Supplementary Materials for

Human skin long non-coding RNA *WAKMAR1* regulates wound healing by enhancing keratinocyte migration

Dongqing Li, Lara Kular, Manika Vij, Eva K. Herter, Xi Li, Aoxue Wang, Tongbin Chu, Maria-Alexandra Toma, Letian Zhang, Eleni Liapi, Ana Mota, Lennart Blomqvist, Irène Gallais Sérézal, Ola Rollman, Jakob D. Wikstrom, Magda Bienko, David Berglund, Mona Ståhle, Pehr Sommar, Maja Jagodic, Ning Xu Landén*

*Correspondence to: N.X.L. (ning.xu@ki.se)

This PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplemental experimental procedures

Human wound samples Two centers provided human samples for this study: normal wounds (No. 1-14 in Table S1) and venous ulcer samples (VU, No. 1-10 in Table S2) from Caucasian donors were collected at Karolinska University Hospital (KUH, Stockholm, Sweden); normal wounds (No. 15-22 in Table S1) and diabetic foot ulcers (DFU, No. 1-29 in Table S3) from Asian donors were collected at the Second Hospital of Dalian Medical University (Dalian, China). The DFU and VU patients were always compared with the healthy donors with same ethnicity.

We enrolled patients with non-healing VU or DFU that, despite conventional therapy, persisted for more than two months. Patients with apparent soft tissue infection and need of systemic antibiotics, patients taking systemic antibiotics one day prior to biopsy, as well as immunocompromised patients were excluded. Tissue samples were taken using a 4 mm biopsy punch at the non-healing edges of chronic wounds after local lidocaine injection (Fig. S2).

The exclusion criteria for healthy donors were diabetes, skin disease, unstable heart disease, infections, bleeding disorder, immune suppression, and any on-going medical treatments. One or two full-thickness excisional wounds were created using a 4 mm biopsy punch for each donor and the wound-edge skin was collected using a 6 mm biopsy punch 1 and 7 days later (Fig. S2). Local lidocaine injection was used for anesthesia while sampling.

To establish human *ex vivo* skin wound model, human skin was obtained from abdominal reduction surgeries (No. 23-28 in Table S1) at KUH, Sweden.

Written informed consent was obtained from all donors for the collection and use of clinical samples. The study was approved by the Stockholm Regional Ethics Committee (Stockholm, Sweden) and the Ethics Committee of the Second Hospital of Dalian Medical University (Dalian, China). The study was conducted according to the Declaration of Helsinki's principles.

Cell culture and treatments Adult human epidermal keratinocytes were cultured in EpiLife serum-free keratinocyte growth medium supplemented with Human Keratinocyte Growth Supplement (HKGS) at a final Ca^{2+} concentration of 0.06 mM and Pen Strep (100 units/mL Penicillin and 100 µg/mL Streptomycin) (ThermoFisher Scientific) at 37 ℃ in 5% CO_2.

To determine which RNA polymerase transcribes WAKMAR1 and to study its stability, α-amanitin (5 µg/mL, Sigma-Aldrich) or actinomycin-D (5 µg/mL, Sigma-Aldrich) were used to treat keratinocytes for 2-8 hours. qPCR were performed to analyze gene expression. RNA quality was measured by 2100 Bioanalyzer Instrument (Agilent).

To study the mechanism regulating WAKMAR1 expression, keratinocytes were treated with different cytokines and growth factors (Table S4) or PBS as control for 24 hours. WAKMAR1 expression was analyzed by qPCR. 15 µM TGF-β receptor inhibitor SB431542 (Tocris) and/or 5 µM DMH1 (Sigma) was applied 15 minutes before adding TGF-β2 and/or BMP2 to the cells.

To study the biological function of WAKMAR1, third passage keratinocytes at 60-70% confluence were transfected with 20 nM LNA-long RNA-GapmeR-antisense-oligonucleotide (GapmeR) targeting WAKMAR1 or negative control oligos (Exiqon) for 24 hours with Lipofectamine™ 2000 (ThermoFisher Scientific). Keratinocytes were transfected with 20 nM gene-specific siRNAs for 24 hours to deplete CDK6, HMMR, E2F1, KIF11, FOS, RAD21, NIBPL (ON-Target plus Human siRNA-smart pool; Dharmacon) or SMAD1, SMAD3, SMAD4 (Silencer Select; ThermoFisher Scientific) (Table S4).

