with 4% formaldehyde (F8775, Sigma) for 10 min at room temperature, washed twice with PBS, and permeabilized with 0.25% Triton X-100 (T8787, Sigma) for 5 min. After washing twice with PBS, cells were incubated with primary antibodies in PBS with 5% BSA (1:200) for one hour at room temperature. Afterward, Alexa594-conjugated secondary antibody was applied for an additional one hour after washing in PBS. Nuclei were visualized with DAPI (1:1000, Invitrogen) and mounted with Prolong Diamond Antifade Mountant solution (P36962, Thermo Fisher Scientific, Waltham, MA USA). Confocal microscopy was carried out with an LSM 880 microscope equipped with ZEN software (Zeiss, Oberkochen, Germany). The antibodies used were anti-E-cadherin (610181, BD Bioscience, San Jose, CA, USA), Vimentin (11-254-C100, Exbio, Vestec, Czech Republic), Alexa Fluor™ 488 Phalloidin (A12379, Invitrogen), DAPI (Invitrogen), and Alexa Fluor 594 goat anti-mouse IgG (A-11005, Invitrogen, Waltham, MA USA).

3.6.1.6 SA- β -GAL Assay

NMuMG cells recovering from TGF β 3 were transfected with siCntrl or siElf3_10, as described before. Seventy-two hours later, the cells were washed with PBS. SA- β -Gal activity was measured by a Senescence Detection Kit (K320, BioVision, Milpitas, CA, USA) according to the manufacturer's instruction. As a positive control, Huh7 cells were used. Induction of senescence was performed with 100 nM Doxorubicin (Dox; A4361, Applichem, Darmstadt, Germany). Huh7 cells were grown in appropriate media containing Dox for three days and an additional six days after Dox withdrawal. The presence of senescent cells was detected using an Olympus CKX41 microscope equipped with a DP25 camera.

3.6.1.7 RT-PCR

Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. Total RNA (1 μ g) was converted to cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA USA). cDNA was then diluted 1:20, and 2 μ L was used for PCR amplification of target genes in a 50 μ L reaction containing 0.2 μ M of forward and reverse primers (Table 5), 200 μ M of dNTPs, 1x Standard *Taq* (Mg-free) Reaction buffer, 1.5 mM MgCl₂, and 1.25 units of *Taq* DNA polymerase (M0320S, New England Biolabs, Ipswich, MA, USA). PCR conditions were 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; 72 °C for 5 min. Amplified DNA was visualized by electrophoresis on a 2% agarose gel.

Table 5. Primers used in RT-PCR experiments.

Primers	Forward	Reverse
Casz1_RT	TCTACTACCACGGCTGCCAC	TCGTTGCTGGATTCCTCGTG
Zfp750_RT	CGACTCCAGCAAGCTGAGCA	GGCGTTGCTTGCATACATGG
Elf3_RT	GTCTGGAGGGCAAGAAGAGC	CCAACCTCTTCTTCCTTCCA
Tbp_RT	AGCCTCAGTACAGCAATCAAC	GAACTTCACATCACAGCTCC

3.6.1.8 Wound Healing Assay

For wound healing assay experiments, NMuMG cells recovering from TGF β 3 were transfected with either siCntrl or siElf3_10, as described before. Six hours later, cells were scratched with a sterile pipette tip and cultured for an additional 48 h. Microscopic evaluations in 24h intervals were carried out using an Olympus CKX41 microscope equipped with a DP25 camera. Scratch width was determined by measuring the distance in pixels between the edges of the scratch in ImageJ. Several measurements were taken from each slide, and the average was calculated for further analysis.

3.6.1.9 Bioinformatics Analysis

The promoter sequences of interest were obtained in FASTA format using the ‘UCSC Genome Browser’ database; scanned with the ‘CLC Sequence Viewer’ and promoter elements were identified. 1 kb promoter regions of different species were then compared by using the mVista tool. ‘JASPAR’ database was used to determine putative transcription factor binding sites.

3.6.1.10 ChIP-Seq Data Analysis

In obtaining ChIP-sequencing data of *Grhl3* and *Ovol2* transcription factors, fastq-dump (version 2.8.1) command (fastq-dump --clip --dumpbase --gzip - skip-technical) belongs to "Sequence Read Archive", (SRA) toolkit were used. The raw sequencing data obtained from the website “iGenome” were aligned to the mm10 Mus musculus reference genome in fasta format [URL: ftp://igenome:G3nom3s4u@usd-ftp.illumina.com/Mus_musculus/UCSC/mm10/Mus_musculus_UCSC_mm10.tar.gz]. For this process, bwa-aln command of the software "Burrows-Wheeler Aligner", (BWA) (version 0.7.15-r1140) was used. Following (SAM) index files creation with "Sequence Alignment

Map", index files were translated into SAM alignment files using bwa-aln command. SAM files were converted to "Binary Alignment Map" (BAM) files with the (samtools view -b -o) command in Samtools (version 1.5). Then, short readings in BAM files obtained using samtools sort command (samtools sort -o) were aligned according to their genomic coordinates. Repeated readings in sorted BAM files was removed with the command "PicardCommandLine MarkDuplicates", (version 2.8.1) (PicardCommandLine MarkDuplicates I = "BAM input", O = "BAM output", M = "repeat conditions", REMOVE_DUPLICATES=TRUE VALIDATION_STRINGENCY=LENIENT) and the cleaned alignment files reordered with samtools.

MACS2 (version 2.1.1.20160309) callpeak command (macs2 callpeak -t "antibody reads", -c "control reads", --SPMR -g mm - nomodel -p 0.05 -B --to-large --fe-cutoff 2) was used for the identification of the Grhl3 and Ovol2 DNA binding sites in BAM files from which the PCR duplicates were removed. Treatment_pileup and control_lambda bedGraph files obtained by callpeak command were compared using bdgcmp command of MACS2 software (macs2 bdgcmp -t "antibody bedGraph", -c "control bedGraph", -S "ratio of reads between files, -m FE --o-prefix) to display the binding point peaks on the genome and a new bedGraph file was created containing the results of the comparison.

AnnotatePeaks.pl command (annotatePeaks.pl peaks.bed mm10 -go - genomeOntology), one of the programs "HOMER", (version v4.9) was used in the annotation of DNA binding sites. Comparing the gene lists belonging to the obtained binding sites was performed with a script prepared in R programming (version 3.4.2). RStudio (version 1.0.153) interface was used in all calculations performed in R (version 3.4.2) environment. ChIPpeakAnno (version 3.8.9) R package was used when determining the distance of the peaks taken from the two gene lists and for the annotation and Venn diagram representations of the common peaks. It was accepted that the peaks in the two lists should be at a maximum of 200 nucleotide distance from each other.

3.6.1.11 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. For Figure 14A, the One-Way ANOVA test was performed. Data are presented as the mean of at least three

independent experiments done in triplicates. ChIP experiments were carried out at least three times, qPCR was performed in triplicates. Error bars represent the standard error of the mean.

3.7 Planning and Calendar of the Study

March 17 - Nov 17	Sep 18 - Feb 19	Feb - June 19	Sep - Oct 20
Literature reading	In-vitro analysis	In-silico analysis	Writing

3.8 Limitations of Study

Results are based on one non-tumorigenic mouse cell-line model. In order to generalize the study's outputs, in-silico analyses of different cell types, regardless of cell type and origin, were carried out. However, the transformation of the in-silico data to other human mammary epithelial cells or cancer cells would be valuable. It is also not likely to verify all transcriptional nodes within the thesis's scope, which limits the scope of this study.

3.9 Ethical Committee

The experiments performed during the study do not need any ethical approval since only a commercial non-tumorigenic mouse mammary gland cell line was used during the course of the experiments.

4. RESULTS

We have previously identified that transcription factor *Grhl3* controls E-cadherin expression during MET by acting on both *Hnf4α* and *Cdh1* (Alotaibi, Basilicata et al. 2015) (Figure 7). Thus, we hypothesized the existence of a core transcriptional network controlling the balance between epithelial and mesenchymal states. It is likely to conclude that the expression of such transcription factor(s) essential for MET progression could also correlate with *Cdh1*.

4.1 Identification of Transcription Factors Correlated with the Epithelial State

To select candidate transcription factors, publicly available four microarray datasets (GSE130548, GSE77551, GSE55711, and GSE55072) with 20 samples containing distinct epithelial and mesenchymal samples were initially analyzed. We applied several criteria for

transcription factor selection, as described in detail in the previous section. The most stringent criterion was to select datasets containing samples with distinct epithelial and mesenchymal states. Correlation analysis of Z-score derived from the datasets determined 1469 transcription factors correlated with epithelial state. *Cas21*, *Zfp750*, *Elf3*, *Sp6*, and *Grhl3* are highly expressed in the epithelial phenotype, while transcription factors such as *Zeb1*, *Zeb2*, *Snail*, and *Twist2* were found overexpressed in the mesenchymal phenotype (Sengez, Aygun et al. 2019) (Figure 9). *Grhl3* expectedly emerged in the top positively correlated gene list due to its role in the regulation of *Cdh1*. The expression levels of top positively correlated genes were checked by RT-PCR in our EMT-MET model cell line NMuMG. *Elf3* appeared as the highly expressed transcription factor in NMuMG cells. It is also indicated that the expression levels of *Elf3* are relatively stable compared to those of *Cdh1* during the course of EMT/MET in NMuMG cells (Figure 10).

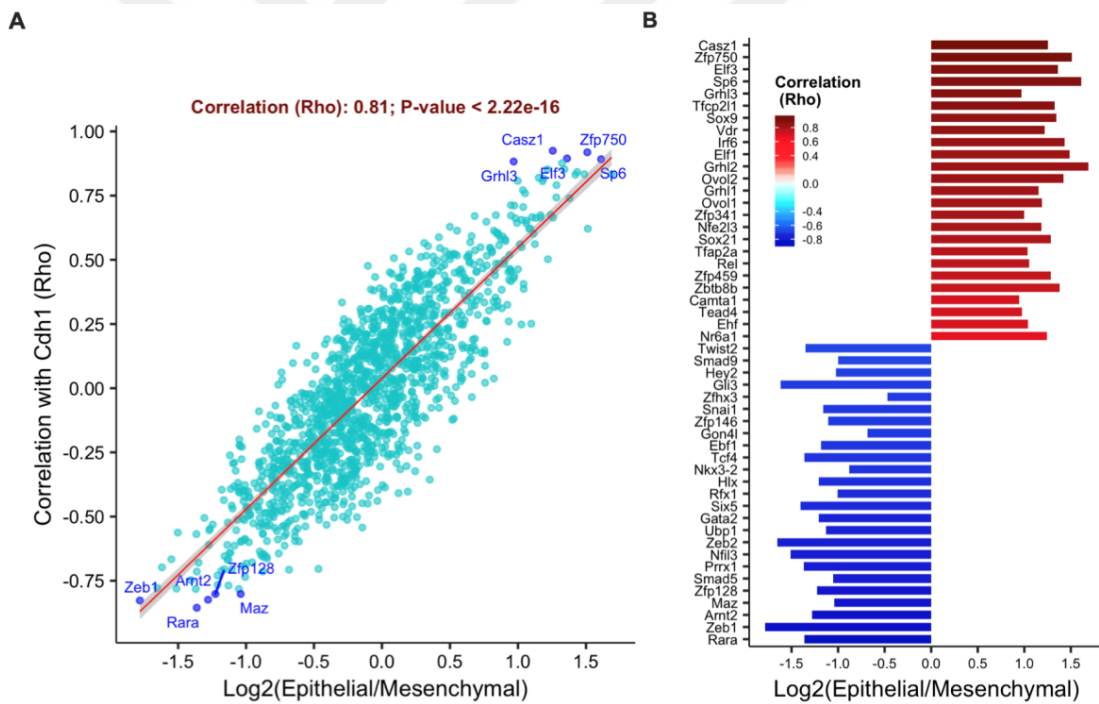


Figure 9. The transcription factors *Elf3* and *Grhl3* are highly correlated with the epithelial state. A scatter plot showing the correlation of 1469 transcription factors with the epithelial phenotype—microarray-based screen for epithelial-specific transcription factors co-regulated with E-cadherin. One thousand four hundred sixty-nine transcription factors were analyzed for differential expression in epithelial compared to mesenchymal samples, and Spearman correlation was calculated between each transcription factor and E-cadherin across all data sets. The top 5 correlated genes with either the epithelial or the mesenchymal phenotypes are labeled (A). The ranking plot displays the top 25 correlated transcription factors in the epithelial and mesenchymal states. The x-axis represents the differential expression of each transcription factor in epithelial versus mesenchymal samples (B).

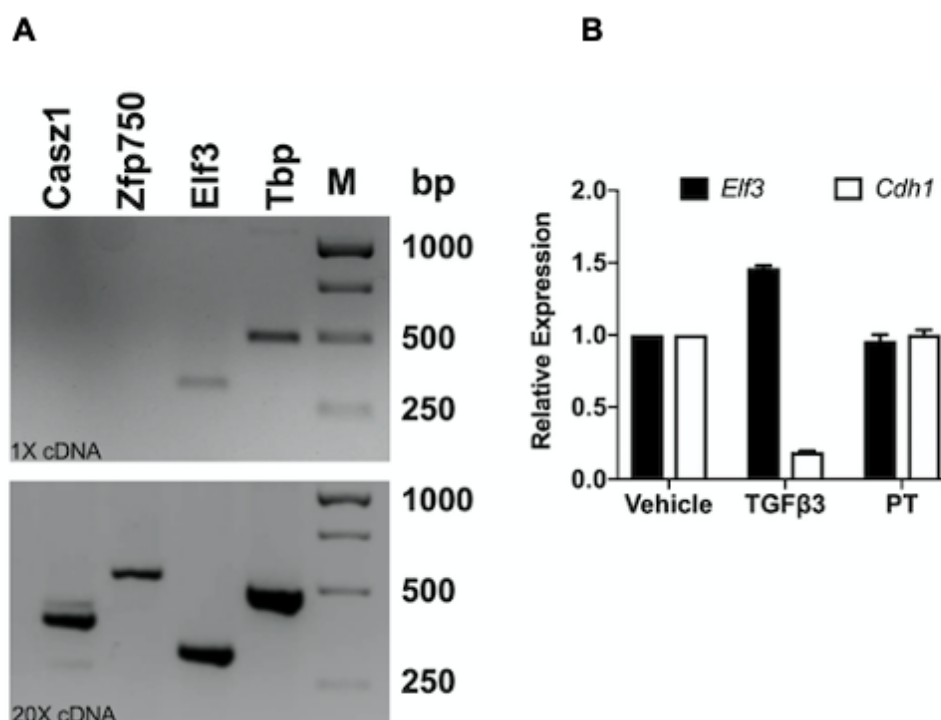


Figure 10. The expression of Elf3 in NMuMG cells and during MET. One microgram of total RNA was isolated from NMuMG cells and used to prepare cDNA for RT-PCR experiments. The expression of Casz1, Zfp750, and Elf3 was visualized on agarose gels. Tbp was used as an internal control. In the upper gel, 2 μ l of 1:20 diluted cDNA was used for PCR amplification; in the bottom gel, 2 μ l of undiluted cDNA was used (A). The mRNA expression levels of Elf3 are relatively stable compared to those of Cdh1 during the course of EMT/MET in NMuMG cells (B).

Afterward, to understand how the MET process is organized transcriptionally in our cell model NmuMG, a time-course microarray experiment was performed. For this purpose, RNAs were obtained at different time points from NMuMG cells during the course of EMT-MET (Veh, B3, and PT6h, PT12h, PT18h, PT24h, PT48h, PT72h). To validate proper EMT-MET transitions, the expression levels of the critical genes such as *Cdh1*, *Hnf4a*, and *Grhl3* were determined by the real-time quantitative PCR method (Figure 11). It was found that the *Cdh1* level decreased significantly in the mesenchymal cells and then increased gradually during the MET process. Likewise, the change in the expression levels of the *Hnf4a* and *Grhl3* transcription factors can be clearly observed during the course of EMT-MET as expected (Figure 11). The verified two sets of RNA were sent to Ayka Medikal, Istanbul, for microarray analysis. Hybridization was performed using the platform "GeneChip Mouse Gene 2.0 ST Array" (Affymetrix, Inc.). Raw data were analyzed as described in the 'Materials and Methods' section.

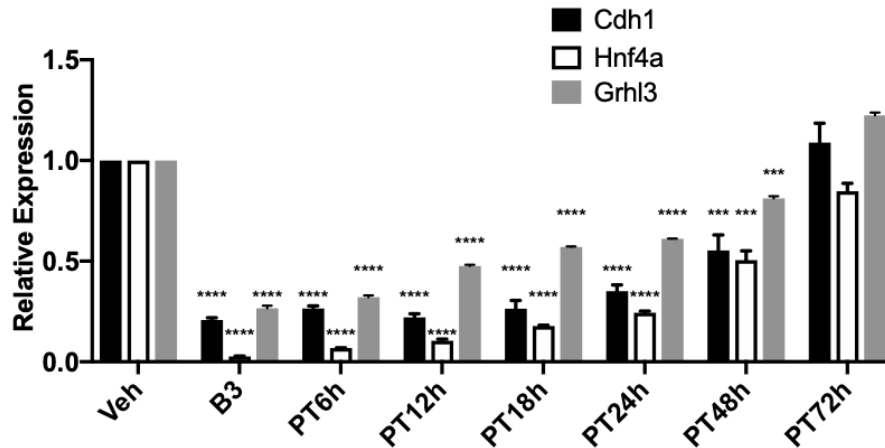


Figure 11. Validation of EMT-MET transitions. One microgram of total RNA from each time point was isolated from NMuMG cells and used to prepare cDNA for qPCR experiments. The mRNA expression levels of *Cdh1*, *Hnf4a*, and *Grhl3* are downregulated during EMT and restored during the course of MET. (* $p=0,01$, ** $p=0,001$, **** $p=0.0001$).

Expression profiling of NMuMG cells during MET by microarray analysis showed a marked decrease in mesenchymal related gene signature; in contrast, epithelial gene signature showed a significant upregulation. Then, we compared our gene list containing transcription factors co-regulated with E-cad derived from microarray analysis to the list of transcription factors curated from the in-silico analysis. As a result, we defined an epithelial state-specific transcription factor subset that is co-regulated with E-cad during the process of EMT-MET. This list is composed of 54 transcription factors (Genes of interest is marked as bold):

Ahr, *Ankrd1*, *Atf6b*, *Brca2*, *Carm1*, *Cdca7*, **Cebpa**, *Ctbp2*, *Dbp*, *Dhx9*, *E2f8*, **Ehf**, **Elf3**, *Esrra*, *Ezh2*, *Foxa2*, *Foxg1*, **Grhl3**, *Gata6*, *Hells*, *Hhex*, *Hmgb3*, **Hnf4a**, *Htatip2*, *Jarid2*, *Klf13*, *Klf4*, *Klf5*, *Litaf*, *Med12*, *Mkl1*, *Mtf2*, *Nfib*, *Nfix*, **Nfya**, *Nr1d2*, **Ovol2**, *Pawr*, *Pbx1*, *Pbx3*, *Rbl1*, *Rere*, *Rora*, *Runx2*, *Sfpq*, *Smarca4*, *Smarca5*, *Smarcc1*, *Sox11*, *Tcf7*, *Tgif1*, *Thra*, *Tle4*, *Wdr77*.

As expected, the list included transcription factors from our previous work such as *Grhl3* and *Hnf4a* and the transcription factors from in-silico analysis of publicly available datasets such as *Elf3* and *Ovol2*. We then examined the association of the transcription factors derived from our microarray analysis by ‘RegNetwork-Regulatory Network Repository’ database and ‘ChiBE’ (Chisio BioPAX Editor) software. We identified that transcription factors such as *Ahr* and *Nfya* abundantly interacts with each other and other transcription factors in the list (Figure 12), which indicated the possible significance of them within the network.

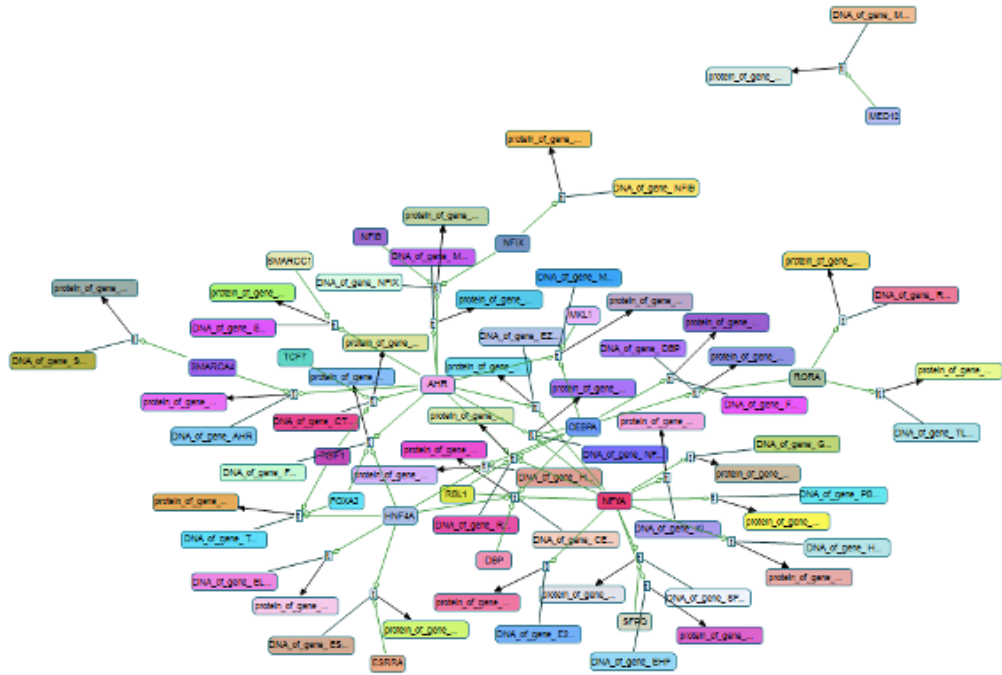


Figure 12. Representation of transcription factors obtained from microarray data analysis by the ‘RegNetwork’ database and ‘ChiBE’ software. Complex association of the genes within the epithelial state-specific transcription factor list were indicated through publicly available software.

