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Identification of novel TGF-β regulated genes with pro-migratory roles

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ABSTRACT

Transforming growth factor-β (TGF-β) signaling plays fundamental roles in the development and homeostasis of somatic cells. Dysregulated TGF-β signaling contributes to cancer progression and relapse to therapies by inducing epithelial-to-mesenchymal transition (EMT), enriching cancer stem cells, and promoting immunosuppression. Although many TGF-β-regulated genes have been identified, only a few datasets were obtained by next-generation sequencing. In this study, we performed RNA-sequencing analysis of MCF10A cells and identified 1166 genes that were upregulated and 861 genes that were downregulated by TGF-β. Gene set enrichment analysis revealed that focal adhesion and metabolic pathways were the top enriched pathways of the up- and downregulated genes, respectively. Genes in these pathways also possess significant predictive value for renal cancers. Moreover, we confirmed that TGF-β induced expression of MICAL1 and 2, and the histone demethylase, KDM7A, and revealed their regulatory roles on TGF-β-induced cell migration. We also show a critical effect of KDM7A in regulating the acetylation of H3K27 on TGF-β-induced genes. In sum, this study identified novel effectors that mediate the pro-migratory role of TGF-β signaling, paving the way for future studies that investigate the function of MICAL family members in cancer and the novel epigenetic mechanisms downstream TGF-β signaling.

1. Introduction

TGF-β signaling plays a fundamental role in cell proliferation, differentiation, organ morphogenesis, and tissue homeostasis [1]. The growth inhibitory function of TGF-β suggests it functions as a tumor suppressor; however, TGF-β signaling can also promote cancer development. For example, TGF-β induces an epithelial-to-mesenchymal transition (EMT), which is considered a mechanism underlying cancer metastasis [1,2]. In fact, EMT is also associated with cancer stem cells (CSCs), which are characterized as $CD44^{high}CD24^{low}$ cells [2]. Moreover, TGF-β also exerts systematic immune suppression and inhibits host immunosurveillance [3]. Mechanistically, TGF-β signaling is initiated when a ligand binds to transmembrane TGF-β receptor kinases, which subsequently phosphorylate and activate SMAD2/3 proteins. The SMAD2/3 protein form a complex with SMAD4, and this SMAD protein complex translocates into nucleus and interacts with other transcription factors and chromatin regulators to regulate transcription [1,2,4].

In the past decade, tremendous effort has been made to identify the target genes of TGF-β signaling pathway. Using microarray analysis, rapid gene expression changes that occurred due to treatment with TGF-β (i.e., within 12 h of treatment) were identified in lung cancer A549 cells and non-malignant lung epithelial (HPLID) cells [5]. In addition, a signature of 153 TGF-β-induced genes was obtained from MDA-MB-231, HaCat, HPL1, and MCF10A cells treated with TGF-β for 3 h [6]. Extended treatment with TGF-β for 72 h in A549 cells revealed over 3000 genes that were differentially regulated [7]. With continuous annotation of mammalian reference sequences (refseq) [8], many novel TGF-β-regulated genes, such as *GPR68* and *SLAMF8* were identified from the non-small lung cancer cell line, H358, and from MCF10A cells treated with TGF-β for 7 days [9]. Combined ChIP-seq (chromatin immunoprecipitation followed by deep sequencing) analysis and transcriptomic data revealed that in specific type of cells, master

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transcription factors determine the genes that are bound by the SMAD protein complex, i.e. Oct4 in embryonic stem cells, MyoD1 in myotubes, and PU.1 in pro-B cells selectively interact with SMAD proteins to regulate cell-type-specific transcriptome [10]. Recently, the synergistic action of master transcription factors during EMT, namely ETS2, HFN4A, and JUNB, were identified through RNA-seq analysis in A549 cells [11]. Using combined RNA-seq and SMAD3 ChIP-seq from MCF10A MII cells, a critical role for JUNB was identified governing a feed-forward network for TGF-β signaling and aggravating breast cancer invasion [12]. Although these studies have advanced our understanding of TGF-β signaling, the cell-specific genes regulated by this pathway, and their impact on cellular function, remains to be determined.

TGF-β signaling heavily cross-talks with chromatin regulators to regulates transcription [13]. For example, Enhancer of Zeste Homolog 2 (EZH2), a polycomb group histone methyltransferase that targets trimethylated histone 3 lysine 27 (H3K27me3), plays an essential role in mediating transcriptional repression by Sox4 in TGF-β-induced EMT [14]. In addition, KDM6B/JMJD3, a histone demethylase acting on H3K27me3/2, was on the list of TGF-β-upregulated genes [6] and recent studies demonstrated that KDM6B interacts with SMAD3 to regulate expression of TGF-β-responsive genes in neural stem cells [15]. Recently, we demonstrated that the histone demethylase PHF8, which acts on multiple methylated histones such as H3K9me2, H3K27me2 and H4K20me1 [16], is a co-activator of *SNAI1*, whose transcriptional activation is induced by TGF-β [17]. We also showed that, post-transcriptionally, TGF-β signaling regulates PHF8 through the c-MYC-microRNA22 regulatory axis in non-malignant cells. Specifically, TGF-β represses c-MYC, which represses microRNA22, leading to de-repression of PHF8 expression [17]. In fact, the downregulation c-MYC by TGF-β is a key event in the growth inhibitory function of TGF-β signaling in non-malignant cells [18]. Beyond histone methylation, TGF-β also promotes global changes in DNA methylation in ovarian cancer cells [19], inducing both the expression and the activity of DNA methyltransferases, DNMT-1, -3A and -—3B [19]. In contrast, TGF-β downregulates the DNA demethylases TET2 and TET3 in melanoma cancer cells [20]. The loss-of-function of TET2 or TET3 induces an EMT-like process, suggesting an inhibitory function of these DNA demethylases in TGF-β-induced EMT [20]. However, whether there are additional epigenetic factors critical for the transcriptional regulation of TGF-β signaling remains to be determined.

In this report, we analyzed the transcriptome induced by TGF-β in MCF10A cells and identified over one thousand genes that were differentially regulated. Beyond gene enrichment analysis, our study confirmed that TGF-β regulates MICAL1 and histone demethylase KDM7A and revealed their critical roles in the pro-migratory functions of TGF-β signaling. Thus, these findings identify novel effectors that mediate the pro-migratory effects of TGF-β signaling and novel epigenetic mechanisms downstream TGF-β signaling.

2. Materials and methods

2.1. Cell culture and reagents

A549 and MCF10A cells were obtained from ATCC (American Type Culture Collection). A549 cells were cultured in RPMI1640 medium containing 10% FBS. MCF10A cells were cultured in DMEM-F12 supplemented with 20 ng/ml Epidermal Growth Factor (EGF; Sigma), 100 ng/ml cholera toxin (Sigma), 10 g/ml insulin (Sigma), 500 ng/ml hydrocortisone (Sigma), and 5% horse serum. All cell lines used in this study were maintained in the specified medium supplemented with 1 × Penicillin–Streptomycin (GIBCO) and incubated in 5% CO2 at 37 °C. TGF-β1 (Protein Tech) was added into the medium directly with final concentration of 5 ng/ml. For induction of shRNA expression, doxycycline (Sigma) was added to the culture medium at 0.5 μg/ml for 72 h. Antibodies for γ-TUBULIN (MA1-850) from Thermo Fisher; p-SMAD2 (Thr 220, sc-135644), FN1 (H-300, sc-9068), ZEB1 (H-102, sc-25388) and Vimentin (E-5, sc-373717) from Santa Cruz; β-ACTIN (Ab8287) from Abcam; and CDH1 (24E10), CDH2 (D4R1H) from Cell Signaling Technology were used for Western blotting analysis.