RNA extraction and qPCR Skin biopsies were homogenized using TissueLyser LT (Qiagen) prior to RNA extraction. Total RNA was extracted from human tissues and cells using the miRNeasy Mini kit (Qiagen) or Trizol reagent (ThermoFisher Scientific). Reverse transcription was performed by using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Gene expression was determined by SYBR Green expression assays (ThermoFisher Scientific) and normalized based on the values of the housekeeping gene 18S or RPLP0. Information for all the primers used in this study is listed in Table S4.

Magnetic cell separation After washed in PBS, the skin and wound tissues were incubated in dispase II (5 U/mL, ThermoFisher Scientific) at 4 ℃ overnight, and epidermis and dermis were separated. The epidermis was cut into small pieces and digested in Trypsin/EDTA Solution at 37°C for 15 min. CD45- and CD45 + cells were separated by using CD45 Microbeads together with MACS MS magnetic columns according to the manufacturer's instructions (Miltenyi Biotec). The dermis was incubated with enzyme mix from human Whole Skin Dissociation Kit (Miltenyi Biotec.) at 37°C for 3 hours and processed by Medicon tissue disruptor (BD Biosciences). Dermal cell suspension was pelleted and resuspended in PB buffer. CD90 (fibroblasts), CD14 (macrophages) and CD3 (T cells) Microbeads were incubated with the cell suspension and separated with MACS MS magnetic columns.

In situ hybridization WAKMAR1 probe was designed and synthesized by Advanced Cell Diagnostics (ACD). The keratinocytes were cultured on slides and fixed in cold 4% formaldehyde for 15 minutes. After dehydrate with 50%, 70% and 100% ethanol, the slide was incubated with Protease III (ACD) at room temperature for 20 min. Then the slide was incubated with WAKMAR1 probes for two hours at 40 degree in HybEZ™ II Hybridization System by using RNAscope® Multiplex Fluorescent Reagent Kit v2. The hybridization signals were amplified via sequential hybridization of amplifiers and label probes. WAKMAR1 signals were visualized on Zeiss LSM800 confocal microscopy.

CRISPR-SAM CRISPR/Cas9 Synergistic Activation Mediator (SAM) plasmids, i.e., sgRNA (MS2) cloning backbone (Plasmid #61424), MS2-P65-HSF1_GFP (Plasmid #61423) and dCAS9-VP64_GFP (Plasmid #61422), were purchased from Addgene. 1000-bp region upon WAKMAR1 transcription starting site was used to design sgRNAs by using gene activation

sgRNA design tool (http://crispr.mit.edu/). Individual SAM sgRNA expression plasmid was constructed by first annealing each pair of oligos and then ligating them to BbsI-linearized sgRNA (MS2) cloning backbone according to SAM target sgRNA cloning protocol (http://sam.genome-engineering.org) (1). Correct insertion was verified by sequencing. The sequences of sgRNAs were shown in Supplementary Table S4. The third passage keratinocytes at 60-70% confluence were transfected with these three plasmids (200ng each plasmid per well in 24-well plate) for 48 hours with Lipofectamine™ 2000 (ThermoFisher Scientific). The expression of WAKMAR1 was confirmed by qPCR.

Analysis of cell motility Human primary keratinocytes were plated in Essen ImageLock 96-well plates (Essen Bioscience) at 15,000 cells per well and transfected with 20 nM WAKMAR1 GapmeRs or 20 nM siRNAs or 50 ng WAKMAR1 CRISPR-SAM plasmids for 24 hours. Cells were pretreated with Mitomycin D and confluent cell layers were scratched using Essen wound maker to generate wounds with approximately 800 μm width. After washed with PBS, the ImageLock 96-well plates were placed into IncuCyte (Essen Bioscience) and imaged every 2 hours. The photographs were analyzed by using the IncuCyte software (Essen Bioscience).