Of note, transcription factors that represent complex connections with other factors often tend to be involved in regulating the other genes. Identifying transcriptional regulatory nodes, including these factors, is crucial to better understanding the proposed network within this context. The relationship between many of the transcriptional factors planned to be investigated within MET transcriptional nodes is also a matter of EMT study (Rico-Leo, Alvarez-Barrientos et al. 2013, Jia, Jolly et al. 2015, Zhao, Guo et al. 2016, Zheng, Xu et al. 2018, Dolfini, Andrioletti et al. 2019), which highlights the importance of the possible nodes of MET as well.

4.2 Transcriptional Nodes of MET

Our previous study revealed that *Grhl3* is a key transcription factor for the initiation of MET due to its critical role in the regulation of *Hnf4α* and *Cdh1* (Alotaibi, Basilicata et al. 2015). Together with *Grhl3*, transcription factors such as *Elf3* and *Ovol2* also emerged in the top positively correlated gene list with the epithelial state, which indicates that there might be other transcription factors (s) crucial for the MET process. In the light of both in-silico analysis of publicly available datasets and our microarray analysis, priority has been given to some transcription nodes that might be included in the MET core network. Several transcriptional

nodes, including transcription factors *Grhl3*, *Elf3*, *Ovol2*, *Ahr*, and *Nfya*, were further analyzed for their contribution to the proposed network.

4.2.1 'Elf3-Grhl3-Hnf4a-Cdh1', Transcriptional Node

To establish the relevance of the highly correlated transcription factors to MET, we first analyzed the expression levels of the top correlated genes in NMuMG cells by RT-PCR. While *Cas21* and *Zfp750* were barely detectable in NMuMG cells (only detectable when 20 times more cDNA was used), *Elf3* had comparably higher RNA levels. Together with *Grhl3*, *Elf3* showed a strong correlation with the epithelial state and a strong co-regulation with *Grhl3* (Sengez, Aygun et al. 2019) (Figure 13). Our previous lab member also identified that *Elf3* also interacts with the *Grhl3* promoter and activates its expression. Thus, *Elf3* was initially selected for further transcriptional node analyses.

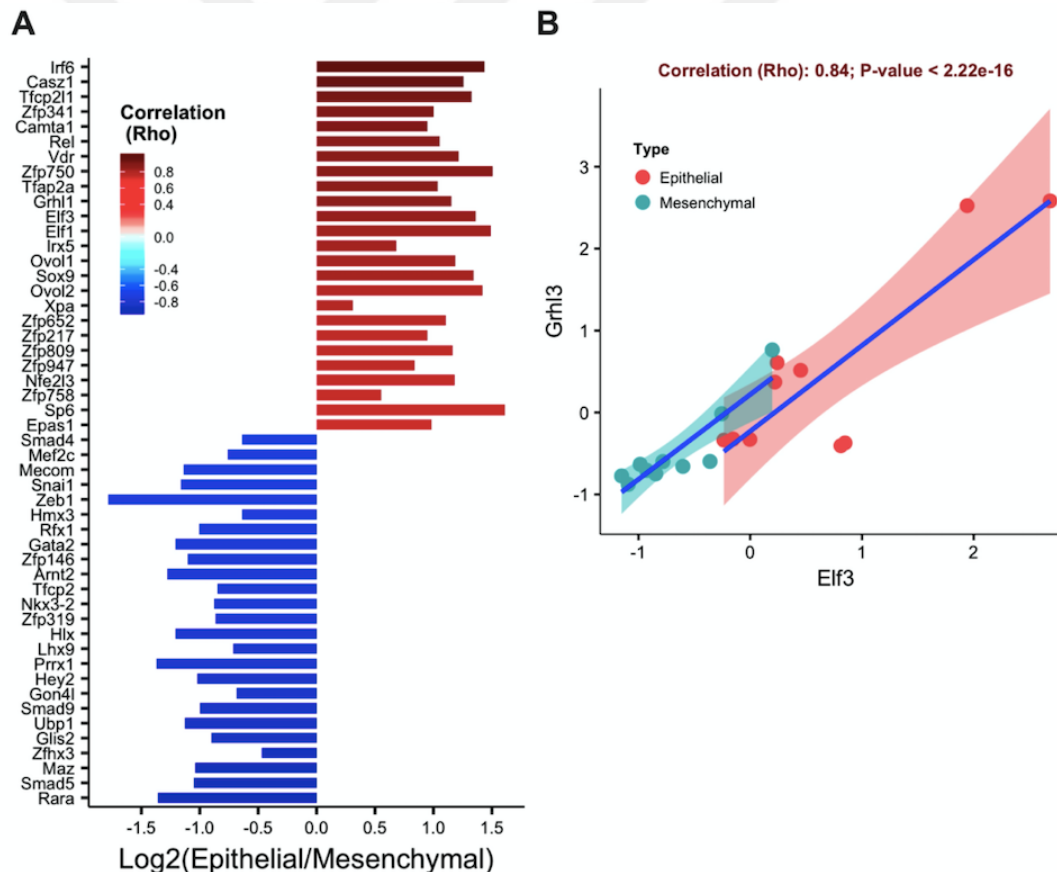


Figure 13. Genes correlated with Grhl3. A plot showing the top 25 Grhl3 correlated transcription factors in the epithelial and in the mesenchymal states. The x-axis represents the differential expression of each transcription factor in epithelial versus mesenchymal samples (A). Grhl3 and Elf3 are highly correlated. The expression of Grhl3 and Elf3 was evaluated in each data set, and the Spearman correlation was calculated across all samples. Grhl3 and Elf3 showed a strong correlation and a strong co-regulation with the epithelial phenotype (B).

We used siRNA mediated silencing of candidate genes at the onset of MET in NMuMG cells. We followed the progression of MET both morphologically and expression levels of epithelial and mesenchymal defining genes within the scope of transcriptional node analysis.

We initially examined the expression pattern of *Elf3* in NMuMG cells during the progression of EMT-MET. We have noticed that unlike the *Cdh1* expression pattern, *Elf3* RNA levels were relatively stable (Figure 10B). We then used several siRNAs targeting *Elf3* and validated their knockdown efficiency in NMuMG cells by measuring *Elf3* RNA levels (Figure 14A). As a result, we noticed that all siRNAs used produced a similar phenotype with varying yet substantial knockdown efficiencies. In cells transfected with nontargeting siRNA, we did not notice any morphological changes; on the other hand, *Elf3* targeting siRNAs caused a MET failure evident by the enlarged and flattened cell morphology (Figure 14B). Confocal images of siElf3 transfected cells revealed oversized cells, with actin present as stress fibers, absence of E-cadherin at the plasma membrane, and an increased Vimentin expression (Figure 15). These results confirmed the MET failure and suggested that this aberrant MET was, in part, due to the absence of E-cadherin at the plasma membrane. The enlarged cellular morphology was reminiscent of senescent cells. Thus, NMuMG cells recovering from TGF β 3 were transfected with either control or *Elf3* targeting siRNAs. Huh7 cells were simultaneously treated with Doxorubicin for 48 hours and used as a control. As a result, it was shown that phenotypic changes in *Elf3* depleted cells are not associated with senescence, suggesting a different cause for the enlarged cells.

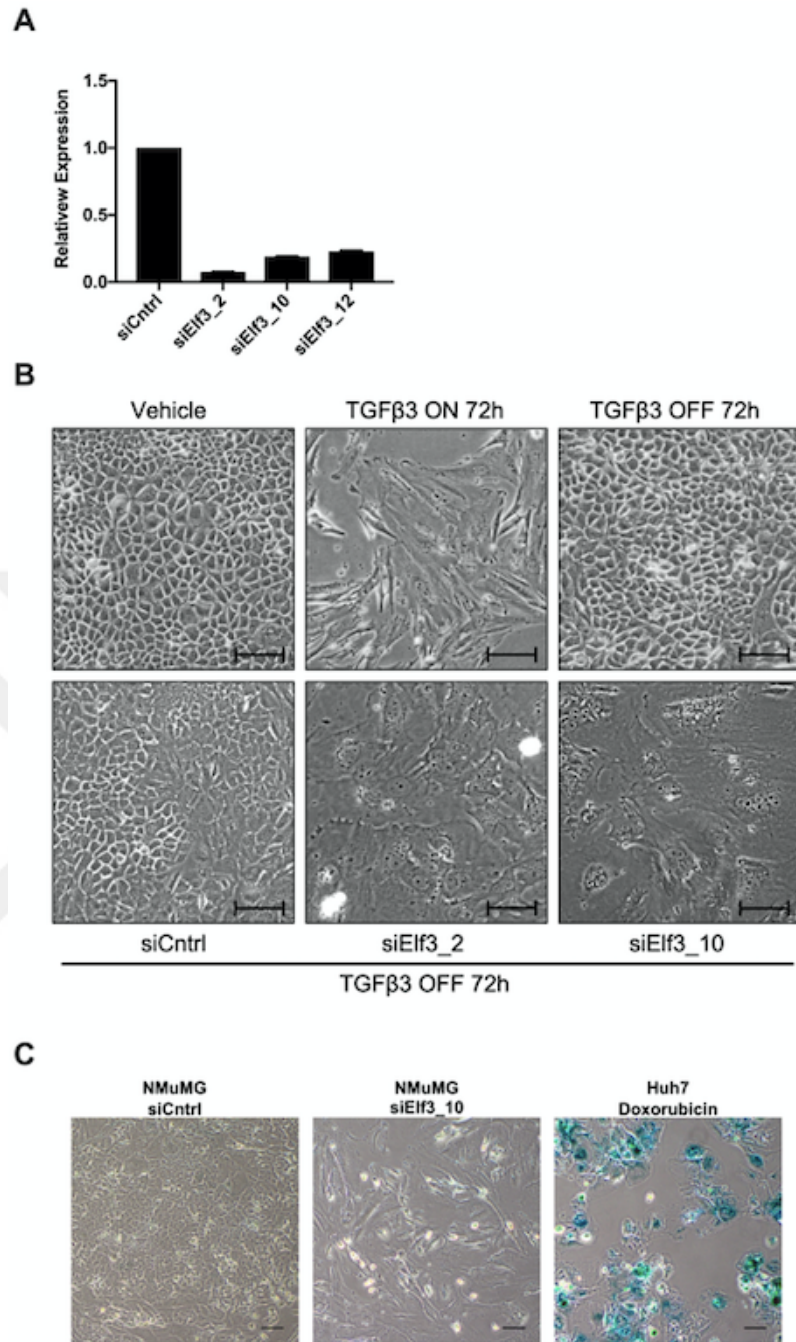


Figure 14. The siRNA mediated silencing of Elf3 results in an impaired MET. The efficiency of silencing was measured by qPCR analysis of RNA collected from NMuMG cells recovering from TGF β 3 treatment after Elf3 knock-down using three different siRNAs (A). Phase-contrast images of NMuMG cells showing the morphological changes that accompany the TGF β 3 mediated EMT- MET (upper panel), and the morphological changes associated with the silencing of Elf3 (siElf3) as compared to control siRNA (siCntrl) at the onset of MET (lower panel) (B). phase-contrast images of NMuMG cells and Huh7 cells, showing that the phenotypic changes in Elf3 depleted cells are not associated with senescence. NMuMG cells recovering from TGF β 3 were transfected with either control or Elf3 targeting siRNAs, Huh7 cells were treated with Doxorubicin for 48 hours (C). All cells were subjected to SA-BGal staining. The scale bar is 100 μ m.

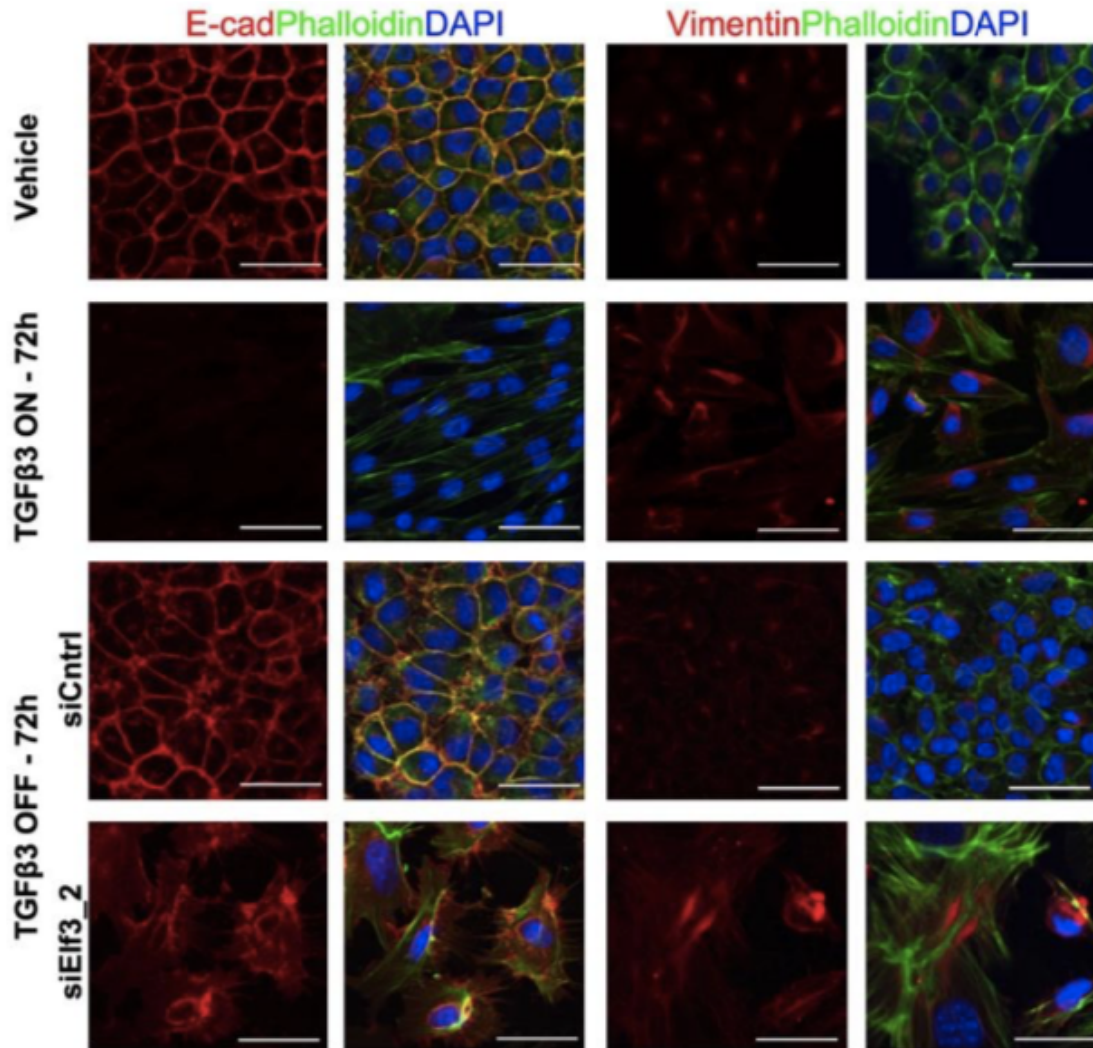


Figure 15. The siRNA-mediated silencing of *Elf3* results in an impaired MET. Confocal images showing the immunofluorescence staining of NMuMG cells visualizing changes in expression and intracellular distribution of E-cadherin, Vimentin, and Actin during TGF β 3 treatment, withdrawal, and also during the silencing of *Elf3*. Actin distribution (Phalloidin, green) and detection of E-cadherin (red) and Vimentin (red) are shown. Nuclei are labeled with DAPI. Scale bar, 50 μ m.

These observations were then confirmed by studying RNA expression levels of genes relevant to the EMT and MET programs. We selected *Ahr*, *Cebpa*, *Ovol2*, *Exosc9*, and *Ehf* for their potential involvement in the process of maintaining epithelial integrity, regulating the dynamic switch between epithelial and mesenchymal states, and their key roles in metastasis (Albino, Longoni et al. 2012, Roca, Hernandez et al. 2013, Huan, Wen et al. 2016, Xue, Fu et al. 2018). NMuMG cells undergoing MET were transfected with different siRNAs targeting *Elf3*, and gene expression levels were evaluated by qPCR analysis. There was a significant downregulation of several transcription factors, such as *Ahr*, *Cebpa*, *Ehf*,

and *Ovol2* (Figure 16A). On the other hand, the expression levels of some mesenchymal markers showed an evident increase in the absence of *Elf3*; we noticed a significant increase in the classical EMT inducers such as *Snai1/2* and *Zeb2* (Figure 16C), which was accompanied by an increased expression of *Cdh2* and *Fn1* (Figure 16D).

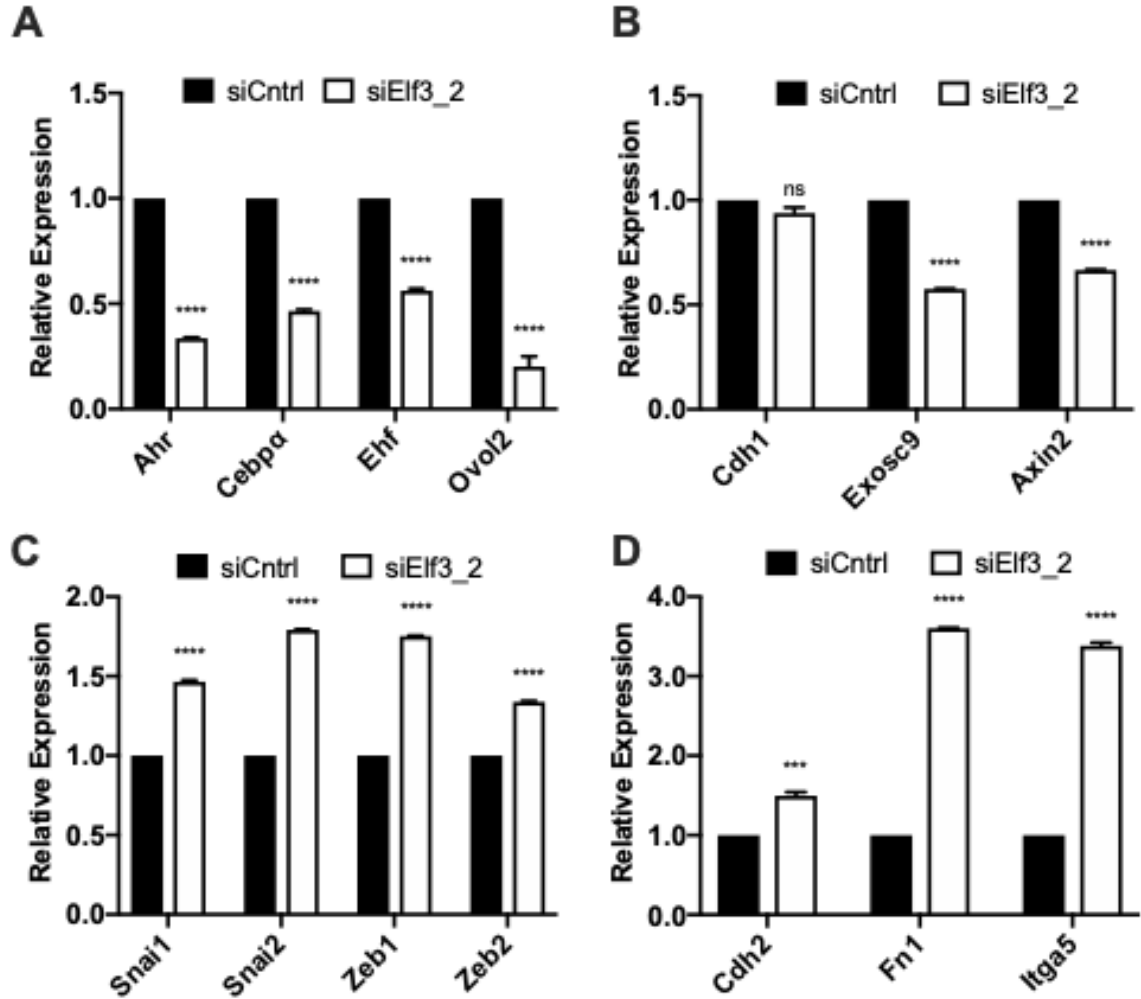


Figure 16. Loss of Elf3 preserves the mesenchymal state. NMuMG cells recovering from TGF β 3 treatment were transfected with either control or Elf3 targeting siRNA. After 72h, cells were collected, RNA was isolated, and changes in gene expression were measured by qPCR for EMT/MET relevant genes (A, B) and mesenchymal markers (C, D). Results represent the averages of at least three independent experiments in triplicates.