2.2. Bioinformatic analysis

Sequence read archive files for GSE74377, GSE125280, GSE83788, GSE69667, and GSE90567 were downloaded and converted to fastq files using the SRA toolkit. RNA-seq raw fastq files were quantified using kallisto pseudo alignment [21] and the GRCh38 human transcriptome. Differential expression utilized the sleuth R package (v0.3.0) [22] with the parametric Wald test to determine significance and log2-fold change. *p* values were adjusted for multiple comparisons using the Benjamini-Hochbery method.

Gene set enrichment analysis (GSEA) for KEGG signaling pathway enrichment was analyzed using Enrichr [23,24]. The association with cancer prognostic markers was performed using the GSEA software [25,26]. Dataset files were developed based on normalizing the expression intensities by DESeq2. Specifically, the log2-fold change values were compared for two phenotypes in our analysis: MCF10A-0 h versus MCF10A-TGF-β-24/48/72 h. GSEA was executed using the default settings. Gene sets of favorable and unfavorable prognostic markers for each type of cancer were obtained from The Human Protein Atlas for enrichment analysis [27].

2.3. Transwell assay

The Transwell assay was performed using Corning 96 Well HTS Transwell Permeable Support with 8.0 μm pore size PET (polyester) Membrane (Corning 3374). The inserts were then washed with PBS, and 100 μl of serum-free cell culture medium with 1×10^4 cells were seeded onto the insert. 100 μl of complete cell culture medium were added to the well underside of the insert for 24 h at 37 °C and 5% CO_2. After incubation for 24 h, cells were fixed, stained with crystal violet for 10 min and microscopically analyzed.

2.4. Wound healing assay

For wound healing assays, after incubating with complete medium with 5 ng/ml TGF-β for 48 h, cells were reseeded in 6-well plates. 24 h later, confluent cells were uniformly scraped with a pipette tip across the well. Following wounding, culture medium was washed with PBS and replaced with fresh serum-free medium, and cells were continually exposed to 5 ng/ml of TGF-β for the indicated time periods. After incubation for 24 h, cells were fixed, stained with DAPI for 30 min and the number of migrated cells into free space were counted using a microscope.

2.5. Chromatin immunoprecipitation assays

ChIP was performed as described previously [16,28]. Briefly, after treatment of 5 ng/ml TGF-β for 48 h, formaldehyde-crosslinked cells were lysed and sonicated to shear the DNA. The sonicated DNA-protein complexes were immunoprecipitated (IP) with rabbit IgG (A01008, GenScript) and anti-H3K27ac (ab4729, Abcam) antibodies. The immuno-complexes were collected using protein A/G agarose beads. The eluted DNA and 10% of the respective input DNA were reverse cross-linked at 65 °C overnight. DNA was extracted and used for qPCR analysis using the SYBR Green qPCR mix and a CFX96 instrument (Bio-Rad).

2.6. Cloning of CRISPR/Cas9 and guide RNA design

Three single guide RNAs (gRNAs) were used to target *MICAL1*. gRNAs were designed using DNA2.0 gRNA Design Tool (https://www.

dna20.com/eCommerce/cas9/input), as detailed in the table below. gRNAs were cloned into the lentiCRISPR v2 vector (Addgene plasmid # 52961) [29], utilizing the *BbsI* restriction site according to the method described previously [30]. lentiCRISPR v2 were gifts from Feng Zhang [29,30].

2.7. Oligonucleotides

All oligonucleotides were synthesized by IDT, and the sequences are shown below:

Oligonucleotides names	Sequence
ChIP-qPCR primers	
CDH2 TSS F	AGTAGGTCTCTCGCGCCT
CDH2 TSS R	CTGGGAGTTATCCTGCCTCAC
CDH2 UTR F	TCTGACAACAGCTTTGCCTCT
CDH2 UTR R	ATGCTACGCGTTTCAATGGTG
FN1 TSS F	GGACCCTAAAGCCGACCAAA
FN1 TSS R	GAGTCCTCGTCCTCCAATGC
FN1 UTR F	CTTCTTCCCTCCACCTTTCCTT
FN1 UTR R	TAGCTGGGAGTTTATCAAGCCA
SNAI1 TSS F	GGCCTAGCGAGTGGTTCTTC
SNAI1 TSS R	CCAACGCACCTGGATTAGAG
SNAI1 UTR F	CTCTGAGCGGTGAGGGTTAG
SNAI1 UTR R	CCCAAAATAGAGCCCTGTGA
RT-qPCR primers	
RPL13A qPCR F	CCTGGAGGAGAAGAGGAAAGAGA
RPL13A qPCR R	TTGAGGACCTCTGTGTATTTGTCAA
ZEB1 qPCR F	CTGATTCTACACCGCCCAAA
ZEB1 qPCR R	AGCGCTTTCCACATTTGTCA
CDH2 qPCR F	CGACGAATGGATGAAAGACC
CDH2 qPCR R	CATAGTCCTGCTCACCACCA
SNAI1 qPCR F	CCCTCAAGATGCACATCCGAA
SNAI1 qPCR R	TGGCACTGGTACTTCTTGACATCTGA
MICAL2 qPCR F	CTTCTTCCACCGGGAGTGTT
MICAL2 qPCR R	CTTCAACTCTGCCCGTCTCT
MICAL1 qPCR F	ACCATCCAACGGCGACTAAA
MICAL1 qPCR R	GCTGTTCTGGGGAACTGCTC
KDM7A qPCR F	CCACCAAGAGACCGGCATCAA
KDM7A qPCR R	ACTTAAGGATCTTCCCAAGACGTT
SLAMF9 qPCR F	TGTCAGAGCCCCAGATCACT
SLAMF9 qPCR R	CAGAAGCATAGTTAGGATCTGCAT
ABAT qPCR F	TGCTCCCTACCGGATCTTCA
ABAT qPCR R	ACCCAACACCACACCTTTGT
MOB3B qPCR F	CCACTGAACCCTCCACAACA
MOB3B qPCR R	ATGCCACCCTGGGAGAATTG
FAM214B qPCR F	ACATTGAGGGCTTCACAGCA
FAM214B qPCR R	AGATCCACGATGCCCAGGAA
CCNG2 qPCR F	GTGAAAGTGAGGACTCTTGTGA
CCNG2 qPCR R	GCACAGTGTTTGTGCCACTT
NALCN qPCR F	ATCCGCATGTGGCTCAAGAA
NALCN qPCR R	CTGGCTGCTTGTCTCAGGTT
COA4 qPCR F	GTACAGAGGATCCCCAACCG
COA4 qPCR R	AGGTATGGCCTTGAGGGACT
FYB qPCR F	GATGGCTCCACACTACAGGTT
FYB qPCR R	AGTTCCTTGACTCATCTCTGCT
Oligonucleotides for interfering target genes	
sh*KDM7A*-1F	CCGGGAGGAAACTTCGAGATCATAACTCGAGTTATGATCT CGAAGTTTCCTCTTTT
sh*KDM7A*-1R	AATTAAAAAGAGGAAACTTCGAGATCATAACTCGAGTT ATGATCTCGAAGTTTCCTC
sh*KDM7A*-2F	TCGAGGTCCCTTGATCTCCAAGTAGTTTCAAGAGAACT ACTTGGAGATCAAGGGTTTTTG
sh*KDM7A*-2R	GATCCAAAAACCCTTGATCTCCAAGTAGTTTCAAGAGAA CTACTTGGAGATCAAGGGACC
MICAL1 gRNA1 F	CACCGAAGGCCTGCTCACCGGTGGC
MICAL1 gRNA1 R	CAAGGCCTGCTCACCGGTGGCCAAA
MICAL1 gRNA2 F	CACCGAGAAAACATGCATCGCAATG
MICAL1 gRNA2 R	CAGAAAACATGCATCGCAATGCAAA
MICAL1 gRNA3 F	CACCGGGAGCTGCTACGCTGGTGCC
MICAL1 gRNA3 R	CGGAGCTGCTACGCTGGTGCCCAAA