Transwell migration assay was performed using an 8 μm BD Chamber (BD Falcon). Keratinocytes were transfected with 20 nM WAKMAR1 GapmeRs or control oligos for 24 hours. 1×10^5 transfected keratinocytes in serum-free Epilife medium were placed into the upper chamber of the insert. Medium containing HKGS was added to the lower chamber. 24 hours later, the cells migrating through the chamber membrane were stained with 0.1% crystal violet and counted under microscope.

Analysis of cell proliferation and viability Keratinocytes were transfected with WAKMAR1 GapmeR or WAKMAR1 sgRNA for 48 hours. 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay was carried out to assess keratinocyte proliferation by using Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Cell viability was accessed by using the MTS Assay Kit (Abcam) according to the manufacturer's instructions.

Human *ex vivo* wound model Human skin was obtained from abdominal reduction surgeries (healthy donor No. 23-28 in Table S1). A 2 mm biopsy punch was used to make an excisional wound on the epidermal side of the skin. The injured skin was excised using an 8 mm biopsy punch and cultured in DMEM medium plus 10% fetal bovine serum and antibiotics (penicillin 100 units/mL and streptomycin 100 μg/mL, ThermoFisher Scientific). 0.1 nmol WAKMAR1 GapmeRs or control oligos (Exiqon) were dissolved in 30% pluronic F-127 gel (Sigma-Aldrich) and topically applied on the wounds immediately following injury and then on every other day. Wound samples were collected for histological and qPCR analysis at the indicated time points after injury.

Gene expression microarray and analysis Expression profiling of primary keratinocytes transfected with either control GapmeR or WAKMAR1 GapmeR1 for 24 hours (n = 3 per group) was performed using Affymetrix Genechip HuGene-2_1-st at the core facility for Bioinformatics and Expression Analysis at Karolinska Institutet. In brief, total RNA was extracted using the miRNeasy Mini Kit (Qiagen) and RNA quality and quantity were determined using Agilent 2200 Tapestation with RNA ScreenTape and Nanodrop 1000. 150 ng of total RNA were used to prepare cDNA following the GeneChip WT PLUS Reagent Kit labelling protocol. Standardized array processing procedures recommended by Affymetrix including hybridization, fluidics processing and scanning were used. Genes showing at least 1.3-fold regulation and P value less than 0.05 were considered to be differentially expressed. Gene set enrichment analysis (GSEA) was performed using public software from Broad Institute (2). Cytoscape (Version 3.0.1) was applied to visualize the WAKMAR1-regulated gene network. MetaCore software (Thomson Reuters) was used to analyze transcription factors with overrepresented targets in this gene network. Heatmaps were generated with Multiple Experiment Viewer software.

Laser capture microdissection (LCM) Frozen wound biopsies were embedded in Tissue-Tek O.C.T. Compound (ThermoFisher Scientific) and cut into 8-µm sections. After stained with hematoxylin, laser capture microdissection was performed with Leica LMD7000 (Leica). RNA was purified using the miRNAeasy mini Kit (Qiagen).

Cell fractionation Cytoplasm and nucleus of keratinocytes were separated by using Nuclear Extract Kit (Active Motif) following the manufacturer's instructions. RNA was extracted from these fractions using Trizol (ThermoFisher Scientific). qPCR were performed to analyze the WAKMAR1, MALAT1 and HPRT expression.

WAKMAR1 polyadenylation study Total RNA was extracted from keratinocytes by using Trizol (ThermoFisher Scientific). Poly(A)+ and poly(A)- RNA was separated by Dynabeads™ mRNA Purification Kit (ThermoFisher Scientific) according the manufacturer's instructions. Briefly, total RNA was incubated with the Dynabeads®/binding buffer suspension at room temperature for 3-5 minutes and the reaction tubes were placed on a magnet until solution was clear. The supernatant containing poly(A)- RNA was saved. The beads with poly(A)+ RNA were washed three times with washing buffer. RNA was extracted from the supernatant and beads respectively using Trizol. qPCR were performed to analyze the WAKMAR1, ACTB and HIST1H1D expression in the Poly(A)+ and poly(A)- RNA fractions.