NMuMG cells were then transfected with either siCntrl or siElf3 and wound healing assay was performed. Cells were monitored and documented at regular intervals (each 24h). We observed a five-fold increased migration compared to control cells in wound healing assay

(Figure 17B). The mesenchymal state observed at RNA levels was thus confirmed by the increased migratory capacity of *Elf3* depleted cells.

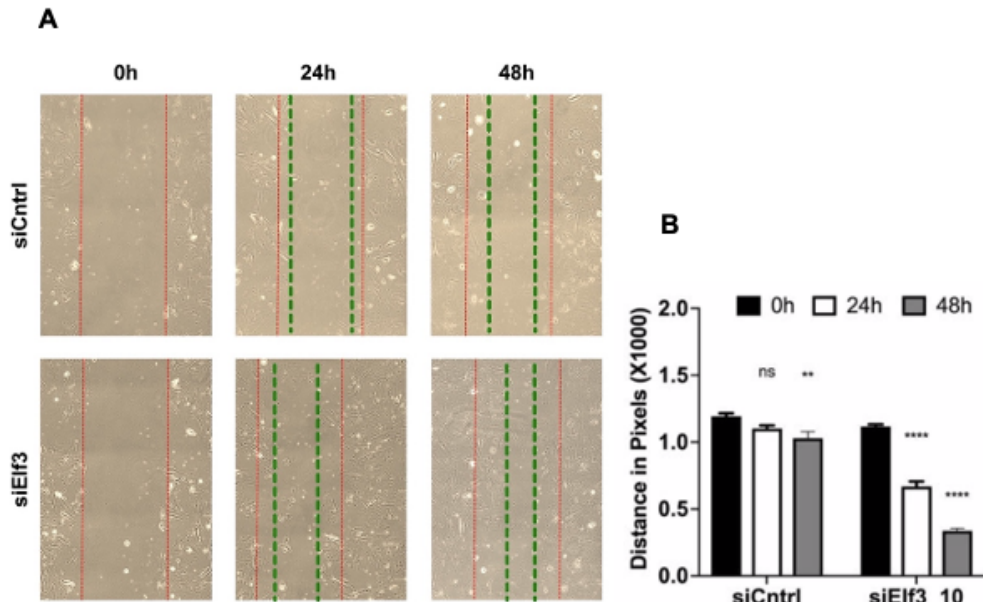


Figure 17. The absence of *Elf3* during MET preserves the mesenchymal state. NMuMG cells were transfected with either siCntrl or siElf3 and wound healing assay was performed. Cells were monitored and documented at regular intervals. The red vertical lines define the original width of the scratch, and the green vertical lines show the edges of the migrating cells (A). Summary of the wound healing assay results following *Elf3* silencing measured as the distance between the edges of migrating cells (B).

To address the potential transcriptional regulatory relationships, the transcription factor binding sites were primarily defined via the JASPAR tool (Figure 18).

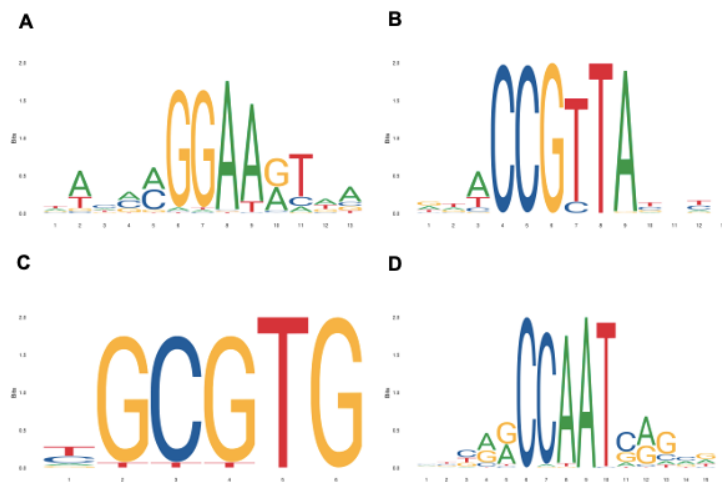


Figure 18. Transcription factor binding site profiles from JASPAR. *Elf3* (A), *OVOL2* (B), *Ahr* (C), and *Nfya* (D)

The minimal promoter region of *Grhl3* (1 kb proximal to TSS, range: chr4:135129340-135130535) was analyzed with regard to conserved sequences and *Elf3* binding sites by our previous lab member. Of the 17 *Elf3* binding sites in the *Grhl3* promoter, six were present in the first conserved sequence (CNS1: 210 bp upstream of TSS) (Figure 19), four of which were found conserved among different mammalian species (Figure 20).

```

-1000 tgcctagcac agagcactta cagtgtactg gccagctaag actcctgagg caaccacctt
-940 caacttttcc atcctttctg ggtggccag aggcattgtg tctgcccaca tcccgttcag
-880 attggcatcg cacaagatga aaggctgccc tgccctaatt ccctgattcc ccaccccatc
-820 tctctctctc tctctctctc tctctctctc tctctctcac acacacacac acacacacac
-760 acacactcat gcacattggg ggaggggggg ctctctctga atagaaacca gcattttctt
-700 tcccgcgtat tgccccagaa ccagagcgga ggagaccttg cacactcggg acttagtaaa
-640 tgtccctgtg caactgaggc caccaacttc aactcaagga gtagctagtc tcccagatga
-580 ctgaacgggt ctctgacact ggctctcacc cttaggcaag gcacctgcc tctccaacct
-520 acatttccct gatcactgtg agagggcatg gggttggtg ggggtggaag gagagttgac
-460 agaaagtcc tccctagctg ttactgggag aatagggaag aatcacttag ctaacaagac
-400 tagaacctgt ggtgtttggt acccgaagg gaaagtctg gagaaaagag aggggactca

      Elf3
-340 cagagagcta cctgctctc tccgtgaaat ttccgggta gagaagggtg ctataaagag
-280 atatggtgtc tgtggggaga tgtttgtggg atccaggatc cgagcgatga gatggacaga
-220 gatttccctt tgagccaagt acctgtctgt attttccctc tagttctccc gttcccttcc
-160 aacgtttgtc tctcccttt tccctcgtct ccacccctc acccctcag ctaagaagga

      Sp1
-100 attggccggc tccaccagc cagcccccg gagctccgcc cctgctggtg ggccaaaagt

      TSS
-40 taacatctgc acctgctgtg gtctagctgc agtcggcacc TGTGCTTCCA GCCAATCAGC
  30 GCCACCGCAG CCGGGGACCG CTGTCAGCAA AATCTCAACA TCCAAAGCGC AACGTAGAGC
  90 AAACGCTTCC CCGGGCAGGA AGGGAATGTC TGTGTCAGAG GAGAATTAAG AGACGAGTGC
 150 TCAGCAGCGC CTGCGAGCCA ACCAGAGACG GATCGCTGGA ACCTCGGAGA AGGAAG

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Figure 19. *Grhl3* promoter. The sequence of the *Grhl3* promoter (1 kb) showing the location of the predicted TATA Box, SP1 sequence, and the transcription start site. Sequences in red represent the conserved regions, and blue bases represent putative *Elf3* binding sites.

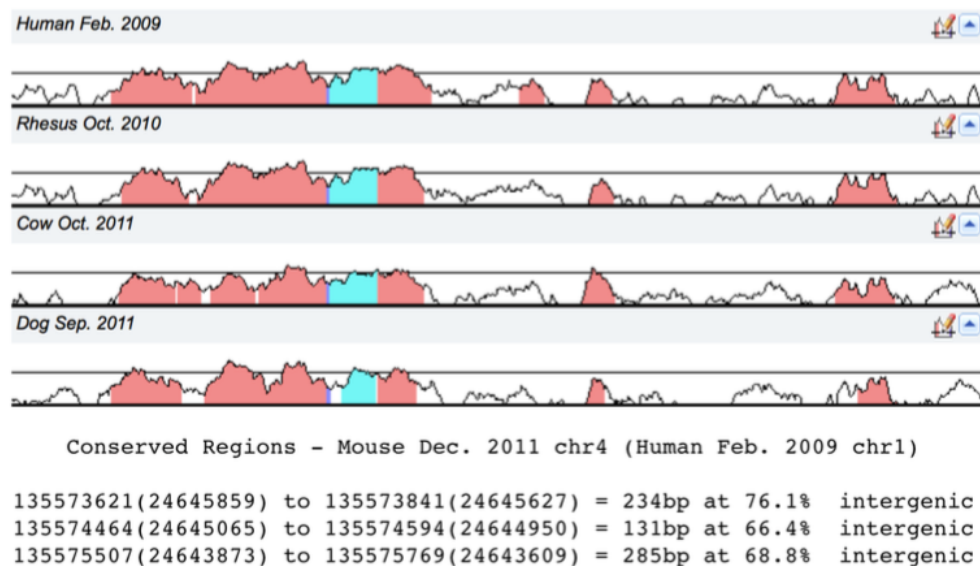


Figure 20. Conserved regions in the *Grhl3* promoter. The pink represents the conserved regions, and the blue represents the UTR (untranslated region) region.

The binding of *Elf3* at the *Grhl3* promoter was confined to CNS1 during MET (Figure 21A); we could not detect similar binding using primers outside of this conserved sequence (Figure 21B).

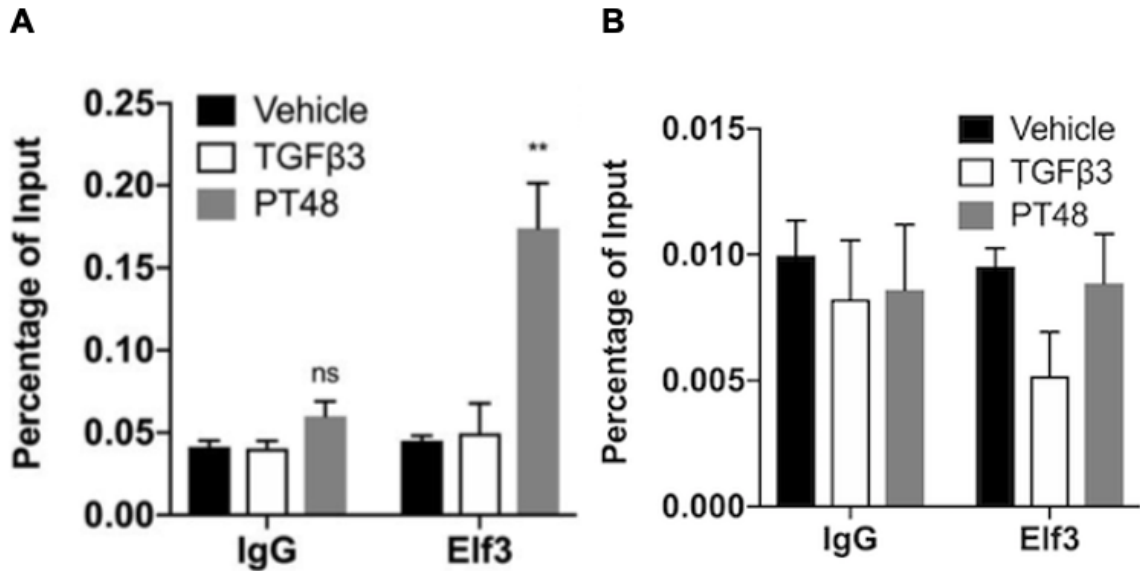


Figure 21. ELF3 binding at the promoter of *Grhl3*. NMuMG cells were treated with either vehicle or TGFβ3 for 72 h. TGFβ3 treated cells were then washed with PBS and continued incubation for an additional 48 h to initiate MET (post-treatment: PT48). Cells were cross-linked, and chromatin immunoprecipitation was performed. ChIP DNA was used in qPCR to measure the occupancy of ELF3 compared to control antibodies (A, B).

4.2.2 '*Nfya-Ahr-Ovol2-Grhl3*', Transcriptional Node

Correlative to *Grhl3* and *Elf3*, *Ovol2* showed a strong correlation with the epithelial phenotype. In-silico analysis and our microarray data revealed that transcription factors *Ahr* and *Nfya* are potential regulators within the proposed transcriptional network, as well.

In order to investigate whether these relationships have a direct regulatory role, it is planned to examine the 1 kb promoter regions of desired genes, whether they have putative transcription factor binding sites. When the 1 kb *Nfya* promoter (range: chr17:48539838-48550145) was examined, the *Grhl3* binding site was not determined within the 1 kb region. However, one *Grhl3* binding sequence was detected in the first intron, but the determined site was not in the conserved sequences. Also, three *Ahr* binding sites were determined in the established region of the promoter. Of the 3 *Ahr* binding sites in the *Nfya* promoter, all were present in the conserved region (Figure 22A), and they were highly conserved among different mammalian species (Figure 22C).

A

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-300 tcccgctacc gctcaggeat gcgtaaccgag atgccgaccc cgactggaga agaagagcgc
-240 ttgcgcattc ggccccagag ggtgggtccc agtggacgct tagaaatagg tgggcagggg
      Ahr          TATA Box
-180 ggcggtgtcag cgctcggtg gtatatataatt cagggaggtg ttttaagcagc agcgtacgga
-120 gcggtgtgaaa ggcgcctgcg taggcctttg gcgggagggc gtgtcaaadc cagggaagtgg
                        Sp1
-60 gtgtggcctc tggaacgcct gcgcggtgct cttcctaggg ggcgggggcca ggctggccgg
      TSS
  1  GGTATATGGGG CGTGGTCTCG AAGGCGCTTG CGCGAAGGCG GTTGGAGGGA GGCCCGATTC
 61  CCCTTTGTTC AGATTGCGCA TTTTGCAGAG CAGCGGCAGT GGCGGCGGCA GCGGCGGCTG
121  GAGCCTCTGA TTGGGTTTCG GGGTCCGGTA CTGGAGCCAA TCAGCGCGGG CAGCGAACCG
181  GGGGAGCGAG GCACGgtgag tatgaggagc caatatccag cggcccagag ccggccccag
241  cgccccgatt ggcgggtctc gctgaccact caggagaggc ccaggcgctc gtcgagccccg
301  ggagtcgagc cgaggctgcc cgagctgggt agccagtagc cccggctcgg agttgggtgga
361  ggctgcaggg ggcgcgtccag cgttgggacg gcgtcctgga gccaggcttt tcgggatctg
                        Grhl3
421  gatcgtctcc gggggccggt ggcggtatgcg caccgggtcc ccgacccctc gtcccagagag
481  gtcaggtggc tgaccgcca gccacggacc gtggcggttt cccacgctc ttccagcctc
541  atcctacggc ctgtccttgt gtgtccccac attccgggac ccgacgaggc ccgtgtgtcc

```

B

Conserved Regions - Mouse Dec. 2011 chr17 (Human Feb. 2009 chr6)

48409821(41040628) to 48410079(41040361) = 270bp at 70.4% intergenic
48409471(41040979) to 48409625(41040824) = 156bp at 91.7% intron

C

		Ahr*		Ahr**	
mouse	CAGCGCCT	GCGTG	GTA...//...ACGGA	GCGTG	TGAAA
human	TAGCGCCT	GCGTA	GTA...//...CTGGC	GCGTC	TGGAA
chimp	TAGCGCCT	GCGTA	GTA...//...CTGGC	GCGTC	TGGAA
cow	TAGCGCCT	GCGTG	GTG...//...CTGGC	GCGCC	TGGAA
rhesus	TAGCGCCT	GCGTA	GTA...//...CTGGC	GCGTC	TCGAA
Identity	*** **	****	**	***	* **

Figure 22. Nfya promoter. The sequence of the Nfya promoter (1 kb) and part of the first intron showing the location of the predicted TATA Box, SP1 sequence, and the transcription start site. Sequences in red represent the conserved regions, and green bases represent putative Ahr binding sites, and orange bases represent putative Grhl3 binding sites (A). 70% identity when considering the intergenic sequences and 90% identity when considering the intron (B). Ahr binding sites are highly conserved among different species (C).

In consequence of *Nfya* depletion during MET, the cells could not return to the epithelial state. *Cdh1* mRNA level slightly decreased. NMuMG cells recovering from TGFβ3 treatment were transfected with either control or *Nfya* targeting siRNAs, and changes in gene expression levels were measured by qPCR for epithelial and mesenchymal markers (Figure 23). We demonstrated a significant decrease in the expression of EMT-MET relevant genes such as *Grhl3* and *Ehf* by the depletion of *Nfya*. Confocal images of siNfya transfected cells revealed oversized cells, with actin present as stress fibers, absence of E-cadherin at the plasma

membrane (Figure 23C). The same cellular response was encountered when *Elf3* was silenced during the course of MET. These results confirmed the MET failure due to the absence of E-cadherin at the plasma membrane.

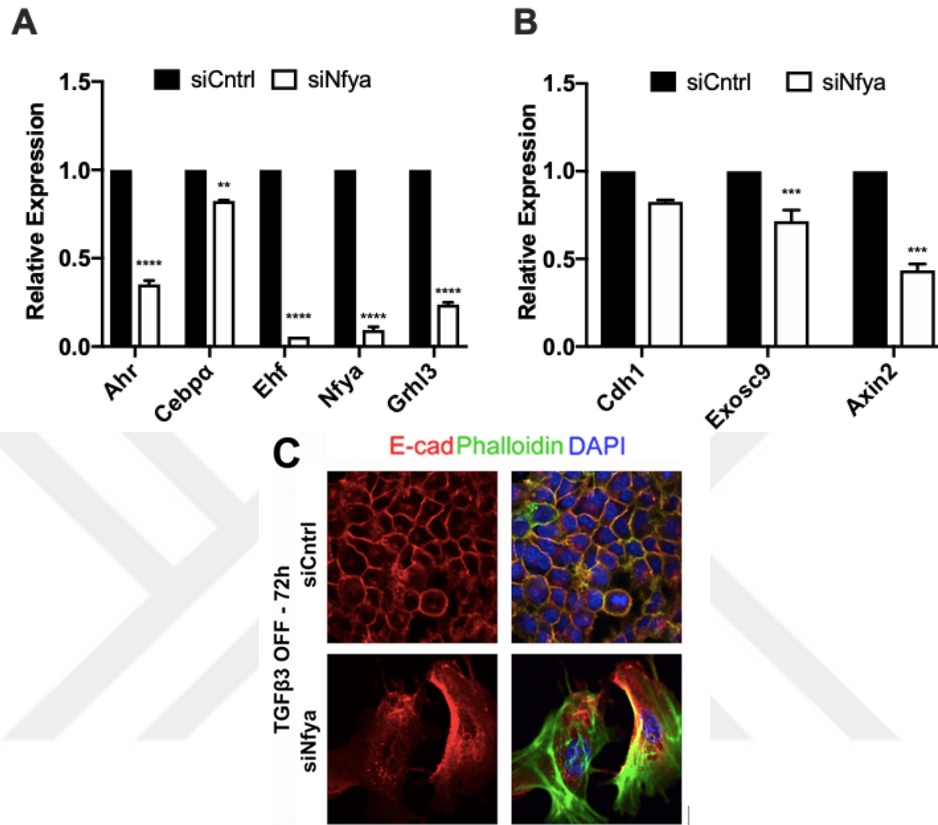


Figure 23. Loss of *Nfya* preserves the mesenchymal state. NMuMG cells recovering from TGFβ3 treatment were transfected with either control or *Nfya* targeting siRNA. After 72h, cells were collected, RNA was isolated, and changes in gene expression were measured by qPCR for EMT/MET relevant genes (A, B). Results represent the averages of at least three independent experiments in triplicates. Confocal images showing the immunofluorescence staining of NMuMG cells visualizing changes in expression and intracellular distribution of E-cadherin, Vimentin, and Actin during TGFβ3 treatment, withdrawal, and also during the silencing of *Nfya*. Actin distribution (Phalloidin, green) and detection of E-cadherin (red) are shown (C). Nuclei are labeled with DAPI. Scale bar, 50 μm.

The siRNA-mediated silencing of *Ahr* also results in an impaired MET. *Ahr*'s loss preserves the mesenchymal state shown by the changes in the expression levels of EMT-MET relevant genes and mesenchymal markers (Figure 24). Significant downregulation of the expressions of such factors, including *Ahr*, *Ehf*, *Grhl3*, and *Ovol2*, was observed except *Cebpa*. Changes in the mesenchymal signature was also determined by the upregulation of *Cdh2* and *Zeb1/2*. Confocal images of siAhr transfected cells revealed the same phenotypic changes

encountered when *Elf3* and *Nfya* were silenced during the course of MET in NMuMG cells (Figure 24D). Although there was no significant decrease in the mRNA levels of *Cdh1*, the E-cadherin once again was localized in the cytoplasm (especially around the nucleus) instead of the plasma membrane, which may explain why the cells cannot return back to the epithelial state.