3. Results

3.1. TGF-β-regulated genes in MCF10A cells are differentially associated with cancer prognostic markers

Using RNA-seq analysis, we recently discovered that overexpression of the histone demethylase PHF8 in MCF10A cells reprograms the transcriptome toward EMT-like features, including upregulation of *SNAI1*, a transcription factor that is critical for TGF-β-induced EMT [17]. To identify novel TGF-β-regulated genes, we further analyzed RNA-seq data from the control MCF10A cells treated with TGF-β for 24, 48, and 72 h (GSE74377). We also carried out RNA-seq from MCF10A cells treated with TGF-β for 1.5 h to identify genes that are regulated early after TGF-β exposure (GSE125280). All RNA-seq data were analyzed with kallisto pseudo alignment [21] and differential expression was determined using the sleuth R package [22]. Differentially regulated genes for each time point were obtained using an actual fold-change cutoff of 1.5 (Log2 FC > 0.58 or < −0.58) and adjusted p value < 0.05 (Fig. 1 and Supplemental Table 1). After treatment with TGF-β for 1.5 h, we identified 67 upregulated and 7 downregulated genes compared with over several hundred genes that were differentially regulated by prolonged treatment (Fig. 1 and Supplemental Table 1). These findings are consistent with a recent study demonstrating differential expression of a large number of genes at later timepoints following TGF-β treatment [12]. Many genes that were upregulated 1.5 h after treatment with TGF-β, such as *CTGF*, *SNAI1*, *VEGFA* and *NEDD9*, were also among the 153-gene TGF-β response signature (TBRS) obtained from four cell lines treated with TGF-β for 3 h [6]. With the inclusion of the non-redundantly regulated genes from all four time points of treatment, we concluded that TGF-β upregulated 1166 genes and downregulated 861 genes, respectively (Supplemental Table 1). Notably, the change in expression of a large number of genes (557 upregulated and 305 downregulated genes) remains altered 24 to 72 h after treatment (Fig. 1B). The regulation of select genes is shown in Fig. 1C. MYC was included because of its critical roles in regulating the growth inhibitory function of TGF-β signaling [18].

In MCF10A cells, complete EMT is induced by prolonged TGF-β treatment (6 days) [9,31]. In the cells relevant to MCF10A, genes that were upregulated rapidly (i.e., within 3 h following TGF-β treatment) [6,7,12] or at later time points (i.e., 7 days following TGF-β treatment) [9] were identified. Thus, in our study, assessing those genes that were differentially regulated between 1.5 and 72 h following TGF-β treatment shed light on how the transcriptome transits from the early response to an intermediate transition and toward the final stages of EMT. Gene Set Enrichment Analysis (GSEA) analysis using Enrichr [23,24] revealed that the 1166 upregulated genes are enriched in the Kyoto encyclopedia genes and genomes (KEGG)-defined pathways of focal adhesion, proteoglycans in cancer, pathways in cancer, regulation of actin-cytoskeleton and PI3K-AKT signaling pathway (Fig. 2A). Importantly, genes that remain upregulated from 24 to 72 h are enriched in 4 of these 5 KEGG pathways, and of the later time point, the ECM-receptor interaction ranks fourth (Supplemental Table 2), indicating consistent activation of these pathways along TGF-β treatment. In contrast, the early responsive genes (1.5 h of TGF-β treatment) are enriched in TGF-β, amphetamine addiction, pluripotency of stem cells, Hippo, and HIF-1 pathways (Supplemental Table 2). Such differential functions of genes regulated by TGF-β at different time points have also been revealed by other studies [9,12]. Beyond focal adhesion, pathways in cancer, regulation of the actin cytoskeleton, and the TGF-β signaling pathway have been found in cell lines from mammary gland [12], lung [32] and kidney [33]. The genes enriched in the PI3K-AKT pathway, or pathways involving proteoglycans and microRNAs in cancers may provide to novel insight into the mechanisms underlying the cross-talk between these pathways and TGF-β signaling. Among the total downregulated genes, the top 5 signaling pathways enriched are metabolic pathways and those associated with RNA polymerase, pyrimidine metabolism, ribosome biogenesis, and