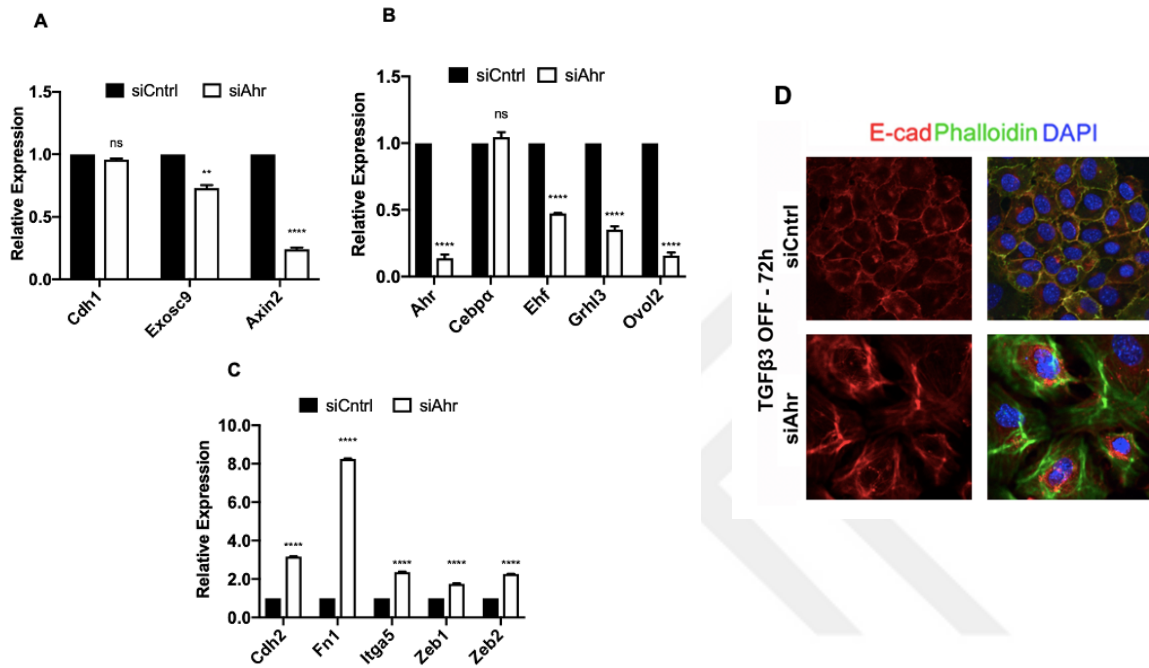


Figure 24. Loss of Ahr preserves the mesenchymal state. NMuMG cells recovering from TGFβ3 treatment were transfected with either control or Ahr targeting siRNA. After 72h, cells were collected, RNA was isolated, and changes in gene expression were measured by qPCR for EMT/MET relevant genes (A, B) and mesenchymal markers (C, D). Results represent the averages of at least three independent experiments in triplicates. Confocal images showing the immunofluorescence staining of NMuMG cells visualizing changes in expression and intracellular distribution of E-cadherin, Vimentin, and Actin during TGFβ3 treatment, withdrawal, and also during the silencing of Ahr. Actin distribution (Phalloidin, green) and detection of E-cadherin (red) are shown (E). Nuclei are labeled with DAPI. Scale bar, 50 μm.

Similar to *Grhl3*, *Elf3*, *Ahr*, and *Nfya*, depletion of *Ovol2* preserves the mesenchymal state as well. Although the knockdown efficiency of *Ovol2* is not comparable to the other transcription factors within the network, the EMT relevant genes such as *Ehf* and *Grhl3* were significantly downregulated. A significant increase was observed in the expression levels of selected mesenchymal markers, especially *Twist1*. Confocal imaging represented E-cadherin localization in the cytoplasm, not in the plasma membrane, such as examples of *Elf3*, *Ahr*, and *Nfya* (Figure 25E).

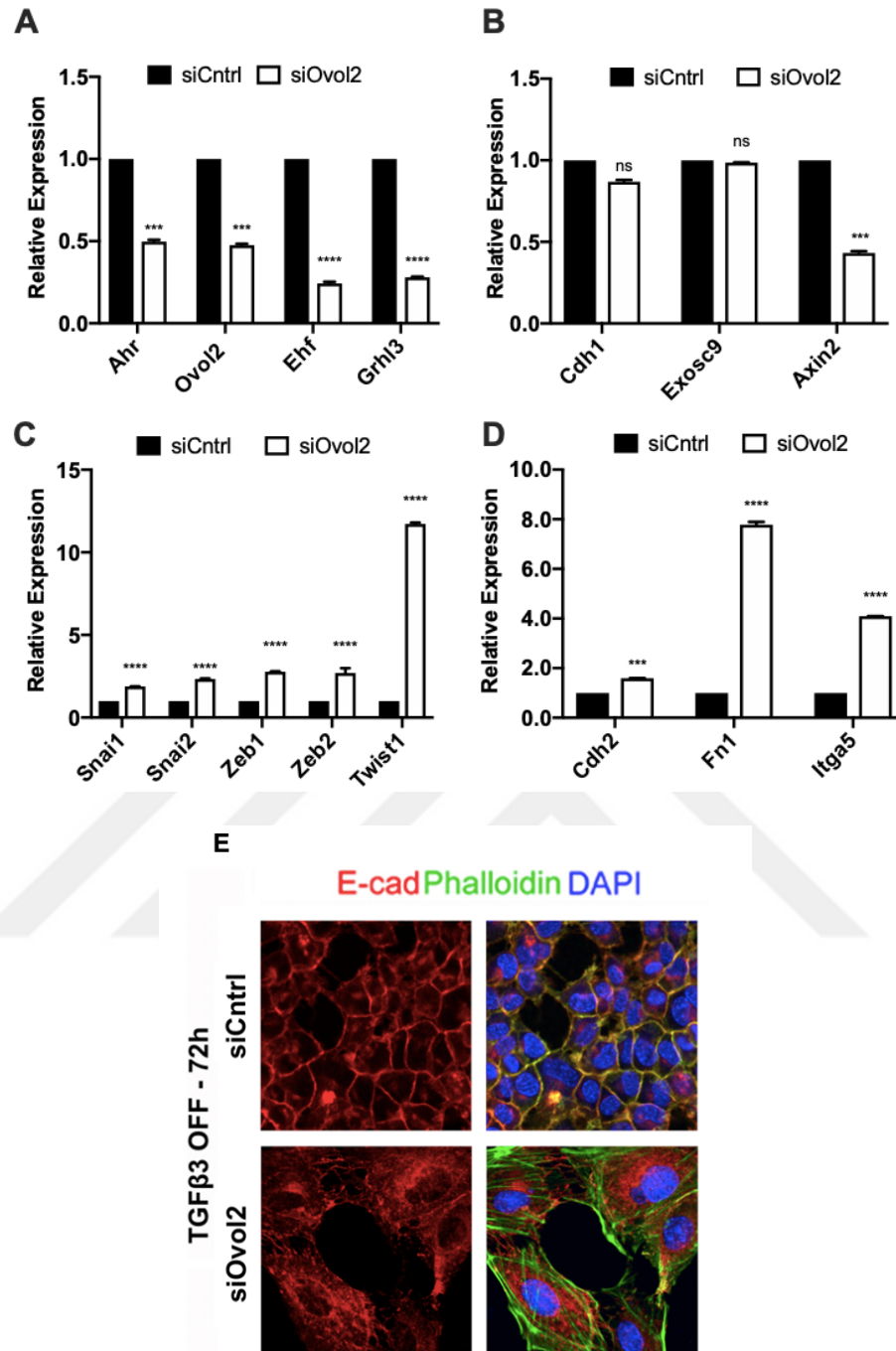


Figure 25. Loss of Ovov2 preserves the mesenchymal state. NMuMG cells recovering from TGFβ3 treatment were transfected with either control or Ahr targeting siRNA. After 72h, cells were collected, RNA was isolated, and changes in gene expression were measured by qPCR for EMT/MET relevant genes (A, B) and mesenchymal markers (C, D). Results represent the averages of at least three independent experiments in triplicates. Confocal images showing the immunofluorescence staining of NMuMG cells visualizing changes in expression and intracellular distribution of E-cadherin, Vimentin, and Actin during TGFβ3 treatment, withdrawal, and also during the silencing of Ovov2. Actin distribution (Phalloidin, green) and detection of E-cadherin (red) are shown (E). Nuclei are labeled with DAPI. Scale bar, 50 μm.

Similarly, the 1 kb *Ovol2* promoter (range: chr2:144157597-144158816) was evaluated for the putative binding sites. One *Ahr* and two *Grhl3* sites were determined. All *Ahr* and *Grhl3* binding sites in the *Ovol2* promoter were present in the conserved sequence (Figure 26A). They were found 100% conserved among different mammalian species (Figure 26C).

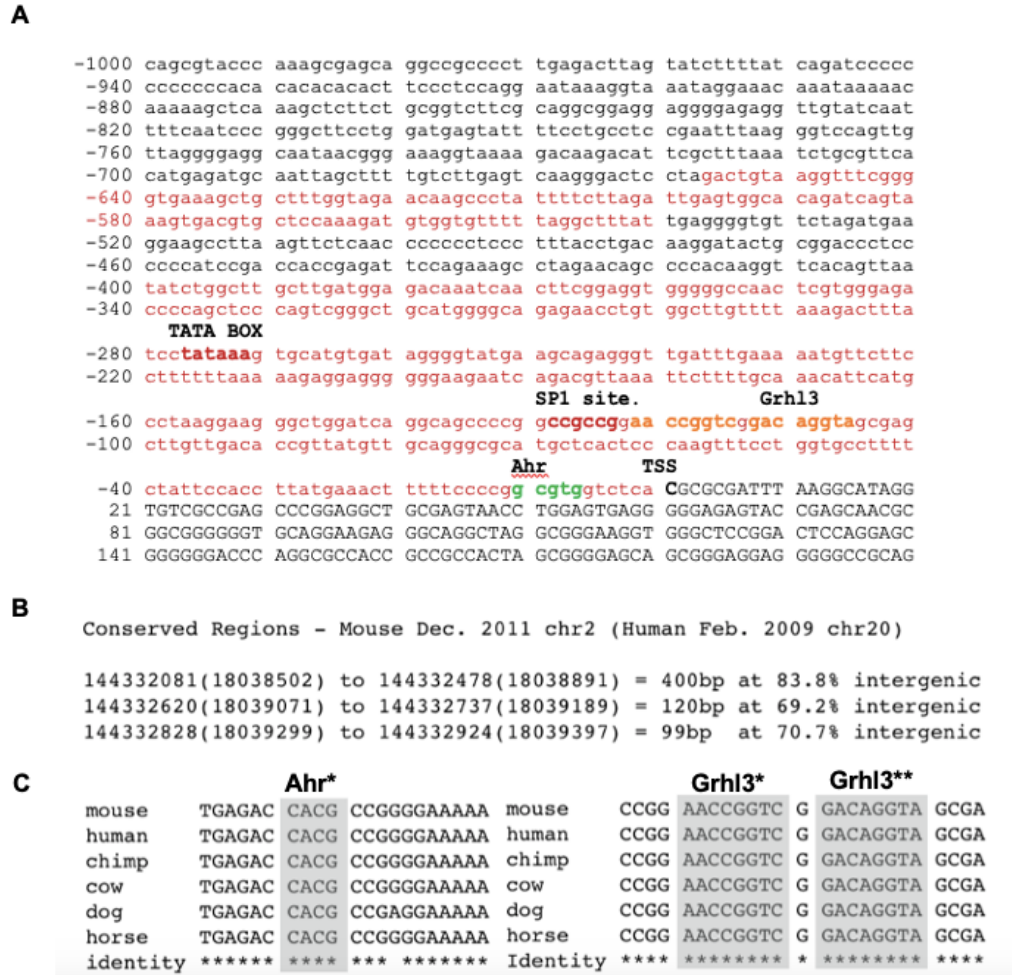


Figure 26. *Ovol2* promoter. The sequence of the *Ovol2* promoter (1 kb) showing the location of the predicted TATA Box, SP1 sequence, and the transcription start site. Sequences in red represent the conserved regions, and green bases represent putative *Ahr* binding sit. Orange bases represent putative *Grhl3* binding sites (A). Approximately 70% identity was determined when intergenic sequences were evaluated (B). *Ahr* and *Grhl3* binding sites were conserved among different species (C).

To provide evidence for direct physical interaction of *Grhl3* and *Ovol2*, ChIP assay was performed. The binding of *Grhl3* at the *Ovol2* promoter was also confined during MET by ChIP analysis (Figure 27). The performance of the assay was evaluated by examining the enrichment of *Grhl3* at the previously published enhancer within the second intron of *Cdh1*.

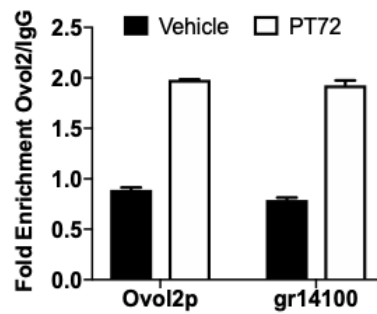


Figure 27. Grhl3 binding at the promoter of Ovov2. NMuMG cells were treated with either vehicle or TGF β 3 for 72 h. TGF β 3 treated cells were then washed with PBS and continued incubation for an additional 72 h to initiate MET (post-treatment: PT72). Cells were cross-linked, and chromatin immunoprecipitation was performed. ChIP DNA was used in qPCR to measure the occupancy of Grhl3 compared to control antibodies.

4.2.3 'Elf3-Ahr-Ovov2', Transcriptional Node

Within the scope of the previous transcriptional node, the relationship between *Ahr*, *Nfya*, *Grhl3*, and *Ovov2* was examined. It was also determined that *Elf3* is the regulator of *Grhl3* during MET (Sengez, Aygun et al. 2019). These observations prompted us to investigate whether *Elf3* is responsible for regulating the other common transcription factors, which may be critical for MET. For this purpose, *Ahr*, *Nfya*, and *Ovov2* expression levels were determined where *Elf3* was silenced during the MET process. While no change was observed in *Nfya* levels, a significant decrease was detected in the expression levels of *Ahr* and *Ovov2* (Figure 28). We have already indicated that by depleting *Ahr*, the expression levels of *Ovov2* and selected EMT-MET relevant genes were significantly decreased (Figure 24).

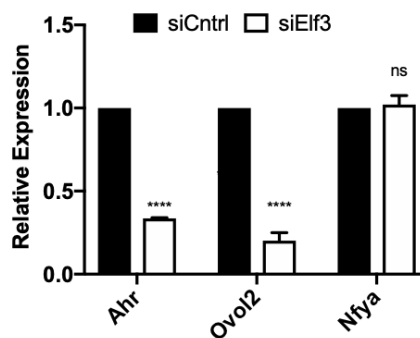


Figure 28. Loss of Elf3 preserves the mesenchymal state. NMuMG cells recovering from TGF β 3 treatment were transfected with either control or Elf3 targeting siRNA. After 72h, cells were collected, RNA was isolated, and changes in gene expression of *Ahr*, *Ovov2*, and *Nfya* were measured by qPCR. Results represent the averages of at least three independent experiments in triplicates.

Next, the 1 kb *Ahr* promoter region (range: chr12:36219296-36220661) was evaluated in terms of putative binding sites. When the 1 kb *Ahr* promoter region was examined, two *Nfya* binding sites and eleven *Elf3* binding sites were identified. The sequences we used in the *Nfya* and *Ovol2* binding site detection was as follows respectively: (a/g)CCAAT(a/c/g) and 5'-A(A/T) (A/T) (C/ A) (T/C)GTTA(T/A) 'AWWMYGTAW'. The sequence was also evaluated for the *Grhl3* binding sites, and two *Grhl3* binding sites were determined (Figure 29). Of the 2 *Grhl3* binding sites in the *Ahr* promoter, one was present in the conserved region (Figure 29A).

A

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-1000 ctacctgact cccaaaattc acttctctgc ctctattctt tgggtggttaa cagcgacctt
-940 ccctcttcct ttatcatctc tgctgtctct tttctctcat ccccatggct gtgccattgg
-880 tcttgacgct ggctgatttc atcaatttcc cagcgattct ctctttccatc tgttttgttc
-820 ctgagtaaca gaatttccac cttttccac agtgactttc ccaagaaaga tggaacatca
                                     Nfya
-760 tcttccaaat tacgccttta aaagctctct gttgtctgca gaatgagacc caatttcttc
-700 attaaatctt tatgatttgt tcctagatta tctttttttt tttttttctt ttccttccca
-640 tcagctctac cgtactacta acagaaactc aggtctctgg tcatagtctc agtacctacc
      Nfya
-580 ctccatttc tcattgctctc agcactctctg tgcttctttc cttctcatct tcctgaagtc
-520 tgatggagga aacgatgttt tattgtcttc tgtagacata aaataaatgt gggagggaaa
      TATA Box Grhl3
-460 aggattataa aactcgtagc cttcaagaca actaactttg cagggcattt ttgaagtttt
-400 ccccttagaa tgctctccag tcgcagttat ttatctacca gctcaatgta aaatctttgt
-340 caaaaattag atgactcaac tctaggatga gctaagctct ttgcttggtg aatcttgatg
-280 tctgggttac aaggaatttg cctaggcata atgtggcctc acaccccttt tgaggctgga
-220 aaacaggtac tgggcgaaca ccacccacc cccagaacct cggactgcaa gaagtgttgg
-160 gagtcactac gtctcgcgtc atcacgtgct gcgaagaggg tggggccctc ctggaaccg
      Sp1          Sp1          Sp1
-100 tgaggggcgg ggccgagcag gtgggcggg gctggagccc ggggcggagc tgccggggt
                                     Sp1 TSS
-40 gcggcgtgcg gaggagcggt ctcgcggcg gcggcagcgt GTGTGTGCGC TCCCTTTGAC
21 GCTCGGTGCC CCACGCGTGT CCCGGAGAGG CTCAGCCCGG AGCGACCCGG AGGACGAACT |
81 GGTGGACGGA CTCGGGGGTG GCTGCCAGGC AGGGTTGTGA GCGACGCCGC GGCACAGCGC
141 GGGAGCCGGG AAGCCCTAGA GCACTGCGGC CCCTCCTGAC CCTCGGGACC GGGCGCGGCT
201 AGCGTGCGGG TTTCTCTCC GCGGCCAGCT CGGGCAGCCC GAGTCTCCTC TGTCGCCCGC

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B

Conserved Regions - Mouse Dec. 2011 chr12 (Human Feb. 2009 chr7)

35534993(17338277) to 35535142(17338096) = 183bp at 65.6% intergenic
35535271(17338023) to 35535452(17337826) = 205bp at 62.4% intergenic

Figure 29. *Ahr* promoter. The sequence of the *Ahr* promoter (1 kb) showing the location of the predicted TATA Box, SP1 sequence, and the transcription start site. Sequences in red represent the conserved regions, and purple bases represent putative *Nfya* binding sites, orange bases represent putative *Grhl3* binding sites, and blue bases represent putative *Elf3* binding sites (A). Considering the intergenic sequences, around 60% identity was determined (B).

Taken together, *Elf3* may seem to have a role in regulating transcription factors *Ahr* and *Ovol2*. Further analyses are needed to determine the physical interaction of those factors.

4.2.4 'Grhl3-Ovol2', Transcriptional Node

Studies conducted within the context of the 'Nfya-Ahr-Ovol2-Grhl3' transcriptional node pointed to the strong association of *Grhl3* and *Ovol2* transcription factors. To understand this relationship in more detail, *Grhl3* and *Ovol2* ChIP-Seq data obtained from publicly available datasets were analyzed. As a result of this analysis, it was determined that *Ovol2* and *Grhl3* were associated with 9360 genes. 8980 of these genes were only *Grhl3*, and 1246 were only *Ovol2* related genes. There are 866 genes within the intersection of both transcription factors. The existence of such a huge number of common genes confirms the importance of this association. It was also determined that 117 of 981 transcription factors in total are common. When the ratio of transcription factors vs. gene list was examined, more than one-tenth of the genes were composed of transcription factors (Table 6).

Table 6. The number of genes and transcription factors that are potentially regulated by *Grhl3* and *Ovol2*.

Transcription Factors	Number of the genes	Number of the transcription factors	Gene/transcription factor ratio
Grhl3	8980	949	~ %11
Ovol2	1246	149	~ %12
Grhl3-Ovol2	866	117	~ %13.5

It has also been determined that these two factors act together in many processes by analyzing ChIP-Seq data. Some of the main processes in which both factors act are:

- Regulation of gene expression
- Chromatin organization and epigenetic regulation
- EMT, cell migration and motility
- Cellular responses to DNA damage and stress
- Apoptosis and programmed cell death
- Development and morphogenesis
- Differentiation

Using the 'Integrative Genomics Viewer (iGV)' software, *Ovol2* occupancy at *Grhl3* gene region was determined. Analysis revealed a large peak apart from the promoter region,

which indicates the possibility of enhancer mediated regulation (Figure 30). Then, Grhl3 occupancy at the Ovol2 gene region was identified likewise.

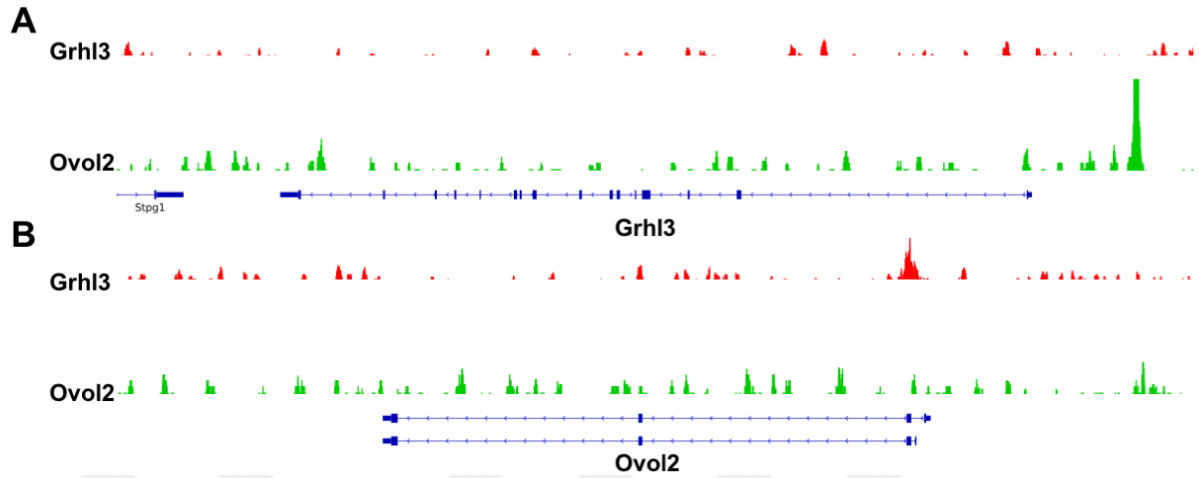


Figure 30. IGV visualization of Ovol2 occupancy at the Grhl3 gene region (A) and Grhl3 occupancy at Ovol2 gene region (B).

These data, which support our studies during MET, indicates that *Grhl3* and *Ovol2* tend to regulate each other. When the common gene list was examined, it was clearly seen that many EMT-related genes such as Notch target gene *Hey2*, Forkhead transcription factors, *Atf3* gene, Smad genes, and EMT inducers such as *Snail1*, *Snai2*, *Twist1* were on the list. Also, in our previous studies, a common change was observed in the expressions of some of the genes that appeared in the common gene list created by ChIP analysis by the depletion *Grhl3* and *Ovol2* during MET. One of these factors was *Zeb1*, a very well-known EMT core regulator. A significant increase in *Zeb1* level was detected when *Grhl3* and *Ovol2* were silenced during MET in our cellular model NMuMG (Figure 31).

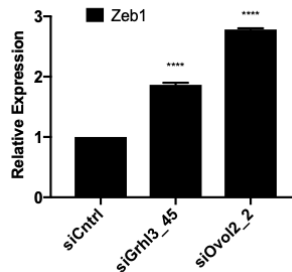


Figure 31. Expression levels of Zeb1 by the depletion of Ovol2 and Grhl3. NMuMG cells recovering from TGF β 3 treatment were transfected with either control or Grhl3 or Ovol2 targeting siRNA. After 72h, cells were collected, RNA was isolated, and changes in the gene expression of Zeb1 measured by qPCR. Results represent the averages of at least three independent experiments in triplicates.

To investigate whether *Grhl3*-*Ovol2* factors act together in the regulation of *Zeb1*, occupancy of Grhl3, and Ovol2 transcription factors at the *Zeb1* gene were examined by IGV software as well. The presence of two peaks sitting in the same region explains that these two factors are highly likely to act together in the regulation of *Zeb1* (Figure 32A). The possible association of *Grhl3* and *Ovol2* on other EMT inducers such as *Twist1* and *Snai2* are also shown in Figure 32B and Figure 32C.

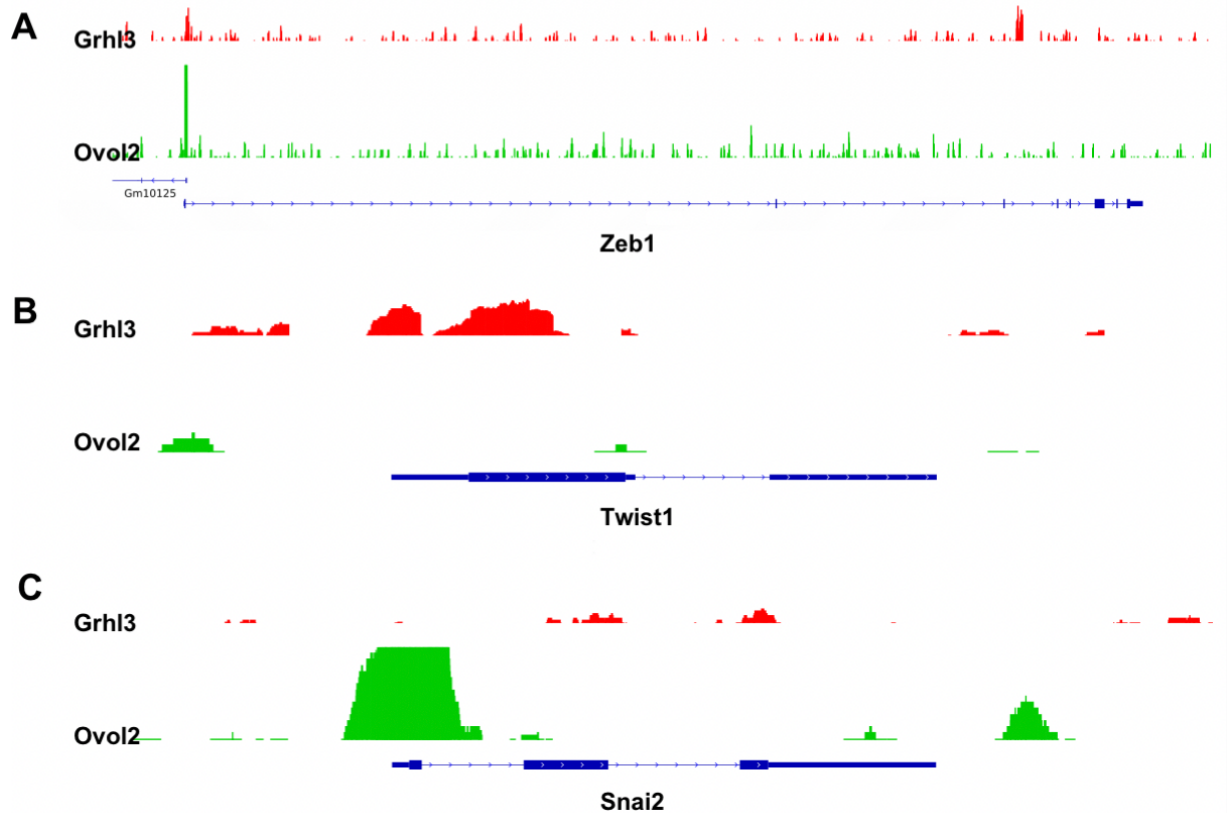


Figure 32. iGV visualization of Grhl3 and Ovol2 occupancies at Zeb1 (A), Twist 1 (B), and Snai1 gene regions (C).

NMuMG cells undergoing MET were also transfected with siRNAs targeting *Grhl3*, and gene expression levels of EMT relevant and mesenchymal genes were evaluated by qPCR analysis. There was a significant downregulation of the expression levels of *Ahr*, *Nfya*, *Cebpa*, *Ehf* (Figure 33A). Together with the data obtained by the in-silico analysis of Grhl3-Ovol2 ChIP-Seq, our results highlight our hypothesis's depth.

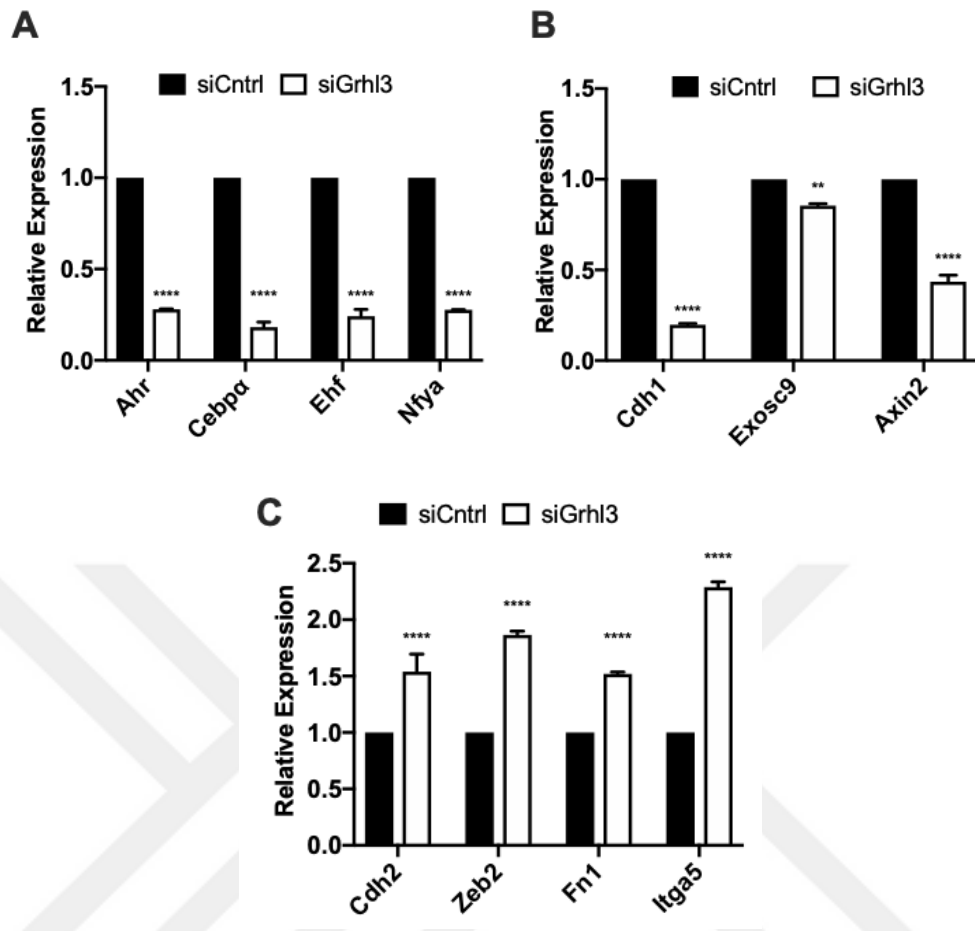


Figure 33. Loss of Grhl3 preserves the mesenchymal state. NMuMG cells recovering from TGF β 3 treatment were transfected with either control or Grhl3 targeting siRNA. After 72h, cells were collected, RNA was isolated, and changes in gene expression were measured by qPCR for EMT/MET relevant genes (A, B) and mesenchymal markers (C, D). Results represent the averages of at least three independent experiments in triplicates.

Following all these analyzes, the transcription factor list obtained by microarray analysis during the EMT-MET process in NMuMG cells was compared with the common transcription factor list obtained from Grhl3 and Ovol2 ChIP bioinformatics analysis. All of the transcription factors obtained by microarray analysis were found in the *Grhl3-Ovol2* common gene list. This proves the consistency of our experimental data and bioinformatics analysis.

5. DISCUSSION

The adhesion molecule E-cadherin expression is indispensable for the epithelial state, and its regulation is tightly and dynamically controlled during several developmental and physiological processes. EMT and MET programs are characterized by the switching of cadherin gene expression. Positive regulation of *Cdh1* expression is a limiting factor for an

efficient MET, suggesting that regulators of *Cdh1* are, in fact, regulators of MET and the epithelial state. Here, by using in silico methods comparing several datasets with distinct epithelial and mesenchymal phenotypes and time-course microarray analysis of MET in NMuMG cells, we defined an epithelial-specific set of transcription factors based on their correlation with *Cdh1* expression. We identified several transcription factors with a high correlation with the epithelial phenotype, such as *Elf3*, *Grhl3*, and *Ovol2*. *Elf3*, a member of the Ets family of transcription factors, appeared to be essential for proper MET progression. Its depletion caused a severe MET defect marked by the absence of E-cadherin from the plasma membrane and the downregulation of several epithelial genes, in particular *Grhl3*. Closer examination of the relationship between *Elf3* and *Grhl3* led us to identify ELF3 as a regulator of *Grhl3* expression (Sengez, Aygun et al. 2019). ELF3 was found to be present at the promoter of *Grhl3* in cells undergoing MET. Besides the critical roles of ELF3 in epithelial cell differentiation, gut development, inflammation, and apoptosis, it is also essential for the pathophysiology of cancer epithelial cells such as in breast, lung, and prostate cancers (Tymms, Ng et al. 1997, Longoni, Sarti et al. 2013, Gajulapalli, Samanthapudi et al. 2016). ELF3 was recently identified as a candidate transcriptional regulator involved in human urothelial cytodifferentiation together with GRHL3; its depletion in urothelial cells result in a declined expression of some transcription factors such as FOXA1 and GRHL3, which are known to be involved in urothelial differentiation (Bock, Hinley et al. 2014). It was reported that IRF6 and GRHL3 function downstream of RIPK4 to promote *ELF3* gene expression in keratinocytes (Scholz, Sulaiman et al. 2016). A recent publication also revealed that ZEB1 and ELF3 transcriptionally regulate *IRF6* (Li, Cheng et al. 2019). The role of *Elf3* was also indicated in animal models. *Elf3* knockout results in embryonic lethality in ~30% of mice models at E11.5. Born *Elf3*^{-/-} mice develop a wasted phenotype characterized by watery diarrhea, lethargy, and malnourishment (Ng, Waring et al. 2002).

The studies in literature accentuated the importance of ELF3 in epithelial cells; this is in agreement with the findings presented here; *Elf3* tops the highly correlated transcription factors with the epithelial phenotype. The fact that *Grhl3* was also among the first factors correlated with the epithelial phenotype was not surprising; it holds a critical role in MET (Alotaibi, Basilicata et al. 2015). The close association between *Elf3* and *Grhl3* was reported in earlier studies (Bock, Hinley et al. 2014), and hence, we deduced their regulatory relationship. The transcriptional relationship between *Elf3* and *Grhl3* was not limited to NMuMG cells since

results obtained from MEFs and keratinocytes provided compelling proof (Sengez, Aygun et al. 2019). Similar to the loss of *Grhl3*, loss of *Elf3* during MET failed the epithelial transition. However, unlike *Grhl3*, lack of which resulted in the downregulation of *Cdh1*, *Elf3* depletion did not cause a reduction in *Cdh1* mRNA or its protein levels. Instead, E-cadherin was absent from the plasma membrane, which is a significant obstacle preventing MET progression favoring a mesenchymal phenotype. The implications of a deregulated E-cadherin expression, as well as its absence from the plasma membrane, have been described before. Analysis of tumor samples suggested that E-cadherin's aberrant expression (or no expression) significantly correlates with poor prognosis and promoted metastasis (Onder, Gupta et al. 2008, Elston, Gill et al. 2009, Sayan, Griffiths et al. 2009). However, the association of *Elf3* with an aberrant E-cadherin expression is a new finding but with comparable observations from *Drosophila*. The *Drosophila* *aop* (anterior open, also named Yan) and *Eip74EF* (Ecdysone-induced protein 74EF) are two genes found in the FlyBase (Thurmond, Goodman et al. 2019) as orthologs for several mammalian Ets transcription factors, such as *Elf3*, *Ehf*, and *Elf5*. In particular, *aop* was reported to control the border cell migration in the *Drosophila* egg chamber by altering *shg* (DE-Cad) expression and its localization (Schober, Rebay et al. 2005). Therefore, a dramatic level of evolutionary conservation of the *Elf3* function cannot be ruled out.

In *Drosophila*, grainy head (*grh*) drives postembryonic neuroblast function and proliferation by promoting DE-cadherin expression (Bray and Kafatos 1991, Almeida and Bray 2005). This transcriptional relationship between *grh* and *shg* is conserved; the mouse orthologs of *grh*, *GRHL2*, and *GRHL3* have been reported to regulate the transcription of *Cdh1* in mouse cells (Werth, Walentin et al. 2010, Alotaibi, Basilicata et al. 2015).

Although the ETS transcription factors have a novel role in various diseases, the function of these transcription factors, particularly in cancer, remains largely unclear. *ELF3* depletion during MET was not limited to the aberrant expression of *Cdh1*; our data confirmed the role of *ELF3* as a regulator of epithelial identity, reflected by the dramatic changes in the expression of selected EMT and MET relevant genes such as *Ahr* and *Ehf*.

Ahr (Aryl hydrocarbon receptor) is a transcription factor activated by a ligand and acts as an environmental pollutant sensor (Rothhammer and Quintana 2019, Vogel, Van Winkle et al. 2020). It generates biological responses to the toxic effects of environmental contaminants. It functions as a sensor for endogenous and xenobiotic signals (Rothhammer and Quintana 2019).

Although *Ahr* knockout mouse models survive, they show many physical abnormalities such as liver shrinkage, suppressed immune system, and reproductive problems (Moreno-Marin, Barrasa et al. 2017, Shinde and McGaha 2018). *Ahr* shuttles between the cytoplasm and the nucleus to dimerize the AhR nuclear transporter (ARNT) and regulate the expression of target genes by binding to xenobiotic response elements in enhancer regions (Ikuta, Eguchi et al. 1998, Kuramoto, Baba et al. 2003). It is known that they can interact with many coactivators such as p300, CBP, RIP140 (Kollara and Brown 2009). It has also been determined that they play a crucial role in biological processes related to cell cycle and immune responses (Yin, Sheng et al. 2016, Xue, Fu et al. 2018). It is thought that they participate in controlling the cell cycle by directly binding to p300 (Tohkin, Fukuhara et al. 2000). It is also known that the expression of *Ahr* is suppressed by epigenetic mechanisms such as DNA hypermethylation (Winans, Nagari et al. 2015). Recent publications revealed that *Ahr* expression is strongly associated with EMT (Rico-Leo, Alvarez-Barrientos et al. 2013, Duan, Li et al. 2018). *Ahr* promotes EMT and metastasis via its ligands l-kynurenine (l-Kyn) and d-kynurenine (d-Kyn) (Duan, Li et al. 2018, Xue, Fu et al. 2018). *Ahr* is also capable of modulating the expression of EMT inducers, including *Snail* and *Slug* (Ikuta and Kawajiri 2006, Rico-Leo, Alvarez-Barrientos et al. 2013). Our transcriptional node analyses indicated the possible transcriptional relationship between *Ahr* and other transcription factors within the epithelial-specific gene set obtained through our analyses. *Ahr* seems to interact with more than half of the factors in the list.

NFY (Nuclear factor Y) is one of the most common transcription factors in mammals and recognizes the CCAAT sequence (Gurtner, Manni et al. 2003, Xu, Fu et al. 2012). It is known as one of the leading factors that assist transcription factors by facilitating accessibility to the chromatin (Oldfield, Yang et al. 2014, Lu, Liu et al. 2016). Therefore, it was defined as a promoter activator. NFY can act as both activator and repressor depending on the cofactor it interacts with (Peng and Jahroudi 2002, Zhu, Wang et al. 2012). Activation occurs by binding to histone acetylase, such as p300 or helping the TFIID bind to the main promoter (Frontini, Imbriano et al. 2002). It was found that NFY suppresses the expression of some genes by directly recruiting HDACs (Peng and Jahroudi 2002). The NFY protein is trimeric and has three subunits: NFYA, NFYB, and NFYC (Romier, Cocchiarella et al. 2003). Of these subunits, only NFYA contains the DNA binding site and contributes to the regulation of several genes, such as stress-induced ones. Early embryonic lethality is observed in *Nfya* knockout mouse models (Yamanaka, Tosaki et al. 2014), so NFYA is thought to play an important role in the early

developmental process (Lu, Liu et al. 2016). In mammals, a physical binding has been established between p53 and NFYA. In the case of DNA damage, p53 forms a complex with NFYA on the CCAAT containing promoter regions, invoking HDACs and releasing HATs, causing the suppression of cell cycle-related gene promoters (Imbriano, Gurtner et al. 2005). It is known that many different transcription factor families such as p53, p63, p73, Cebpa, and SMAD2 /3 interact with NFYA as well (Dolfini, Gatta et al. 2012). DNA binding of NFY is very important for the formation of post-translational modifications through H3K4me3, H3K79me2, and H3K36me3 (Nardini, Gnesutta et al. 2013). Recent studies indicated that the NFYA gene is needed to maintain hematopoietic stem cells (Bungartz, Land et al. 2012). Of note, the available evidence seems to indicate the value of NFYA on promoting EMT in some cancer cells (Gurtner, Manni et al. 2017, Cao, Zhao et al. 2018, Dolfini, Andrioletti et al. 2019). Increased *NFYA* expression levels are associated with hindering EMT in breast cancer (Dolfini, Andrioletti et al. 2019). Although direct regulation of the factors in the network by *Nfya* has not been determined yet through this thesis, close association of *Nfya* and the factors crucial for chromatin infrastructure and cellular homeostasis strengthen our proposed transcriptional node.