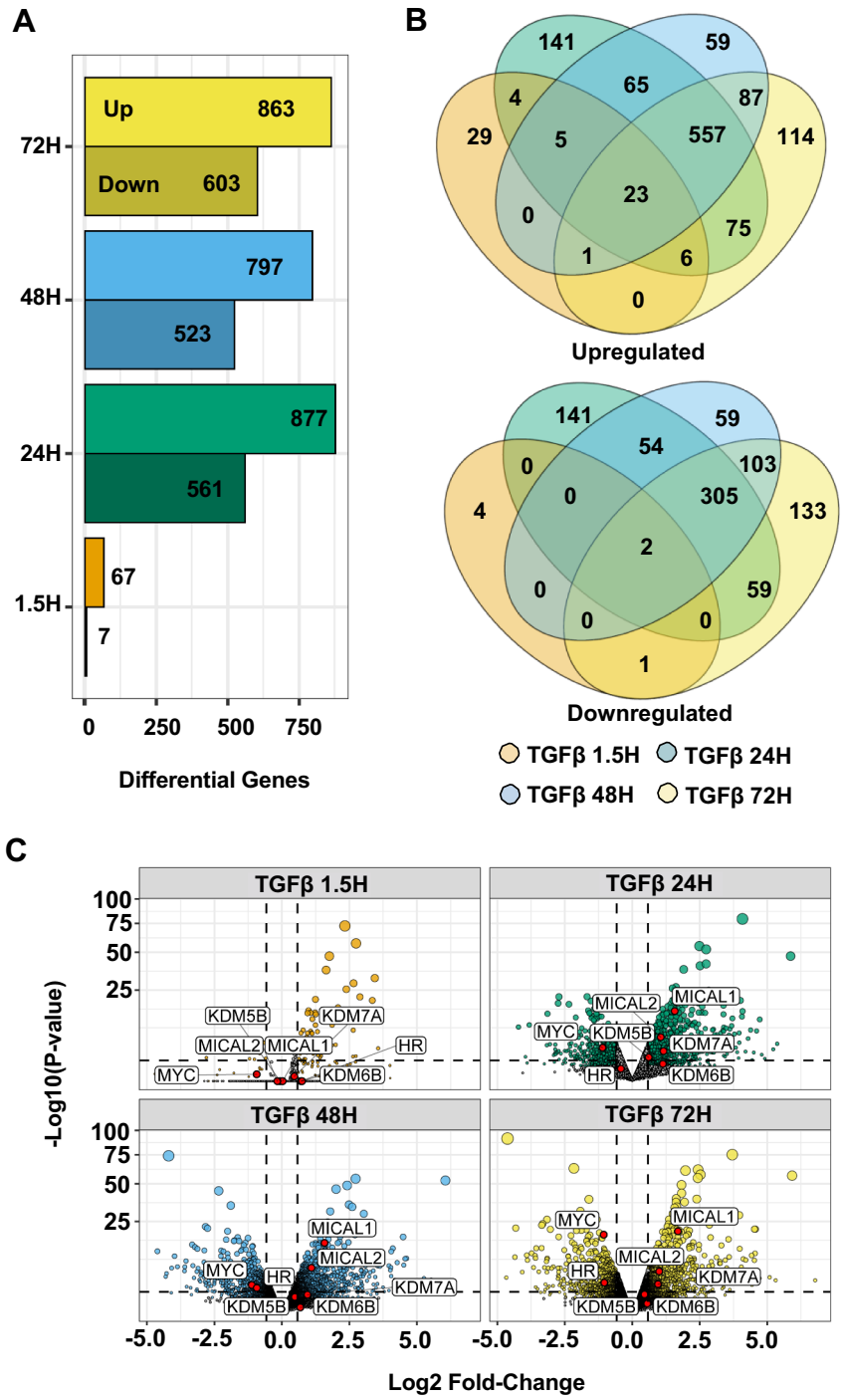


Fig. 1. Identification of novel TGF-β-regulated genes in MCF10A cells. A. RNA-seq analysis of MCF10A cells to identify genes that are differentially-regulated by TGF-β at the indicated hours (H) after treatment with TGF-β. Genes were defined as differentially regulated by the cut-off value of actual fold change of 1.5 (Log2 FC > 0.58 or < −0.58) and adjusted *p* value of < 0.05. B. Distribution and intersection of genes that are differentially-regulated by TGF-β for the duration of treatment. Upper panel shows TGF-β-upregulated genes; lower panel shows TGF-β-downregulated genes. C. The regulation pattern of selected differentially-regulated genes across all time points. Y-axis is the -Log10 (adjusted *p* value) scaled using a square root function for easier visibility.

purine metabolism (Fig. 2A). These pathways most resemble pathways enriched among genes downregulated at 24 h (Supplemental Table 2). Prolonged TGF-β treatment sustains genes enriched in metabolic pathways and increases the rank of pathways associated with histidine metabolism, glycolysis, carbon metabolism and bladder cancer (Supplemental Table 2). Collectively, these data reinforce the role of TGF-β in metabolic signaling pathways [34,35].

We next carried out GSEA analysis to examine how TGF-β-regulated genes associate with cancer prognostic markers. Using datasets from Pathology Atlas with default parameter settings [27], we found a negative association of TGF-β-regulated genes with favorable prognostic markers of renal, lung and colorectal cancers (Fig. 2B). Notably, most upregulated genes and some downregulated genes contribute to this association. In the case of renal cancer, most genes that are downregulated by TGF-β contribute to a positive association with prognostic markers of unfavorable outcomes (Fig. 2B). A similar association was also observed for head and neck cancer. Taken together, these associations support an oncogenic role of TGF-β signaling in cancer progression. Additionally, genes that are both up and downregulated by TGF-β have a positive association with unfavorable prognostic markers for urothelial and stomach cancers (Fig. 2B), indicating cancer type-specific effects of TGF-β-regulated genes.

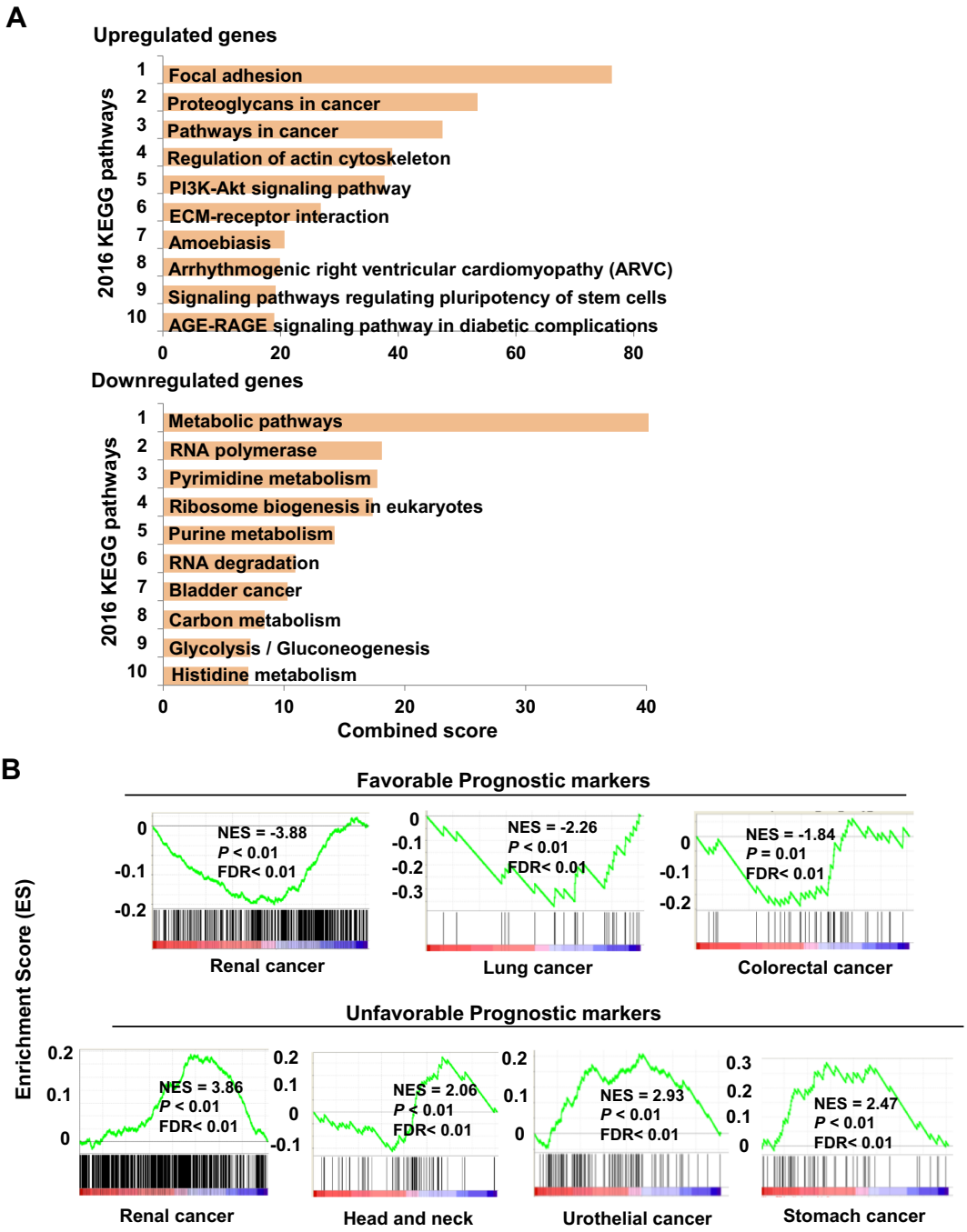


Fig. 2. TGF-β-regulated genes are associated with cancer signaling pathways and cancer prognosis. A. KEGG signaling pathways enriched among TGF-β-regulated genes, as analyzed through Enrichr. B. Association of TGF-β-regulated genes with cancer prognosis was assessed through GSEA. NES: Normalized Enrichment Score; FDR: False Discovery Rate.