Cellular plasticity is another big puzzle during EMT/MET phenotypic switches. Numerous studies have shown that the expression levels of EMT inducers are linked to cell plasticity and stem cell phenotypes. There is no doubt that the dynamic nature of EMT-MET transitions is highly regulated by other transcription factors which can influence known master EMT regulators. Multilevel regulation of some transcription factors defines a harmonic balance between epithelial plasticity and stability in a context-dependent manner. *OVOL2* was recently revealed as a transcriptional repressor of EMT by downregulating *ZEB1* and *TWIST* in various cancer types such as lung, breast, and colorectal cancers (Roca, Hernandez et al. 2013, Wang, Li et al. 2017, Qi, Han et al. 2018). In a recent report, *OVOL2* was identified as a MET inducing transcription factor in fibroblasts, which also enhanced the reprogramming of dermal fibroblasts into keratinocyte-like cell state (Watanabe, Liu et al. 2019). In this thesis study, *OVOL2* was identified following a comprehensive screening approach for transcription factors correlated with *CDH1* in many expression datasets. In support of our findings, it was interesting to find that besides *Elf3* and *Ovol2*, similar transcription factors were also common between the two studies and present among the top correlated genes, such as *Grhl3*, *Irf6*, *Grhl2*, *Grhl1*, and *Ehf*, suggesting a conserved function of these transcription factors in EMT and MET regardless of the organism or cell type studied.

Considering the importance of EMT-MET during development and cancer, a better understanding of these fundamental processes will lead to a better comprehension of the common players in physiological and pathological contexts. In a recent study, *Ovol2* was an indispensable guardian of the epithelial differentiation besides its EMT suppressing ability (Watanabe, Villarreal-Ponce et al. 2014). The findings obtained in this thesis study present novel insights into MET regulation and seek to emphasize the remarkable role of some transcription factors such as *Elf3*, *Grhl3*, and *Ovol2* as a transcriptional gatekeeper of epithelial state. It is also not surprising that physiological and pathological EMT share common attributes in terms of transcriptional regulators. In conclusion, we provided compelling evidence for the involvement of ELF3, AHR, NFYA, and OVOL2 in the regulation of MET by altering the localization of E-cadherin. As is implied by these findings, our understanding of MET will broaden our knowledge of the regulatory partners of MET. Further studies are needed to establish the mechanistic relevance of these findings and their relevance to possible functions of the transcription factors within the MET specific network in the progression of metastasis.

6. CONCLUSION AND SUGGESTIONS

Dynamic fluctuations through cellular plasticity and gene expression patterns are utilized by conserved and complex mechanisms such as EMT and MET, highly coordinated cellular programs that lead to changes in the epithelial architecture. While EMT and MET programs are indispensable duo, they are dissimilar in many ways, particularly in the function of the regulatory partners during the course of the processes. The recent transcriptomic analysis supports the idea that MET is not equivalent to EMT and should be considered separately.

Within the scope of the thesis project, epithelial and mesenchymal expression profiles were compared, and an epithelial-specific gene set was revealed by selecting transcription factors that are more expressed in the epithelial state. Experimental validation of the factors selected by in-silico analyses confirmed the critical importance of the factors investigated during MET. The partnership of *Ovol2*, which is known to have great importance in terms of EMT, with *Grhl3*, which forms the core network, was determined within the thesis studies' scope, and its relationship with other factors was shown. It was also determined that *Elf3* selected by the bioinformatics analysis of the publicly available datasets and our time-course microarray experiment in NmuMG cells could regulate *Grhl3*. Besides, interactions of *Ahr* and *Nfya* transcription factors with each other and especially with the *Grhl3-Ovol2* pair, reflect the

network dynamics. Although direct regulation of the factors in the network by *Ahr* and *Nfya* has not been determined yet through this thesis, close association of *Ahr* and *Nfya* and the factors crucial for chromatin infrastructure, cellular homeostasis, and cell reprogramming programs strengthens the power of our proposed transcriptional nodes. All in one, our data prove the existence of a core epithelial transcriptional network. The close relations of the identified factors with each other form the basis of the complex and dynamic network infrastructure. The intensive interaction between transcriptional nodes in the network identified and analyzed within the scope of the thesis project is shown in Figure 34. Of note, further studies are needed to establish the functional association of these factors within the MET-specific network to better understanding the mechanisms underlying MET regulation.

In conclusion, the data obtained during this thesis study provides in-depth insights required to better understand the MET program regulation. The consistency of experimental data obtained by our EMT-MET cell model NMuMG and in-silico analysis of the publicly available datasets regardless of cell type emphasize the power and the accuracy of our vision about the regulation of MET as well. Although there have been ongoing debates about the contribution of MET to the biological processes, we believe that the core MET transcriptional network should be studied in more detail to fill in the big puzzle of MET, pluripotency, and reprogramming networks that shape the chromatin infrastructure and maintain cellular homeostasis.

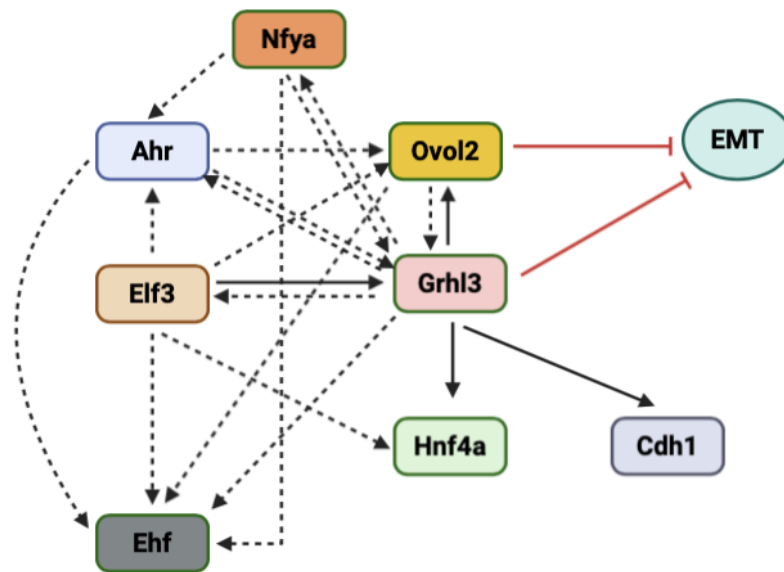


Figure 34. Representation of the proposed MET associated transcriptional network.
(Created by Biorender.com)

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8. APPENDIX

8.1 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 (Biorad)
Bring volume to 50 ml with sterile ddH₂O.

8.2 Curriculum Vitae





BURCU ŞENGEZ

Kişisel Bilgiler

İletişim Bilgileri

İletişim Adresi	Izmir Biyotıp Ve Genom Merkezi Dokuz Eylül Üniversitesi Sağlık Kampüsü
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E-posta	b_sengez@hotmail.com burcu.sengez@ibg.edu.tr
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Öğrenim Bilgileri

01 Mart 2017 - Şu Anda (3 yıl 8 ay)
Doktora, Doktora, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE
İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ, GENOM BİLİMLERİ VE
MOLEKÜLER BİYOTEKNOLOJİ ANABİLİM DALI
Ağırlıklı Genel Not Ortalaması: 3.94 / 4.0

06 Haziran 2006 - 01 Temmuz 2010 (4 yıl 1 ay)
Master Degree, Thesis Program, İZMİR YÜKSEK TEKNOLOJİ ENSTİTÜSÜ, TURKEY
MÜHENDİSLİK VE FEN BİLİMLERİ ENSTİTÜSÜ, MOLEKÜLER BİYOLJİ VE GENETİK
(YL) (TEZLİ)
Tez Başlığı: Investigation Of The Effects Of Hiv-1 Regulatory Genes On African
Green Monkey Cell Lines
Tez Konusu: It Was Aimed To Investigate The Effects Of Hiv-1 Tat And Rev
Proteins On The Gene Expression Of African Green Monkey (agm) Cells Which Are
Known To Be Resistant To Hiv-1 Infection, In An Attempt To Identify Possible Hiv
Restriction Factors Encoded By Endogenous Retroviral Sequences.
Tarih: 2010
Tez Danışmanı: ALPER ARSLANOĞLU

05 Haziran 2001 - 05 Haziran 2005 (4 yıl 1 ay)
Bachelor / Licence Degree, Major/Normal Degree, EGE ÜNİVERSİTESİ, TURKEY
FEN FAKÜLTESİ, BİYOLJİ BÖLÜMÜ
Ağırlıklı Genel Not Ortalaması: 76.0 / 100.0

Deneyim / İşyeri Bilgileri

01 Haziran 2018 - Şu Anda (2 yıl 5 ay) (Yarı Zamanlı)
ARAŞTIRMA GÖREVLİSİ, ARAŞTIRMA GÖREVLİSİ, İZMİR BİYOTIP VE GENOM
MERKEZİ
İdari Görev: DİĞER

Yabancı Dil Bilgileri

İNGİLİZCE (Okuma: İyi, Yazma: İyi, Konuşma: İyi)

Bilimsel Teknolojik Faaliyet Alanları

Bilimsel Teknolojik Faaliyet Alanı Bilgileri

Temel Bilimler -- Yaşam Bilimleri -- Moleküler Biyoloji ve Genetik -- Kanser
Moleküler Biyolojisi

Anahtar Kelimeler

Epitelyal-Mezenkimal Geçiş
Transkripsiyon Faktörleri
Transkripsiyonel Ağlar
Gen Düzenleyici Ağlar

Ar-Ge Yetkinlik

Makaleler

S. ÖZDEMİR, B. ŞENGEZ & A. ARSLANOĞLU, Human immunodeficiency virus type-1 Tat protein induces secretory leukocyte protease inhibitor expression in African green monkey but not human cells, VIRUS GENES, 2020, 0920-8569, 56, 2, 182-193.

B. ŞENGEZ, İ. AYGUN, H. SHEHWANA, N. TOYRAN, S. T. AVCI, O. KONU, M. P. STEMMER & H. ALOTAİBİ, The Transcription Factor Elf3 Is Essential for a Successful Mesenchymal to Epithelial Transition, CELLS, 2019, 2073-4409, 8, 8.

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B. ŞENGEZ & H. ALOTAİBİ, Elf3 is Essential for a Successful Mesenchymal to Epithelial Transition (MET), Davetli Konuşmacı, 14 th Annual EORTC Pathobiology Group Meeting, 12 Eylül 2019, 14 Eylül 2019.

B. ŞENGEZ, Epigenetic Regulation Of Mesenchymal To Epithelial Transition, Davetli Konuşmacı, 7. Multidisipliner Kanser Araştırma&1. Temel Onkoloji Kongresi, 11 Ekim 2018, 14 Ekim 2018, 52 - 52.

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Ödüller

Üniversite, Kurum veya Kuruluşların Verdiği Ödüller, Ulusal, Onkolojide iz bırakanlar zirvesi- Sözel bildiri ödülü, Mezenkimal epitelyal dönüşüm (MET) sürecinde epigenetik yeniden programlamayı etkileyen transkripsiyon faktörlerinin belirlenmesi, Ödül Alınan Kurum: Onkolojide iz bırakanlar zirvesi (Sistemde kayıtlı)

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Awards Honoured By Universities,Institutions or Organizations, National , Hiv-1 Düzenleyici Genlerini Kalıcı Olarak Üreten Afrika Yeşil Maymunu Hücrelerindeki Olası Hiv-1 Kısıtlayıcı Faktörlerin Araştırılması, En İyi 3. Sözlü Sunum Ödülü, Ödül Alınan Kurum: KLİMİK-Türk Klinik Mikrobiyoloji ve Enfeksiyon Hastalıkları Derneği (Non-included institution in the system), TURKEY, 27 Mart 2011.

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Proje Bilgileri

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Panelistlik/İzleyicilik/Raportörlük Sayısı

Panelistlik/Dış Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0
İzleyicilik/Danışmanlık Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0
Raportörlük Sayısı	ARDEB/BİDEB 0	TEYDEB 0	Toplam 0

8.3 Article



Article

The Transcription Factor *Elf3* Is Essential for a Successful Mesenchymal to Epithelial Transition

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Abstract: The epithelial to mesenchymal transition (EMT) and the mesenchymal to epithelial transition (MET) are two critical biological processes that are involved in both physiological events such as embryogenesis and development and also pathological events such as tumorigenesis. They present with dramatic changes in cellular morphology and gene expression exhibiting acute changes in E-cadherin expression. Despite the comprehensive understanding of EMT, the regulation of MET is far from being understood. To find novel regulators of MET, we hypothesized that such factors would correlate with *Cdh1* expression. Bioinformatics examination of several expression profiles suggested *Elf3* as a strong candidate. Depletion of *Elf3* at the onset of MET severely impaired the progression to the epithelial state. This MET defect was explained, in part, by the absence of E-cadherin at the plasma membrane. Moreover, during MET, ELF3 interacts with the *Grhl3* promoter and activates its expression. Our findings present novel insights into the regulation of MET and reveal ELF3 as an indispensable guardian of the epithelial state. A better understanding of MET will, eventually, lead to better management of metastatic cancers.

Keywords: mesenchymal to epithelial transition; *Elf3*; *Grhl3*; Cadherins

1. Introduction

The epithelial to mesenchymal transition (EMT) and the mesenchymal to epithelial transition (MET) has been known to scientists for decades. They are crucial for a variety of biological processes such as embryogenesis, development, and wound healing [1–3], and their aberrant regulation has deleterious outcomes [1,4–6]. For example, tumor cells of epithelial origin can benefit from an EMT event and lose their adherent and polarized morphology and become motile and unpolarized [1,7]. These cells can now relocate and colonize sites in distant organs. To accomplish a metastatic status, relocated tumor cells need to undergo a MET event, after which, they would regain the polarized epithelial-like state, proliferate, and form secondary tumors [1,6,8].

Alongside the global changes in gene expression and cytoskeletal rearrangements, switching the expression of the adhesion molecules E-cadherin and N-cadherin is a hallmark of EMT and MET events [3,6,8–10]. The downregulation of E-cadherin is crucial for a successful EMT [10,11], which is accomplished by a group of transcription factors including but not limited to, *Snai1*, *Snai2*, *Zeb1*, and *Zeb2* [3,12], collectively dubbed ‘The EMT Inducers’. These transcription factors bind to

several E-boxes in the promoter of *Cdh1* and cause the repression of E-cadherin expression [12–15]. The initiation of MET has been mostly explained as a result of the reduction of the levels of the EMT inducers [15,16]; however, MET is not merely the reverse process of EMT and is a fundamental program as well [2,17]. It involves a series of dynamic and tightly regulated events by either transcriptional and 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 [18], which are essential steps for proper entry to MET [10]. MET is primarily described as a loss of the expression of transcription factors such as *Snai1/2* and *Zeb1/2* and gaining epithelial-like features via the initiation of *Cdh1* and *Epcam* expression. Previous studies have shown that E-cadherin internalization and trafficking to and from the plasma membrane is crucial for maintaining epithelial dynamics [18,19] and controlling protein stability at the cell surface and cellular levels of the protein [15].

The hallmarks of transitions between epithelial and mesenchymal states include changes in the cadherin repertoire [20–22]. The *Cdh1* promoter alone, including all known E-box elements, is insufficient to confer a strict cell-type specificity [23]. In contrast, intron 2 carries sufficient information for proper E-cadherin expression [24]. Although the second intron has emerged as an essential regulator of *Cdh1* expression [23–25], very little is known about the molecular determinants controlling the transcriptional activity of the locus. Besides *Klf4* [26,27], Grainy head-like 2 (*Grhl2*), a mammalian homolog of *Drosophila* grainy head, was shown to control epithelial differentiation in several tissues [28–30] and during uretic bud formation by regulating E-cadherin via binding to an element in intron 2 [31].

Moreover, the GRHL3 transcription factor plays a fundamental role during the initiation of MET [25]. It activates the expression of *Hnf4a*, and together they cooperate to activate the expression of *Cdh1* through binding to enhancers in the second intron. Despite the abundance of publications on the regulation of *Cdh1* expression, there still exists limited knowledge on the transcription factors involved in the regulation of *Cdh1* expression. It is then plausible to assume that regulators of *Cdh1* transcription are de facto regulators of MET; thus, a better understanding of how *Cdh1* is transcriptionally regulated would shed light on the regulation of MET.

To gain insights into this, we searched for novel regulators of MET and hypothesized that regulators of MET could be regulators of *Cdh1* transcription or at least have a strong correlation with its expression. Using an in silico approach, we first generated an epithelial-specific set of transcription factors with high correlation to *Cdh1* expression. Moreover, we used the normal murine mammary gland epithelial cells (NMuMG) that undergo a reversible EMT by transforming growth factor β (TGF β) treatment and identified *Elf3* as a compelling regulator of MET and E-cadherin localization to the plasma membrane. Moreover, we provide evidence showing that ELF3 is a regulator of the transcription factor *Grhl3*.

2. Materials and Methods

2.1. Microarray Data Analysis and Correlation Study

Raw data (CEL files) of four gene expression datasets based on the GPL6246 platform (GSE130548, GSE77551, GSE55711, and GSE55072) were downloaded from the Gene Expression Omnibus database. Samples with distinct epithelial and mesenchymal states were selected for analysis. Datasets and samples included in the analysis are shown in Table S1. Data were RMA normalized using Bioconductor package *oligo* [32]. Normalized data were merged and transformed into z-scores using *scale* function in R [33]. The z-score transformation was performed both on the sample and on gene level [34]. Merged z-scores profile was used to evaluate spearman correlation between *Cdh1* and all other probe sets present in the expression profile. Differential expression analysis between epithelial and mesenchymal samples was performed using the Limma package [35] to retrieve log fold changes and p-values. Gene to probe set annotation was performed using the *annotate* package [33] and *mogene10sttranscriptcluster.db* annotation library [36]. List of mouse transcription factors was retrieved from Animal TFDB3.0 [37].

Cdh1 correlation and differential expression profile of transcription factors were extracted from the performed analysis. The Ggplot2 package was used to draw the plots [38].

2.2. Cell Culture and Induction of EMT and MET

NMuMG (ATCC® CRL-1636) cells were cultured in DMEM (11965092, Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, 10500064, Gibco), penicillin-streptomycin (10,000 U/mL, 15140122, Gibco), 1× MEM NEAA (1140035, Gibco), and 10 µg/mL insulin (19278-5 mL, Sigma, Munich, Germany). NMuMG cells were grown at 37 °C in a humid environment with 10% CO₂. MEFs were isolated from C57BL/6J E13.5 embryos and cultured in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin (10,000 U/mL), and 1× MEM NEAA. Cells were grown at 37 °C in a humid environment with 5% CO₂. Keratinocytes were isolated from the back skin of P0 newborn mice as described previously [39], and cultured in Epidermal Keratinocyte Medium (CnT-07, CellnTec, Bern, Switzerland) containing penicillin-streptomycin (10,000 U/mL) and supplements A, B, and C (CellnTec, Bern, Switzerland). Cells were grown at 37 °C in a humid environment with 10% CO₂. Manipulations using animals were approved by the Animal Care and Use Committee of Izmir Biomedicine and Genome Center (iBG, Izmir, Turkey). For EMT induction, cells were maintained in complete medium supplemented with five ng/mL TGFβ3 (100-36E, PeproTech, London, UK) for 72 h (labeled as TGFβ3). The induction of MET was initiated by TGFβ3 withdrawal and washing the plates twice with PBS and incubation for an additional 72 h in fresh medium (labeled PT as in Post-treatment).

2.3. siRNA Knockdown and mRNA Expression Analysis

siRNAs against *Elf3* were purchased from Qiagen for siElf3_2 and siElf3_1-4, and from Dharmacon for siElf3_10 and siElf3_12; sequences are available in Table S2. Cells were transfected with 50 nM siRNA using the Lipofectamine RNAi Max (13778150, Invitrogen, Waltham, MA USA) and collected 72 h later. Total RNA was isolated using the Nucleospin RNA II kit (740955.50, Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. Total RNA (1 µg) was converted to cDNA using the Maxima First Strand cDNA Synthesis Kit (K1642, Thermo Scientific, Waltham, MA USA). cDNA was then diluted 1:20 and used for qPCR with the TaqMan™ Universal Master Mix II (4440040, Applied Biosystems, Waltham, MA USA) and Universal Probe Library probes (Roche, Basel, Switzerland). All qPCR data were normalized to the *Gapdh* gene. Primers and probes used are available in Table S3. Quantification was performed using the $\Delta\Delta C_t$ method.

2.4. Luciferase Reporter Assays

Cells in 48-well plates were transfected with plasmid DNAs using Fugene HD transfection reagent (E2311, Promega, Madison, WI, USA). Transfection was carried out with 100 ng DNA containing five ng pRL-TK (Promega) to normalize for transfection efficiency. We normalized to equal molarity of the plasmid to use equivalent copies of the reporter plasmid. The total DNA content was complemented with promoter-less plasmid DNA. 20 ng of *Elf3* expression plasmid or empty vector (Mock) were transfected. Luciferase reporter activity was measured using Dual-Glo Luciferase Assay (E2940, Promega, Madison, WI, USA) in a Centro XS³ LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany). Firefly luciferase reporter values were normalized to those of the Renilla luciferase control. Fold induction was calculated relative to the values of the empty vector.

2.5. Expression and Reporter Vectors

The *Elf3* expression vector was prepared by amplifying the coding sequence of *Elf3* by PCR, adding restriction sites for *Bam*HI (5′) and *Eco*RI (3′). The PCR product was inserted into a modified version of pcDNA4/TO/Myc-His A (Invitrogen, Waltham, MA USA) containing a sequence coding for a 3× FLAG epitope between *Hind*III and *Bam*HI sites. The full sequence of the plasmid is available upon request.

The *Grhl3* promoter (1 kb; between −1 and −1000 bp relative to the transcription start site) was amplified by PCR in 25 µL reaction volume containing 1× PCR buffer, 1.5 mM MgCl₂, 200 µM dNTP, 10 pmol of each primer (Table S4), 5% DMSO, 1 U of *Taq* DNA polymerase (2900242, 5 PRIME), and 50 ng of C57BL/6J mouse genomic DNA. Thermal cycler conditions were an initial denaturation step at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 sec, followed by a final extension at 72 °C for 10 min. The amplified PCR products were subsequently inserted upstream of the luciferase reporter in pGL4.10 (Promega, Madison, WI, USA) using *Xho*I and *Hind*III restriction sites to produce the reporter plasmid named pGrhl3. The shorter versions of pGrhl3 were prepared by removing sequences flanked by *Xho*I/*Bsa*I for pGrhl3-XB, *Xho*I/*Psp*1406I for pGrhl3-XP and *Psp*1406I/*Hind*III for pGrhl3-PH.

2.6. Chromatin Immunoprecipitation (ChIP)

ChIP was performed as reported previously [40] with the following minor modifications. NMuMG cells were grown and treated in 10 cm plates. Following 10 min cross-linking and lysis, cell lysates were sonicated in the S220 focused-ultrasonicator (Covaris, Woburn, MA, USA) for 30 min (60 s on/60 s off; Peak Power: 140 W; Duty factor: 15; Cycles/burst: 200; water temperature: 4 °C). Immunoprecipitation was performed at 4 °C for 16 h using an antibody against ELF3/ESE-1 (NBP1-30873, Novus Biologicals, Centennial, CO, USA) and a rabbit control IgG (2729S, Cell Signaling, Danvers, MA, USA). Quantitative real-time PCR was performed using the TaqMan Universal master mix II and Universal Probe Library probes (Roche, Basel, Switzerland) and amplification was carried out in the ABI PRISM 7500 Fast qPCR System (Thermo Scientific, Waltham, MA USA) with primers and probes depicted in Table S5 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 percentage of input.

2.7. Immunofluorescence Labeling and Confocal Microscopy

Cells were washed with PBS and fixed with 4% formaldehyde (F8775, Sigma) for 10 min at room temperature, washed twice with PBS, and permeabilized with 0.25% Triton X-100 (T8787, Sigma) for 5 min. After washing twice with PBS, cells were incubated with primary antibodies in PBS with 5% BSA (1:200) for one hour at room temperature. Afterward, Alexa594-conjugated secondary antibody was applied for an additional one hour after washing in PBS. Nuclei were visualized with DAPI (1:1000, Invitrogen) and mounted with Prolong Diamond Antifade Mountant solution (P36962, Thermo Fisher Scientific, Waltham, MA USA). Confocal microscopy was carried out with an LSM 880 microscope equipped with ZEN software (Zeiss, Oberkochen, Germany). The antibodies used were anti-E-cadherin (610181, BD Bioscience, San Jose, CA, USA), Vimentin (11-254-C100, Exbio, Vestec, Czech Republic), Alexa Fluor™ 488 Phalloidin (A12379, Invitrogen), DAPI (Invitrogen), and Alexa Fluor 594 goat anti-mouse IgG (A-11005, Invitrogen, Waltham, MA USA).

2.8. SA-β-GAL Assay

NMuMG cells recovering from TGFβ3 were transfected with siCntrl or siElf3_10 as described before. Seventy-two hours later, the cells were washed with PBS, and SA-β-Gal activity was measured by a Senescence Detection Kit (K320, BioVision, Milpitas, CA, USA) according to the manufacturer's instruction. As a positive control, Huh7 cells were used. Induction of senescence was performed with 100 nM Doxorubicin (Dox; A4361, Applichem, Darmstadt, Germany). Huh7 cells were grown in appropriate media containing Dox for three days and an additional six days after Dox withdrawal. The presence of senescent cells was detected using an Olympus CKX41 microscope equipped with a DP25 camera.

2.9. RT-PCR

Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. Total RNA (1 µg) was converted to cDNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA USA). cDNA was then diluted 1:20 and

2 µL was used for PCR amplification of target genes in a 50 µL reaction containing 0.2 µM of forward and reverse primers (Table S6), 200 µM of dNTPs, 1 × Standard *Taq* (Mg-free) Reaction buffer, 1.5 mM MgCl₂, and 1.25 units of *Taq* DNA polymerase (M0320S, New England Biolabs, Ipswich, MA, USA). PCR conditions were 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; 72 °C for 5 min. Amplified DNA was visualized by electrophoresis on a 2% agarose gel.

2.10. Fluorescence-Activated Cell Sorting (FACS)

TGFβ3 treated cells were prepared as described before. Cells (10 × 10⁶) were harvested, washed in FACS buffer (1 mM EDTA, 25mM HEPES and 1% FBS in PBS), and then stained with E-cadherin antibody (sc-8426, Santa Cruz Biotechnology, Dallas, TX, USA) and goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (A-11001, Thermo Fisher Scientific, Waltham, MA USA) for 30 min at 4 °C. Cells were washed with FACS buffer and stained with DAPI (final concentration: 0.1 µg/µL). Stained cells subsequently collected by BD FACSAria™ III and single E-cadherin-positive and E-cadherin-negative cells were collected in individual wells of a 96-well plate and allowed to grow into colonies.

2.11. Western Blot

NMuMG cells recovering from TGFβ3 were transfected with either siCntrl or siElf3_10 as described before. Seventy-two hours later, the cells were harvested and lysed in RIPA buffer. Protein concentration was determined by TaKaRa BCA Protein Assay Kit (T9300A, Takara, Kusatsu, Japan). Protein samples were then diluted with Laemmli Sample buffer. After electrophoresis, proteins were transferred to a nitrocellulose membrane, blocked for 1 h in blotto (Tris-buffered saline containing 0.5% Tween 20 and 5% nonfat milk powder), and incubated with primary antibodies at 1:1000 dilution (Anti-E-cad: 610181, BD Biosciences and anti-ELF3: NBP1-30873, Novus Biologicals) overnight at 4 °C with shaking. Anti-GAPDH (sc-47724, Santa Cruz Biotechnology, Dallas, TX, USA) was used as a loading control. Proteins were detected with IRDye® 800CW Goat anti-Mouse IgG and IRDye® 680RD Goat anti-Mouse IgG (H + L) antibodies (LI-COR Biosciences, Lincoln, NE, USA) visualized on an Odyssey® CLx Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

2.12. Wound Healing Assay

For wound healing assay experiments, NMuMG cells recovering from TGFβ3 were transfected with either siCntrl or siElf3_10 as described before. Six hours later, cells were scratched with a sterile pipette tip and cultured for an additional 48 h. Microscopic evaluations in 24-h intervals were carried out using an Olympus CKX41 microscope equipped with a DP25 camera. Scratch width was determined by measuring the distance in pixels between the edges of the scratch in ImageJ. Several measurements were taken from each slide, and the average was calculated.

2.13. 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. For Figure S2A One Way ANOVA test was performed. Data are presented as the mean of at least three independent experiments, done in triplicates. Luciferase reporter assays and ChIP experiments were carried out at least three times, qPCR was performed in triplicates. Error bars represent standard error of the mean.

3. Results

3.1. The Transcription Factors *Elf3* and *Grhl3* Are Highly Correlated with the Epithelial State

Previously, we have reported that the transcription factor *Grhl3* is essential for the progression of MET and we showed that it is a crucial regulator of the cell adhesion molecule E-cadherin as well as the

transcription factor *Hnf4a* [25]. In this study, we wanted to reveal other transcription factors essential for the progression of MET. We reasoned that the expression of such transcription factor(s) would correlate with that of *Cdh1*. To this end, we analyzed the expression profiles from several experimental microarray datasets originating from different and unrelated studies but contained distinct epithelial and mesenchymal samples. We had to apply some criteria to be unbiased in our selection, as the NCBI GEO database includes hundreds of examples. One of the data sets used here is an expression profile of primary keratinocytes and E13.5 MEFs previously produced in our lab (GSE130548 prepared with the Affymetrix MoGene-1_0-st chip (GEO platform GPL6246)). Since we wanted to compare epithelial and mesenchymal samples across different studies, using samples coming from the same platform would make the analyses relatively comparable. Second, the selected study should contain both epithelial and mesenchymal samples. The distinction between epithelial and mesenchymal phenotypes within the same study was made based on the expression of *Cdh1*, *Epcam*, *Fn1*, and *Vim* genes. In addition to our dataset (six samples), we were able to identify three datasets (GSE77551, GSE55711, and GSE55072) with 14 samples, ending up with a total of 20 samples.

Correlation analysis of Z-score derived from 4 datasets revealed that the transcription factors *Cas21*, *Zfp750*, *Elf3*, *Sp6*, and *Grhl3* as highly correlated with *Cdh1* gene and highly expressed in the epithelial phenotype (Figure 1A), while transcription factors such as *Zeb1*, *Zeb2*, *Snai1*, and *Twist2* were found overexpressed in the mesenchymal phenotype. Top positively and negatively correlated genes are shown in Figure 1B. Genes highly correlated with *Cdh1* were indeed associated with upregulation in epithelial cell type as indicated by positive fold change, on the other hand, negatively correlated genes elevated in mesenchymal phenotype elicited by negative log fold change.

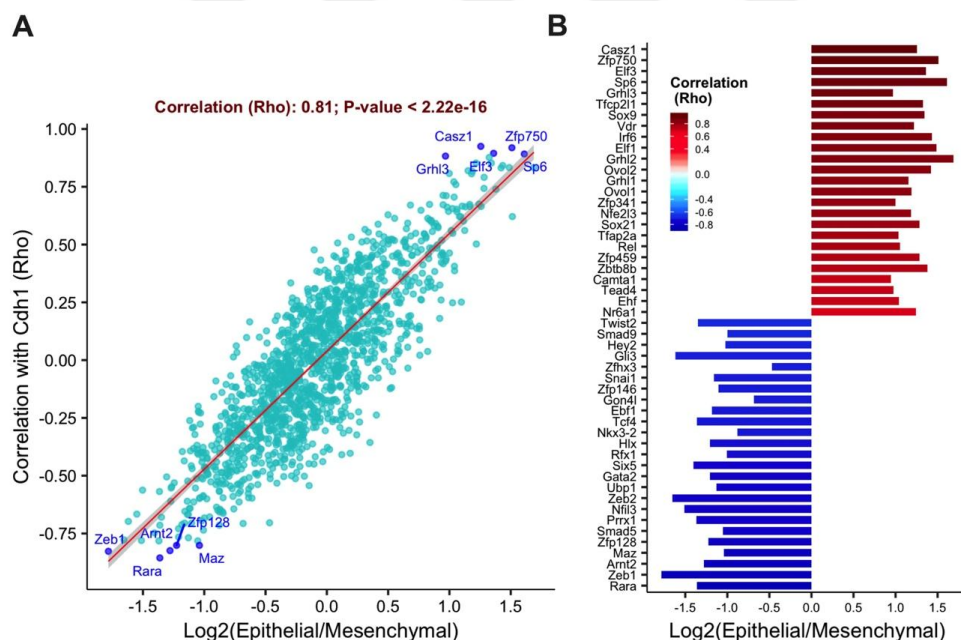


Figure 1. The transcription factors *Elf3* and *Grhl3* are highly correlated with the epithelial state. (A) A scatter plot that shows the correlation of 1469 transcription factors with the epithelial phenotype. Microarray-based screen for epithelial-specific transcription factors coregulated with E-cadherin. One-thousand-four-hundred-and-sixty-nine transcription factors were analyzed for differential expression in epithelial compared to mesenchymal samples, and Spearman correlation was calculated between each transcription factor and E-cadherin across all data sets. The top 5 correlated genes with either the epithelial or the mesenchymal phenotypes are labeled. (B) The ranking plot is displaying the top 25 correlated transcription factors in the epithelial and in the mesenchymal states. The x-axis represents the differential expression of each transcription factor in epithelial versus mesenchymal samples.

3.2. *Elf3* Is Essential for MET

To establish the relevance of the highly correlated transcription factors to MET, we utilized NMuMG cells (an established murine model for the study of EMT and MET). These cells will gain a mesenchymal phenotype once treated with TGF β for three days, and after TGF β withdrawal, the cells will revert to the epithelial phenotype in three days (MET). We first analyzed the expression levels of the top correlated genes in NMuMG cells by RT-PCR (Figure S1A). While *Cas21* and *Zfp750* were barely detectable in NMuMG cells (only detectable when 20 times more cDNA was used), *Elf3* had comparably higher RNA levels, and thus was selected for further analyses. We used siRNA mediated silencing of candidate genes at the onset of MET in NMuMG cells and followed the progression of MET both morphologically as well as by expression levels of epithelial and mesenchymal defining genes.

We first validated MET in NMuMG cells by single-cell sorting of cells treated with TGF β for three days, as well as seeding TGF β treated cells at a very low density (single-cell cloning) to rule out the possibility that remaining epithelial cells within a mesenchymal population could take over due to their faster proliferation rate rather than an actual MET (Figure S1B). As a result, we were able to confirm that single mesenchymal cells were able to form colonies of epithelial cells after the withdrawal of TGF β . Then, we examined the expression pattern of *Elf3* in NMuMG cells during the progression of EMT and MET. We have noticed that unlike the *Cdh1* expression pattern, *Elf3* RNA levels were relatively stable (Figure S1C). We then used several siRNAs targeting *Elf3* and validated their knockdown efficiency in NMuMG cells by measuring *Elf3* RNA levels. As a result, we noticed that all siRNAs used produced a similar phenotype with varying yet substantial knockdown efficiencies (Figure S2A). In cells transfected with nontargeting siRNA, we did not notice any morphological changes; on the other hand, *Elf3* targeting siRNAs caused a failure of MET evident by the enlarged and flattened cell morphology (Figure S2B). Confocal images of si Elf3 transfected cells revealed oversized cells, with actin present as stress fibers, absence of E-cadherin at the plasma membrane, and an increased Vimentin expression (Figure 2). These results confirmed the MET failure and suggested that this aberrant MET was, in part, due to the absence of E-cadherin from the plasma membrane.

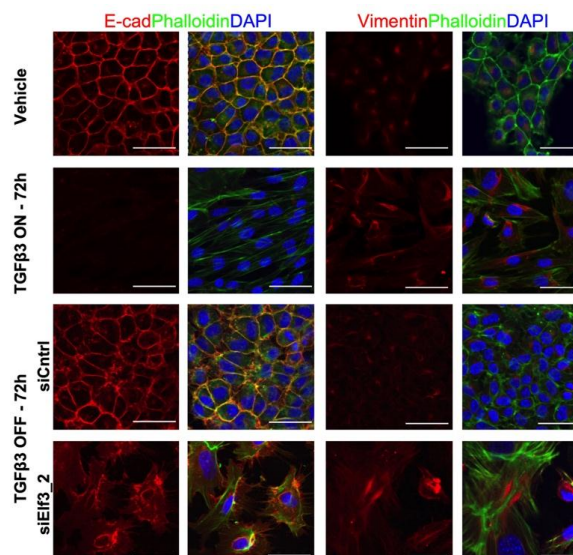


Figure 2. The siRNA-mediated silencing of *Elf3* result in an impaired MET. Confocal images showing the immunofluorescence staining of NMuMG cells visualizing changes in expression and intracellular distribution of E-cadherin, Vimentin, and Actin during TGF β 3 treatment, withdrawal, and also during the silencing of *Elf3*. Actin distribution (Phalloidin, green) and detection of E-cadherin (red) and Vimentin (red) is shown. Nuclei are labeled with DAPI. Scale bar, 50 μ m.

The enlarged cellular morphology was reminiscent of senescent cells, but we could not confirm this using the very well-established SA- β -Gal staining method (Figure S2C), suggesting a different cause for the enlarged cells. We wanted to confirm these observations by studying RNA expression levels of genes relevant to the EMT and MET programs. We selected *Ahr*, *Cebpa*, *Ovol2*, *Exosc9*, and *Ehf* for their potential involvement in the process of maintaining epithelial integrity, regulating the dynamic switch between epithelial and mesenchymal states, and key roles in metastasis [41–45]. NMuMG cells undergoing MET were transfected with different siRNAs targeting *Elf3*, and gene expression levels were evaluated by qPCR analysis. There was a significant downregulation of several transcription factors such as *Ahr*, *Cebpa*, *Ehf*, and *Ovol2* (Figure 3A).

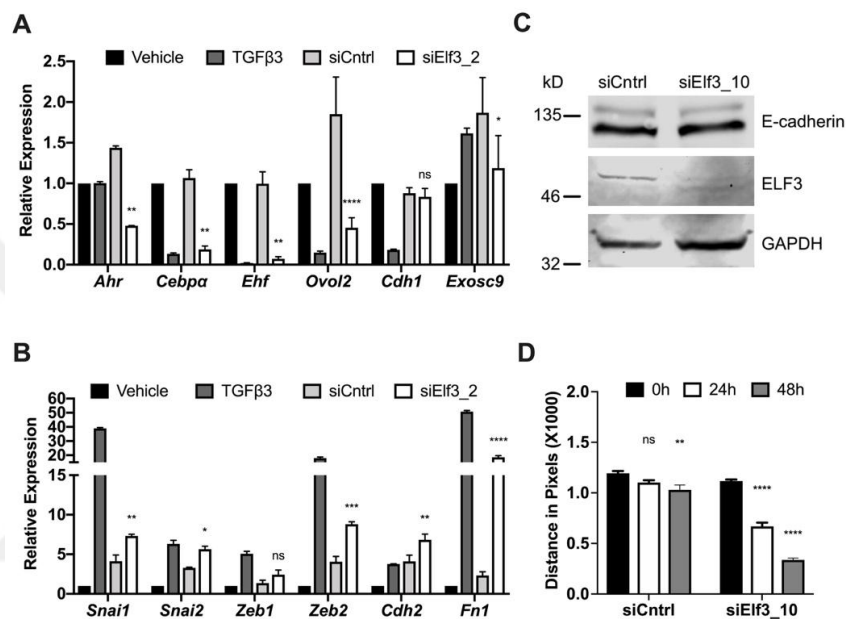


Figure 3. Loss of *Elf3* preserves the mesenchymal state. NMuMG cells recovering from TGFβ3 treatment were transfected with either control or *Elf3* targeting siRNA, after 72 h, cells were collected, RNA was isolated, and changes in gene expression were measured by qPCR for EMT/MET relevant genes (A) and mesenchymal markers (B). (C) Immunoblot showing ELF3 and E-cadherin protein levels in response to *Elf3* silencing by siRNAs. (D) Summary of the wound healing assay results following *Elf3* silencing measured as the distance between the edges of migrating cells. Results represent the averages of at least three independent experiments in triplicates. Paired Student's *t*-test was used to calculate statistical significance. * *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001; **** *p*-value < 0.0001.

On the other hand, the expression levels of some mesenchymal markers showed an evident increase in the absence of *Elf3*; we noticed a significant increase in the classical EMT inducers such as *Snai1/2* and *Zeb2* (Figure 3B), which was accompanied by an increased expression of *Cdh2* and *Fn1* (Figure 3B). In agreement with the data in Figure 2, RNA and protein levels of E-cadherin after *Elf3* depletion were comparable to those of control cells, which can complete the MET transition (Figure 3A,C). These results confirmed once more the MET failure, with a restricted appearance of the cells in a morphology reminiscent of sustained EMT. This mesenchymal state was also confirmed by the increased migratory capacity of *Elf3* depleted cells. We observed a five-fold increased migration as compared to control cells in wound healing assay (Figure 3D and Figure S3A). The observed impact on MET is not a typical feature of the depletion of transcription factors with a strong correlation with the epithelial phenotype. We affirmed this by using siRNAs targeting *Ehf*, another transcription factor from the Ets family, which is also found among the top 25 genes correlated with the epithelial phenotype. We found that the siRNA mediated depletion of *Ehf* did not affect MET progression nor the *Cdh1* RNA levels (Figure S3B,C, respectively).