3.2. MICAL1 facilitates TGF-β-induced cell migration

Although the transcriptome regulated by TGF-β in cell lines from different origins has been extensively studied, due to poorly-defined functions, many genes that are regulated by TGF-β are not assigned to specific signaling pathways. Therefore, the impact of these genes on the cellular functions of TGF-β signaling remains unclear. We next picked up several genes to evaluate their regulations by TGF-β. RT-PCR analysis of MCF10A and A549 cells with TGF-β treatment for 4 and 48 h demonstrated that *ABAT*, *MOB3B*, *FAM214B*, and *CCNG2* are upregulated by TGF-β in both cell lines, whereas, *NALCN*, *COA4* and *FYB1* were downregulated (Supplemental Fig. 1). *SLAMF9* was included due to its higher induction (Log FC 1, 1.7 and 1.7 at 24, 48 and 72 h) despite insignificant adjusted *p* values, and its family member *SLAMF8* was on the gene list regulated by TGF-β [9]. The altered expression of these genes upon TGF-β treatment suggests they may have an impact on the cellular functions of TGF-β signaling.

Focal adhesion and actin cytoskeleton pathways are among the top enriched pathways among TGF-β-upregulated genes in this study. Therefore, we looked at genes known to be involved in cell mobility that were not included in the top enriched pathways. The MICAL (Molecule interacting with CasL) gene family caught our attention as three (*MICAL1*, *MICAL2*, *MICALL2*) of the five gene family members are significantly upregulated by TGF-β (Fig. 3A). Importantly, such

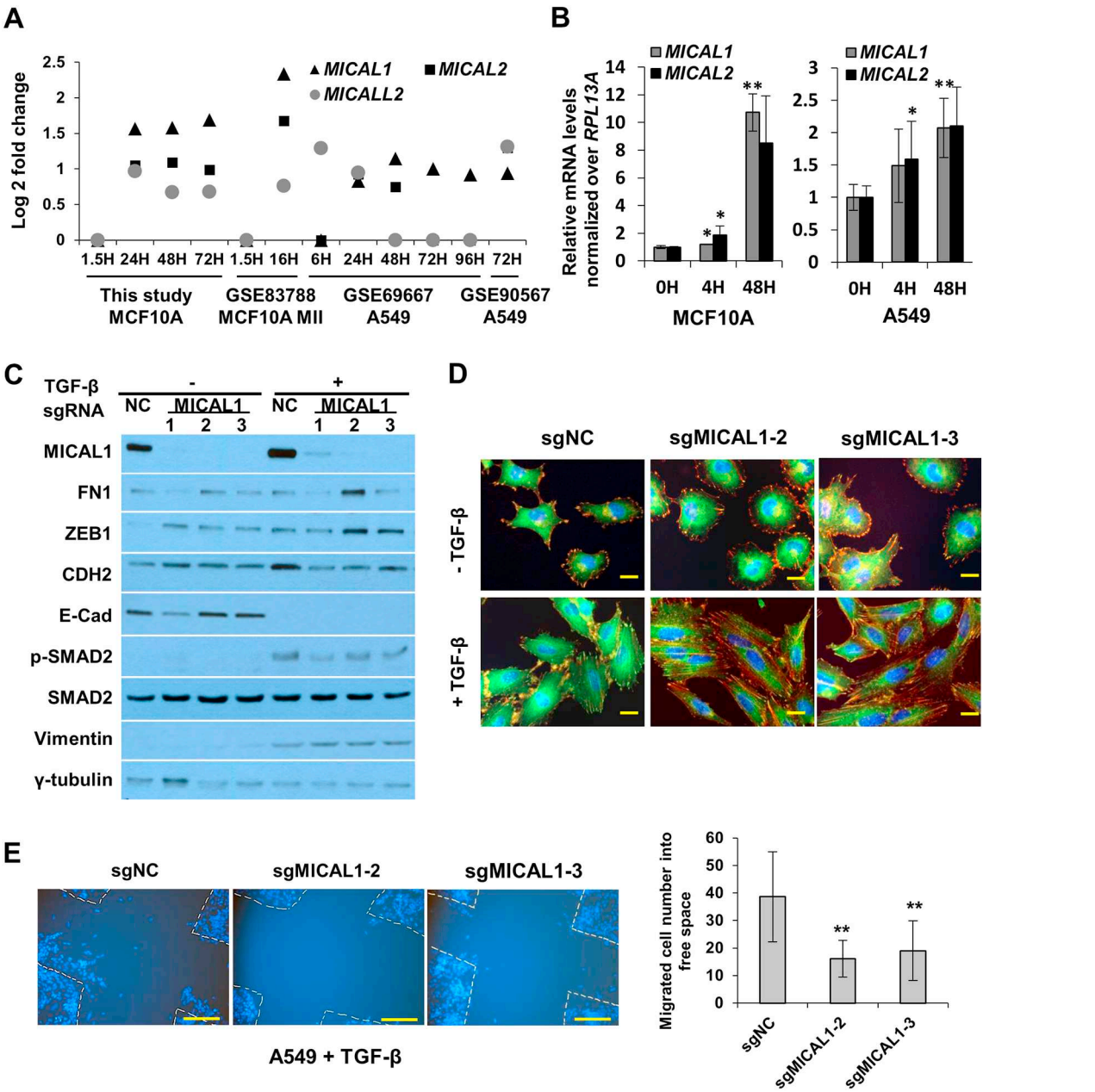


Fig. 3. MICAL1 plays a role in cell migration promoted by TGF-β. A. Upregulation of MICAL gene family members determined by RNA sequencing. Expression is comparable with that retrieved from GEO database of MCF10A MII cells and A549 cells treated with TGF-β. B. *MICAL1* and *MICAL2* RNA expression was verified by RT-qPCR. C. Western blotting indicated the effect of knocking out MICAL1 on EMT markers in A549 cells treated with TGF-β. Stable cell lines with three gRNAs of *MICAL1* are marked as sgMICAL1-1/2/3, respectively. D. Immunofluorescence of F-actin stress fiber in control (sgNC) and MICAL1 knockout A549 cells with and without TGF-β treatment. Bar: 20 μm. E. Cell migration of A549 cells into the free space upon TGF-β treatment for 72 h was examined by DAPI staining. F. Migrated cells into the free space were quantified. Bar: 100 μm. *: $p < 0.05$; **: $p < 0.01$.

upregulation was observed in MCF10A MII cells [12] and, to a certain extent, in A549 cells [11,36] (Fig. 3A). We next confirmed the regulation of *MICAL1* and *MICAL2* by TGF-β in both MCF10A and A549 cells and found that upregulation of each gene was stronger in MCF10A cells than in A549 cells (Fig. 3B), consistent with our RNA-seq data. MICAL proteins possess a conserved monooxygenase domain and participate in F-actin depolymerization via oxidation of actin, thus playing roles in cytoskeleton rearrangement and cell mobility [37,38]. The oncogenic functions of MICAL proteins have been emerging: MICAL1 can activate ROS/AKT signaling and increase cell invasion in breast cancer cells [39], whereas MICAL2 promotes breast cancer cell migration by maintaining EGFR stability and activating EGFR/P38 signaling [40]. Through the Pathology Atlas [27], we found that elevated expression of *MICAL1*, *MICAL2* and *MICALL2* is significantly associated with unfavorable renal cancer prognoses (Supplemental Fig. 2), suggesting that these three MICAL members may mediate the oncogenic role of TGF-β signaling in renal cancer.

We next focused on determining the functional impact of *MICAL1* as it is predominantly upregulated by TGF-β compared to *MICAL2* and *MICALL2* in most treatments (Fig. 3A). Although, we attempted to delete *MICAL1* expression using CRISPR-Cas9 mediated deletion in both MCF10A and A549 cells, we could only establish stable deletion in A549 cells, indicating the requirement of MICAL1 for the viability of MCF10A cells. At steady state, deletion of *MICAL1* in A549 cells did not dramatically affect the regulation of E-cadherin and Vimentin except a slight increasement of ZEB1 (Fig. 3C). Under TGF-β treatment,

attenuation of the upregulation of CDH2 and p-SMAD2 was observed in *MICAL1* knockout cells (Fig. 3C). Interestingly, *MICAL1* knockout enhanced the upregulation of ZEB1 and Vimentin (Fig. 3C). Although, such regulation seems to enhance EMT, loss of *MICAL1* expression dramatically increased the accumulation of F-actin stress fibers (Fig. 3D) and the cell migration of A549 cells upon TGF-β treatment for 72 h (Fig. 3E). Thus, these results support our hypothesis that MICAL1 mediates the pro-migratory role of TGF-β signaling.

3.3. Revealing novel TGF-β-regulated transcription regulators

Transcription regulators, including transcription factors and chromatin regulators, play pivotal roles in regulating transcription downstream of TGF-β signaling [1,13]. Thus, we set out to identify novel transcription regulators that are regulated by TGF-β. Analysis of the 1168 upregulated genes and 861 downregulated genes by TGF-β with GO Molecular Function 2018 at Enrichr [23,24] found these were associated with 685 and 630 GO terms, respectively (Supplemental Table 3). We retrieved genes with GO terms broadly associated with transcriptional regulation: DNA-binding and transcriptional regulatory activities. 76 upregulated and 66 downregulated genes were retrieved from GO terms related to DNA-binding (Supplemental Table 3). Further analysis through GenCLiP2.0 [41,42], which combines published functions, revealed 51 upregulated and 46 downregulated genes with reported DNA-binding (Supplemental Table 3). These genes include well-known TGF-β signaling effectors such as *ZEB1*, *SNAI1*, *MYC*, *JUN*, *JUNB*. Moreover, 25 upregulated and 8 downregulated genes were not associated with the GO term, DNA-binding (Supplemental Table 3), although some of these genes, such as *ZNF175* and *GCFC2*, do have DNA-binding ability. Further analysis with GenCLiP2.0 showed that the relevant cellular functions of many TGF-β-regulated genes discussed above, such as EMT and cell migration, are not well studied (Supplemental Fig. 3).

We next focused on genes associated with transcriptional regulatory activity (Supplemental Table 3). A total of 58 upregulated and 66 downregulated genes fell into this category (Supplemental Table 3). Notably, many transcription factors are included. GenCLiP2.0 analysis of these transcription regulatory factors also revealed that many genes are understudied in the context of the cellular functions of TGF-β (Supplemental Fig. 4). Following our interest in chromatin regulators, we found differential expression of four histone demethylases regulated by TGF-β: *KDM5B* and *KDM7A* are upregulated, *RIOX2/MINA53* is downregulated, and *HR* (Hairless) is upregulated early after treatment with TGF-β and downregulated by extended treatment (Table 1). It is necessary to mention that *KDM6B* has long been known as a TGF-β-regulated gene [6] and such regulation was supported by other studies [43,44]. In addition to the histone demethylases, three methyltransferases (*PRMT3*, *6* and *SETD6*) are downregulated (Table 1) and histone modifying enzymes, *PHF1*, *CDYL2*, *BCOR*, *NCOR2*, and *SMARCA1* are upregulated, whereas, *SMARCD2*, *JADE1*, *NCOA7* are downregulated. Of enzymes that regulate histone methylation, we examined their responses to TGF-β in three published RNA-seq datasets: GSE83788 from MCF10AII [12], and GSE69667 [11] and GSE90567 from A549 cells [36] (Table 1). *KDM6A* and *MYC* were included as positive controls. The regulation of *KDM6B*, *KDM7A*, *KDM5B* and *HR* is conserved in at least three of the four cell lines. However, the regulation of *PRMT3/6* and *SETD6* was found only in MCF10A MII cells. Notably, their regulation is consistent with that of MYC. Therefore, these data do not only reveal the importance of TGF-β signaling in regulating enzymes that modify histone methylation, but also implicate the function of these enzymes downstream of TGF-β signaling.

3.4. KDM7A regulates TGF-β-induced genes and cell migration

Among the histone methylation modifying enzymes, *KDM7A/JHDM1D* caught our attention as it is a transcriptional co-activator with histone demethylation activities on H3K9me2 and H3K27me2 [45]; it also demethylates H4K20me1 in an in vitro assay [16] and its family member, PHF8, is post-transcriptionally regulated by, and plays a critical role in, TGF-β signaling [17]. To reveal the pro-migratory function of KDM7A, we first performed loss-of-function studies in both MCF10A and A549 cells treated with TGF-β. Knockdown of KDM7A by RNAi reduced the cell migration induced by TGF-β in both cell lines with cell migration (Fig. 4A) and scratch assays (Supplemental Fig. 5). We next performed rescue experiments by over-expressing wide type (wt)-KDM7A or a catalytically lethal mutant (mut)-KDM7A (H354A) and at the same time depleting KDM7A by shKDM7A-1(targets ORF) and shKDM7A-2 (targets 3′UTR). Migration assay indicated wt-KDM7A, not the mut-KDM7A, sustained cell mobility reduced by KDM7A knockdown in both cells (Supplemental Fig. 6A). At the mRNA level, we confirmed that *KDM7A* was upregulated by TGF-β treatment and that knockdown of *KDM7A* significantly attenuated TGF-β-induced expression of Fibronectin-1 (*FN1*), N-cadherin (*CDH2*), and *SNAI1* in both MCF10A and A549 cells (Fig. 4B and Supplemental Fig. 7A). Notably, the effects of KDM7A on the induction of *FN1* and *CDH*2 were confirmed at the protein level (Fig. 4C). These findings suggest that KDM7A plays a role in the transcriptional activation downstream of TGF-β signaling. In fact, overexpression of both wt- and mut-KDM7A in MCF10A cells enhances CDH2 protein levels, implicating a histone demethylation-independent function of KDM7A in regulating CDH2 (Supplemental Fig. 6B). Taken together, these data emphases the critical role of KDM7A in TGF-β-induced EMT.