3.3. *Elf3* Regulates the Expression of *Grhl3*

The results from Figure 3 revealed the effect of *Elf3* silencing on genes previously associated with epithelial integrity or EMT. We also wanted to study the influence of *Elf3* depletion on the genes highly correlated with the epithelial phenotype. For this, we examined the expression of the top 10 correlated genes from Figure 1 after *Elf3* silencing at the onset of MET. We noticed a significant downregulation of *Grhl3* expression and a modest downregulation of *Tfcp2l1* (Figure 4A). We were intrigued by the appearance of *Grhl3* in the top 5 genes correlated with *Cdh1* as well as its downregulation in response to *Elf3* silencing because of its importance in MET regulation [25]. For this reason, we measured z-scores for transcription factors correlated with *Grhl3*, and we observed that it also correlates with genes having the highest correlation with the epithelial phenotype, such as *Cas21* and *Elf3* (Figure S4A). We analyzed the bilateral correlation between *Elf3* and *Grhl3* in the datasets from Figure 1 and found a high correlation of 0.84, p -value $< 2.22 \times 10^{-16}$ (Figure S4B). We studied changes in *Grhl3* RNA levels after modulating *Elf3* expression in other cellular models and found that in primary keratinocytes, for example, *Grhl3* was significantly down-regulated upon *Elf3* depletion by siRNAs (Figure 4B). Also, the overexpression of *ELF3* in primary MEFs was effective in increasing the RNA levels of *Grhl3* (Figure 4C), suggesting a potential transcriptional regulatory relationship between the two genes.

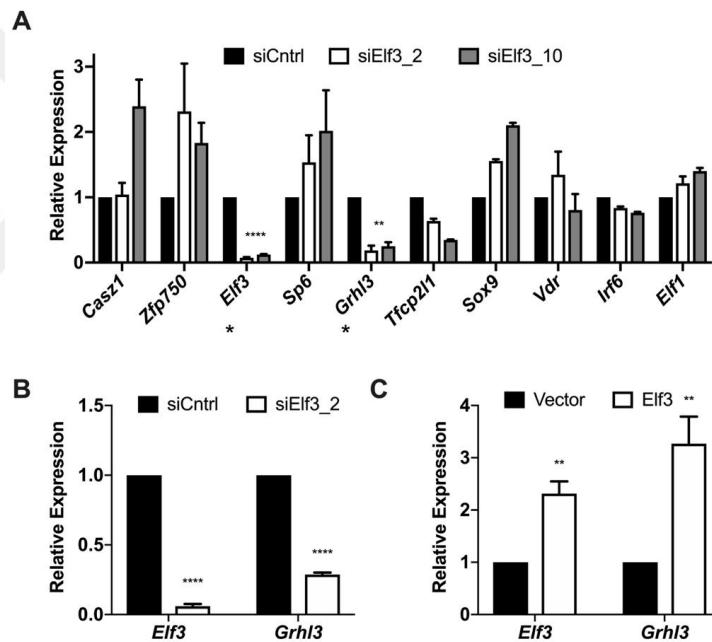


Figure 4. *Grhl3* expression levels change in response to modulating *Elf3* expression in different cellular models. (A) NMuMG cells recovering from TGFβ3 treatment were transfected with either control or *Elf3* targeting siRNAs, after 72 h, cells were collected, RNA was isolated, and changes in the expression of the top 10 genes correlated with the epithelial phenotype were measured by qPCR. The * below gene names indicate genes with efficient downregulation. (B) Primary mouse keratinocytes were transfected with siRNAs targeting *Elf3*. Seventy-two hours after transfection RNA was isolated, and the expression levels of *Elf3* and *Grhl3* were measured by qPCR. (C) MEFs were isolated from E13.5 embryos and transfected with *ELF3* expressing plasmid. Seventy-two hours after transfection cells were collected in RNA lysis buffer, total RNA was isolated and converted to cDNA. qPCR was performed using *Grhl3* specific primers. Relative expression was calculated using the $\Delta\Delta C_t$ method relative to the vector. Data represent the averages of at least three independent experiments. Paired Student's *t*-test was used to calculate statistical significance. ** p -value = 0.005; **** p -value < 0.0001 .

3.4. The Transcription Factor *Elf3* Activates the Promoter of *Grhl3*

To address the potential transcriptional regulatory relationship between ELF3 and *Grhl3*, we analyzed the promoter region of *Grhl3* (1 kb proximal to TSS) for conserved sequences and ELF3 binding sites. The promoter of *Grhl3* was analyzed for conservation using the genome vista tools and aligned to several mammalian species. We were able to identify three conserved sequences from Mouse/Human and Mouse/Chimp alignments, and two conserved sequences from Mouse/Rhesus, Mouse/Cow, and Mouse/Dog alignments (Figure S5A). The sequence of the proximal promoter region is presented in Figure 5A, and some promoter elements are highlighted, the Sp1 site appeared conserved in all comparisons (Figure S5B). We identified a total of 17 ELF3 binding sites in the 1 kb region of *Grhl3* promoter (green nucleotides in Figure 5A). This 1 kb sequence was then cloned in the pGL4.10 vector to measure the reporter activity in response to ELF3 induction (Figure 5B). NMuMG cells were transfected with various extents of the promoter sequence; we observed that the *Grhl3* promoter retained its activity in the absence of CNS2 and 3 but required the presence of CNS1 together with the TATA and Sp1 elements (Figure 5B). Although the functionality of the TATA box per se cannot be deduced from these experiments, the presence of other crucial promoter elements cannot be ruled out.

Moreover, we found that ELF3 expression was sufficient to upregulate the reporter activity. The activation of the *Grhl3* promoter by ELF3 was not confined to in vitro assays, as we were able to detect a significant enrichment of ELF3 at the *Grhl3* promoter in cells undergoing MET (Figure 5C). Of the 17 ELF3 binding sites in the *Grhl3* promoter, six were present in the first conserved sequence (CNS1: 210 bp upstream of TSS), four of which were found conserved among different mammalian species (Figure 5D and Figure S5D). The binding of ELF3 at the *Grhl3* promoter was confined to CNS1 during MET; we were not able to detect similar binding using primers outside of this conserved sequence (Figure S5C).

4. Discussion

The expression of the adhesion molecule E-cadherin is indispensable for the epithelial state, and its regulation is tightly and dynamically controlled during several developmental and physiological processes, during which EMT and MET programs alternate, and is characterized by the switching of cadherin gene expression. Positive regulation of *Cdh1* expression is a limiting factor for an efficient MET, suggesting that regulators of *Cdh1* are in fact regulators of MET and the epithelial state. Here, by using in silico methods and comparing several datasets with distinct epithelial and mesenchymal phenotypes, we defined an epithelial-specific set of transcription factors based on their correlation with *Cdh1* expression. We identified several transcription factors with high correlation with the epithelial phenotype, such as *Elf3*, *Grhl3*, and *Ovol2*.

Elf3, a member of the Ets family of transcription factors, appeared to be essential for proper MET progression, and its depletion caused a severe MET defect marked by the absence of E-cadherin from the plasma membrane and also the downregulation of several epithelial genes in particular *Grhl3*. Closer examination of the relationship between *Elf3* and *Grhl3* led us to identify ELF3 as a regulator of *Grhl3* expression. ELF3 was able to activate the *Grhl3* promoter in reporter assays, and this was further verified as ELF3 was found to be present at the promoter of *Grhl3* in cells undergoing MET.

The Ets family of transcription factors is one of the largest families of transcription factors, consisting of ~30 members (27 in humans and 26 in mice) that can be expressed both ubiquitously and in a tissue-specific manner [46,47]. Ets transcription factors are characterized by conserved winged helix–turn–helix DNA binding domains called ETS domain that binds a typical DNA sequence 5′-GGA(A/T)-3′ [46,48,49]. Members of this family are trans-acting phosphoproteins [50] that can act as upstream and downstream effectors of most signaling pathways, including MAP kinases, Erk1/2, p38, and JNK [51], thus playing crucial roles in a wide variety of biological processes such as differentiation, development, proliferation, apoptosis, tissue remodeling and the epithelial to mesenchymal transition with a suggested involvement in the mesenchymal to epithelial transition [52–56].

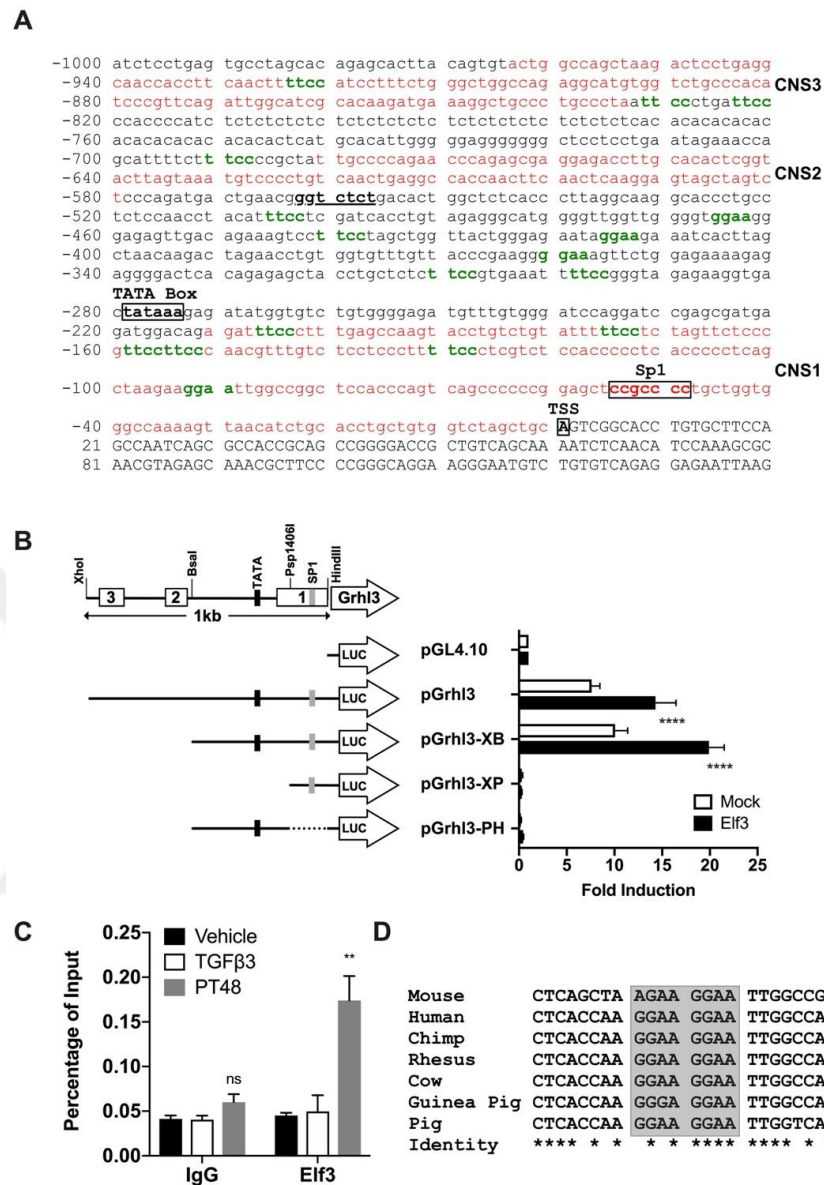


Figure 5. The transcription factor ELF3 regulates the *Grhl3* promoter. (A) The sequence of the *Grhl3* promoter (1000 bp) showing the location of the predicted TATA box, SP1 sequence, and the transcription start site. Sequences in red represent the conserved regions, and green bases represent putative ELF3 binding sites. (B) Luciferase reporter assay showing the activation of the *Grhl3* promoter by ELF3. Relative luciferase activity is calculated as fold induction relative to vector. Drawings on the left side show a schematic representation of the *Grhl3* promoter region (top) and the structure of each reporter plasmid (below). The conserved regions are shown as horizontal boxes labeled 1, 2 and 3, the restriction enzymes used to generate the reporter deletions of the promoter are labeled. (C) ELF3 binding at the promoter of *Grhl3*. NMuMG cells were treated with either vehicle or TGFβ3 for 72 h, TGFβ3 treated cells were then washed with PBS and continued incubation for an additional 48 h to initiate MET (post-treatment: PT48). Cells were cross-linked, and chromatin immunoprecipitation was performed. ChIP DNA was used in qPCR to measure the occupancy of ELF3 compared to control antibodies. (D) Sequence alignment of one putative ELF3 binding site in the *Grhl3* promoter showing conservation among different species. In B and C, data represent the averages of at least three independent experiments. The paired Student's *t*-test was used to calculate statistical significance. ** *p*-value < 0.01; **** *p*-value < 0.0001.

The E74-like transcription factor-3 *Elf3* (also known as ESX, ESE-1, ERT, and JEN), which was first described by Oettgen and colleagues in 1997 [57], is the main Ets transcription factor which is expressed ubiquitously by epithelial-rich tissues, such as stomach, pancreas, uterus, colon, kidney, lung, mammary gland, and skin [58,59]. The observed expression of *Elf3* in our EMT/MET model (Figure S1C) was not restricted to the epithelial states (Vehicle and PT) as for *Cdh1*, but also detected in the mesenchymal cells. While this is contradictory to the high correlation scores between *Elf3* and *Cdh1* in Figure 1, it could be explained by a context-dependent expression of *Elf3* in mesenchymal cells since previous reports indicated that it could also be induced in nonepithelial cells by the proinflammatory cytokines TNF- α and IL-1 β [50,60]. This context-dependent expression of *Elf3* could also imply a context-dependent function of ELF3, the interaction of ELF3 with the *Grhl3* promoter was only detectable during MET, but not in mesenchymal cells.

Besides the critical roles of ELF3 in epithelial cell differentiation, gut development, inflammation, and apoptosis, it is also essential for the pathophysiology of cancer epithelial cells such as in breast, lung and prostate cancers [61–64]. It was demonstrated that ELF3 both activates the type II TGF β receptor gene (*T β R-II*) in epithelial cells and binds *T β R-II* promoter in vivo [65]. For instance, ELF3 controls intestinal epithelial differentiation during development by altering the expression pattern of T β R-II [66]. ELF3 was also recently identified as a candidate transcriptional regulator involved in human urothelial cytodifferentiation together with GRHL3; its depletion in urothelial cells result in a declined expression of some transcription factors such as FOXA1 and GRHL3, which are known to be involved in urothelial differentiation [67]. It was reported that IRF6 and GRHL3 function downstream of RIPK4 to promote *ELF3* gene expression in keratinocytes [68], and considering the data presented here, it is plausible to hypothesize a positive feedback regulatory circuit involving *Grhl3* and *Elf3*. A recent publication also revealed that ZEB1 and ELF3 transcriptionally regulate *IRF6* [69].

The role of *Elf3* was also indicated in animal models. *Elf3* knockout results in embryonic lethality in ~30% of mice models at E11.5. Born *Elf3*^{-/-} mice develop a wasted phenotype characterized by watery diarrhea, lethargy, and malnourishment [66].

The studies mentioned above accentuated the importance of ELF3 in epithelial cells; this is in agreement with the findings presented here; *Elf3* tops the highly correlated transcription factors with the epithelial phenotype. The fact that *Grhl3* was also among the first factors correlated with the epithelial phenotype was not surprising; it holds a critical role in MET [25]. The close association between *Elf3* and *Grhl3* was reported in earlier studies [67], and hence, we deduced their regulatory relationship. The transcriptional relationship between *Elf3* and *Grhl3* was not limited to NMuMG cells since results obtained from MEFs and keratinocytes provided compelling proof.

Similar to the loss of *Grhl3*, loss of *Elf3* during MET failed the epithelial transition. However, unlike *Grhl3*, lack of which resulted in downregulation of *Cdh1*, *Elf3* depletion did not cause a reduction in *Cdh1* mRNA or its protein levels. Instead, E-cadherin was absent from the plasma membrane, which is a significant obstacle preventing MET progression favoring a mesenchymal phenotype. The implications of a deregulated E-cadherin expression as well as its absence from the plasma membrane have been described before. Analysis of tumor samples suggested that aberrant expression of E-cadherin (or no expression) significantly correlates with poor prognosis and promoted metastasis [70–72]. However, the association of *Elf3* with an aberrant E-cadherin expression is a new finding but with comparable observations from *Drosophila*. The *Drosophila* aop (anterior open, also named Yan) and Eip74EF (Ecdysone-induced protein 74EF) are two genes found in the FlyBase [73] as orthologs for several mammalian Ets transcription factors, such as *Elf3*, *Elf*, and *Elf5*. In particular, aop was reported to control the border cell migration in the *Drosophila* egg chamber by altering shg (DE-Cad) expression and its localization [74]. Therefore, a dramatic level of evolutionary conservation of the *Elf3* function cannot be ruled out.

In *Drosophila*, grainy head (*grh*) is driving postembryonic neuroblast function and proliferation by promoting DE-cadherin expression [18,75]. This transcriptional relationship between *grh* and shg is conserved; the mouse orthologs of *grh*, GRHL2, and GRHL3 have been reported to regulate

the transcription of *Cdh1* in mouse cells [25,31]. Interspecies functional conservation would then infer conserved regulation; this, in turn, would suggest conserved *cis*-regulatory elements, such as enhancers. Several reports have indicated that interspecies genome comparisons of non-coding DNA sequences could lead to correct predictions of regulatory sequences [25,40,76,77]. The presence of several conserved ELF3 binding sites in the promoter of *Grhl3* together with the experimental evidence on the regulatory effect of ELF3 on *Grhl3* is in support of the statement mentioned above.

Although the ETS transcription factors have a novel role in various diseases, the function of these transcription factors, particularly in cancer remains largely unclear. ELF3 depletion during MET was not limited to the aberrant expression of *Cdh1*; our data confirmed the role of ELF3 as a regulator of epithelial identity, reflected by the dramatic changes in the expression of selected EMT and MET relevant genes. The ligand-activated transcription factor Aryl hydrocarbon receptor (*Ahr*) promotes EMT and metastasis via its ligands l-kynurenine (l-Kyn) and d-kynurenine (d-Kyn) [45,78]. *Ehf* has a regulatory function in epithelial cell differentiation and gaining stem cell-like properties via EMT [41]. The elevated expression of *Ehf/ESE-3* is associated with poor clinical outcomes and increased recurrence [41,79].

Cellular plasticity is another big puzzle during EMT/MET phenotypic switches. Numerous studies have shown that the expression levels of EMT inducers are linked to cell plasticity and stem cell phenotypes. There is no doubt that the dynamic nature of EMT/MET transitions is highly regulated by other transcription factors which can influence known master EMT regulators. Multilevel regulation of some transcription factors defines a harmonic balance between epithelial plasticity and stability in a context-dependent manner. *OVOL2* was recently revealed as a transcriptional repressor of EMT through downregulating *ZEB1* and *Twist* in various cancer types such as in lung, breast, and colorectal cancers [44,80,81]. In a recent report, *OVOL2* was identified as a MET inducing transcription factor in fibroblasts, which also enhanced the reprogramming of dermal fibroblasts into keratinocyte-like cell state [82]. In this report *OVOL2* was identified following a comprehensive screening approach for transcription factors correlated with *CDH1* in a large number of expression datasets. In support of our findings, it was interesting to find that besides *Elf3* and *Ovol2*, similar transcription factors were also common between the two studies and present among the top correlated genes, such as *Grhl3*, *Irf6*, *Grhl2*, *Grhl1*, and *Ehf*, suggesting a conserved function of these transcription factors in EMT and MET regardless of the organism or cell type studied.

Considering the importance of EMT/MET during development and cancer, a better understanding of these fundamental processes will lead to a better comprehension of the common players in physiological and pathological contexts. In a recent study, *Elf5* emerged as a lineage regulator of mammary gland development and as an inhibitor of EMT in breast cancer via repressing *Snail2/Slug* [83]. Similarly, *Ovol2* was identified as an indispensable guardian of the epithelial differentiation besides its EMT suppressing ability [84]. Our findings present novel insights into the regulation of MET and seek to emphasize the remarkable role of *Elf3* as a transcriptional gatekeeper of epithelial state. It is also not surprising that physiological and pathological EMT share common attributes in terms of transcriptional regulators. Altogether, our findings provide a broader perspective for the management of metastatic cancers.

In conclusion, we provided compelling evidence for the involvement of ELF3 in the regulation of MET by altering the localization of E-cadherin. Moreover, ELF3 regulates the expression of *Grhl3* by binding to conserved sequences in its promoter. Loss of *Elf3* prevented the MET initiation and preserved the mesenchymal state. Further studies are needed to establish the mechanistic relevance of these findings as well as their relevance to possible functions of *Elf3* in the progression of metastasis.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4409/8/8/858/s1>. Figure S1: The expression of *Elf3* in NMuMG cells and during MET; Figure S2: The siRNA mediated silencing of *Elf3* results in an impaired MET; Figure S3: Different influence of *Elf3* and *Ehf* during the progression of MET; Figure S4: Genes correlated with *Grhl3*; Figure S5: Conservation of the *Grhl3* promoter; Table S1: NCBI GEO accession numbers and phenotypes of samples used in correlation studies; Table S2: Sequence information of siRNAs used; Table S3: Primers and corresponding UPL probes used in qPCR experiments; Table S4: Sequences of

oligonucleotides used for the cloning of the Grhl3 promoter; Table S5: qPCR primers and UPL probes used in ChIP experiments; Table S6: Primers used in RT-PCR experiments.

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