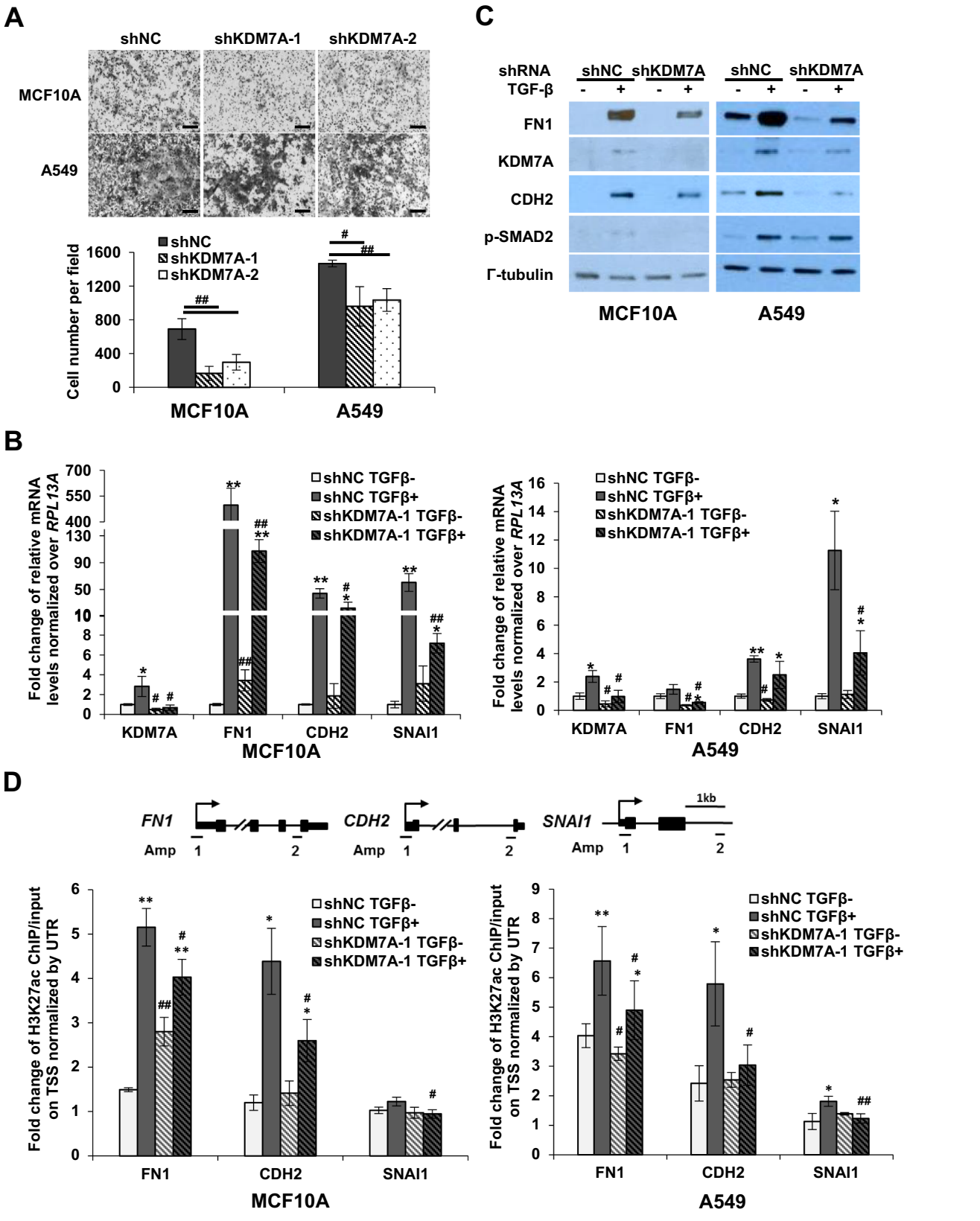
Histone acetylation, particularly at lysine 27 of histone 3, is critical for transcriptional activation in TGF-β-induced EMT [13,46]. Histone demethylation is an essential step to prime the target histones for acetylation. TGF-β upregulates *KDM6B* [43,44], which can cooperate with Smad3 to demethylate H3K27me3 and activate genes induced by TGF-β [15]. KDM6B can also physically interact and collaborate with

Table 1
TGF-β regulates the expression of histone methylation regulators.

TGF-β treatment	This study MCF10A				GSE83788 MCF10A MII		GSE69667 A549					GSE90567 A549
	1.5H	24H	48H	72H	1.5H	16H	6H	24H	48H	72H	96H	72H
KDM5B	–	0.61	–	–	–	0.91	–	–	–	0.62*	0.73*	–
KDM6B	0.75	1.13	–	–	0.93	1.10	1.79	2.24	1.80*	1.96	1.77*	1.84
KDM7A	–	1.16	0.95	0.97	–	1.18	–	0.93*	1.12*	1.05*	1.05*	1.2
HR	0.71	–	−0.93	−1.04	0.61	−0.79	–	–	–	−0.66*	–	−0.97
RIOX2/MINA53	–	−0.63	–	–	–	−0.85	–	–	–	–	–	–
PRMT3	–	−0.93	−0.78	−0.71	–	−1.99	–	–	–	–	–	–
PRMT6	–	−0.74	–		–	−0.97	–	–	–	–	–	–
SETD6	–	−0.71	–	–		−0.79	–	–	–	–	–	–
MYC	−0.93	−1.10	−1.12	−1.06	−1.01	−1.65	–	–	–	–	–	–

* p value > 0.05.

KDM7A to demethylate H3K27, and thus, both proteins are required for transcriptional elongation [47]. Therefore, we hypothesized that elevated expression of KDM7A and KDM6B prime TGF-β-induced genes for H3K27 acetylation by removing methyl groups. To address this, we performed chromatin immunoprecipitation (ChIP) of H3K9me2 (Supplementary Fig. 7B), H3K27me2 (Supplementary Fig. 7C) and H3K27ac (Fig. 4D) in MCF10A and A549 cells with and without *KDM7A* knockdown. PCR amplicons at the 3′ untranslated regions (UTR) of select



(caption on next page)

Fig. 4. KDM7A regulates cell migration and the expression of TGF-β-induced EMT markers. A. MCF10A and A549 cells with control (shNC) and KDM7A knockdown were subjected to a transwell migration assay with TGF-β1 (5 ng/ml) treatment for 48 h. Bar graph (lower panel) shows the numbers of migrated cells after 24 h, quantified by counting 5 random vision fields in a microscope (magnification: ×100). B and C. The mRNA and protein expression of EMT-related markers *SNAI1*, *FN1* and *CDH2* were determined by real-time qPCR and western blotting, respectively. *RPL13A* and γ-*TUBULIN* were used as endogenous controls. D. Enrichment of H3K27ac on the TSSs of *SNAI1*, *FN1* and *CDH2* analyzed by ChIP-qPCR. Relative enrichment represents average fold enrichment of the target promoter in immunoprecipitation (IP) vs. input, normalized to the UTR region of each target respectively. Values are represented by means ± SD from four independent ChIP experiments. Student's *t*-test was performed. * indicates significant difference between TGF-β-treated group and control group within the same stable cell line; # indicates significant difference between the shKDM7A cell line and the shRNA control cell line with the same treatment. * and #: $p < 0.05$, ** and ##: $p < 0.01$. TSS, transcription starting sites; UTR, 3′ untranslated region.

genes were used to normalize the enrichment of H3K27ac at the transcription start sites (TSS). Relative enrichments each histone mark at both TSS and the UTRs were normalized to the input materials. ChIP results are presented as the fold change of enrichment at TSS over UTRs.

In general, the levels of H3K27ac at the TSS of *FN1* and *CDH2* are concordant with their basal expression and the upregulation by TGF-β in MCF10A and A549 cells (Fig. 4D and Supplemental Fig. 7). In contrast, H3K27ac at the TSS of *SNAI1* is not dramatically changed, despite the mRNA levels being drastically induced by TGF-β (Fig. 4D and B), implicating that the upregulation of *SNAI1* may use an alternative mechanism other than upregulation of H3K27ac. Notably, KDM7A knockdown significantly attenuated the TGF-β-induced upregulation of H3K27ac levels at the TSS of *FN1* and *CDH2* (Fig. 4D). Compared with acetylation, di-methylation marks (H3K9me2 and H3K27me2) were not obviously changed by KDM7A depletion (Supplementary Fig. 7B). Decreased H3K9me2 levels upon TGF-β treatment were only observed on TSS of *SNAI1* in MCF10A cells (Supplementary Fig. 7B). In contrast, H3K27me2 reductions upon TGF-β treatment were shown on the TSS of *FN1* and *SNAI1* in MCF10A and A549 cells, respectively (Supplementary Fig. 7C). KDM7A knockdown increased H3K9me2 at the TSS of *FN1*, *CDH2* and *SNAI1* in A549 cells at steady states. However, H3K27me2 levels were not affected by KDM7A knockdown in both cell lines (Supplementary Fig. 7C). These data implicate that KDM7A may regulate H3K27ac through other mechanisms, for example, KDM7A may regulate acetyltransferase. In sum, our data validated the regulation of *KDM7A* by TGF-β, revealed its promigratory role in context of TGF-β signaling and partially explained the mechanisms by which KDM7A indirectly regulates H3K27ac.

4. Discussion

Genome-wide expression analyses to identify TGF-β-regulated genes have greatly advanced our understanding of the molecular mechanisms underlying TGF-β signaling. Recent RNA-seq data from MCF10A [17], MCF10A MII [12] and A549 cells [11,36] generated a large dataset of TGF-β-regulated genes, however, the impact of these genes on the cellular function of TGF-β signaling was not well studied. Here, we used systematic analyses of RNA-seq data from MCF10A cells treated by TGF-β for short (1.5 h) and extended (up to 72 h) time periods to generate a unique dataset that identified novel TGF-β-regulated genes and their pattern of expression, and to determine the conservation of this regulation in different cell types. Indeed, the results of several datasets confirm that TGF-β regulates many genes (including MICAL family members and KDM7A). Importantly, a pattern of regulation was achieved for the *MICAL1* and *MICALL2* genes, which are regulated starting 24 h after TGF-β treatment in MCF10A cells, with similar regulation observed in A549 cells, with the exception for an early response for *MICALL2*. The expression pattern of selected histone modifying enzymes is mostly conserved in MCF10 MII cells, but only *KDM6B*, *KDM7A* and *HR* are regulated in A549 cells. Although MCF10A MII cells (Ha-Ras transformed MCF10A cells) were used mimicking breast cancer cells [12], *MYC* was still downregulated by TGF-β in both cell types, suggesting MCF10A and MCF10A MII cells share a conserved regulatory mechanism. This is supported by the consistent regulation of histone modifying enzymes in both cell lines. Moreover, *KDM6B*, *KDM7B* and *HR* are also regulated by TGF-β in A549 cells, suggesting it is unlikely that these genes are under control of *MYC*.

Our study demonstrates that the regulation by TGF-β of three MICAL family members is conserved in MCF10A, MCF10A MII and A549 cells. The role of MICAL1 in TGF-β-induced cell migration suggests that MICAL2 and MICALL2 are novel effectors downstream TGF-β-signaling. In fact, the tumor-promoting function of MICAL2, especially, its role in EMT, was recently reviewed [37]. Although, MICAL2 can promote breast cancer cell migration by maintaining EGFR stability and activating EGFR/P38 pathway [40], it is possible that in EMT, MICAL2 regulates cell migration by mediating TGF-β signaling. Moreover, the malignancy promoting role of MICALL2 in EMT in ovarian cancer [48] also support our hypothesis that MICALL2 is a novel downstream effector of TGF-β signaling. Taken together, the finding that TGF-β regulates MICAL family members paves the way for further studies that determine the precise downstream function of TGF-β signaling in EMT. Moreover, their functional redundancy in TGF-β signaling needs to be addressed.

The genome-wide reprogramming of genes induced by TGF-β requires coordinated chromatin changes that include a gain in activated histone marks for upregulated genes and a loss of repressive marks for downregulated genes [13]. During EMT, H3K4me3 and H3K27me3 are dynamically regulated histone marks [49,50]. However, how they are regulated is not fully understood. The histone demethylase, KDM6B, acts on H3K27me3/2 and is regulated by TGF-β [6]. It was later found that KDM6B interacts with SMAD3 to regulate TGF-β-responsive genes in neural stem cells [15]. As KDM6B cannot erase all the methyl groups from H3K27, another histone demethylase is still needed to prime H3K27 for acetylation. KDM7A, a histone demethylase, erases H3K27me2 and me1 [45], and its ability to collaborate with KDM6B in transcriptional elongation [47] support our hypothesis that both enzymes are functionally required for gene activation induced by TGF-β. Although, our findings that the loss of KMD7A function attenuated H3K27ac on TGF-β-activated genes support this hypothesis, our data could not prove the demethylation activity of KDM7A on H3K27me2 plays a role. It is possible that KDM7A directly regulates the expression of acetyltransferase. Moreover, KDM7A knockout mouse model will be a valuable tool to further study the pro-migratory role of this gene in vivo. It is important to notice that another KDM7 family member, PHF8, also participates in gene activation under TGF-β signaling [17]. Notably, PHF8 and KMD7A exhibit differential patterns of regulation, e.g. PHF8 is upregulated early and downregulated later following treatment with TGF-β, whereas *KDM7A* is steadily upregulated 24 h after treatment with TGF-β. Therefore, whether and how these two enzymes regulate the same histone methylation marks on the same genes remains to be studied. As both PHF8 and KDM7A have demethylation activity on H4K20me1 and H3K9me2 [16], genome-wide ChIP-seq combined with gene expression analysis are sound methods to determine the detailed mechanisms by which KDM7 family members participate in the transcriptional regulation downstream of TGF-β signaling. Taken together, our study revealed the function of two novel TGF-β-regulated genes, paving the way for future studies that will identify the detailed mechanisms by which they contribute to cancer development.

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Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of competing interest

The Authors disclose no conflicts of interest.

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