Methyl-sensitive restriction enzyme-qPCR (MSRE-qPCR) For DNA methylation analysis, three CpGs giving robust MSRE-qPCR assays (CpG1_chr20:32273183, CpG5_chr20:32273576 and CpG6_chr20:32273924, hg19) were selected (Fig. 6B, Table S4). CpG1 and CpG5 are located within the predicted active promoter of E2F1 gene in keratinocytes

(ENCODE: chr20:32273140-32273739, hg19) while CpG6 is located within a weak promoter-associated region in NHEK cells. The CpG methylation-sensitive digestion of genomic DNA at the promoter region of E2F1 was carried out with the EpiJET DNA Methylation Analysis Kit based on *Msp*I/*Hpa*II digestion (ThermoFisher Scientific) using 150 ng of genomic DNA. Briefly, *HpaII* cuts only unmethylated CCGG motif whereas *MspI* cut both unmethylated and methylated CCGG equally (positive control). Of note, while CpG1 and CpG5 each represents one CCGG motive, CpG6 includes two CCGG motives. The methylation levels were quantified by qPCR on a BioRad CFX384/C1000 Real-Time Detection System with a three-step protocol, SYBR green fluorophore, the 2-ΔCt method, and expressed as the ratio between *HpaII*-digested DNA (target) and input/non-digested DNA.

Bisulfite (BS)-pyrosequencing A region encompassing the differentially methylated CpG1 identified using MSRE-qPCR was selected for bisulfite (BS)-pyrosequencing validation. Bisulfite-pyrosequencing assays of four CpGs, i.e., CpG1-4, within a 100 bp fragment (GRCh37/hg19, chr20:32273175-32273283, Fig. 6B) were designed using PyroMark Design software (Qiagen). Location of CpGs and sequences of the forward, 5'-biotinylated reverse and sequencing primers are listed in Table S4. Of note, the extremely high GC content (79%) and observed-to-expected CpG ratio (0.87) of the CpG island encompassing CpG5 and CpG6 regions targeted by MSRE-qPCR impeded design of robust pyrosequencing assay needed for locus-specific BS-based methodology. Genomic DNA (500 ng) was BS-converted using DNA methylation-Gold Bisulfite Kit (Zymo Research) and eluted in 25 µl of elution buffer. BS-DNA (~15 ng) was applied as a template in the PCR performed with the PyroMarks PCR kit (Qiagen) following manufacturer's recommendations. To verify the efficiency and sensitivity of the pyrosequencing, Epitect unmethylated, partially methylated and fully methylated human BS-DNA samples (Qiagen) were included as controls. The entire PCR product, 4 pmol of the sequencing primer, and streptavidin sepharose high-performance beads (GR Healthcare) were used for pyrosequencing on the PSQ 96 system and PyroMark Gold 96 reagent kit (Qiagen). The PyroMark CpG software 1.0.11 (Qiagen) served for data analysis with results presented as percentage of DNA methylation at each CpG.

RNA immunoprecipitation (RIP) assay RIP assay was performed as previously described (3). Briefly, keratinocytes were resuspended in polysome lysis buffer [100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.0), 0.5% NP40, 1 mM DTT, 100 units/mL RNase Out, 400 µM Ribonucleoside-vanadyl complex (VRC) and protease inhibitor cocktail] for 10 min on ice. The cell lysates were incubated with antibodies or control IgG coated-protein G beads for 4 hours at 4 °C tumbling end over end. The beads were washed with ice cold NT2 buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 0.05% NP40]. RNA was extracted from the beads by using Trizol and qPCR was performed to analyze WAKMAR1 or U1 snRNA expression.

Chromatin immunoprecipitation (ChIP) assay Keratinocytes were transfected with WAKMAR1 GapmeRs or Control oligos for 24 hours, or CRISPR/Cas9-SAM plasmids for 48

hours, then crosslinked with 1% formaldehyde and quenched by 0.125 M glycine. The cells were collected and resuspended with lysis buffer I (5 mM PIPES pH 8, 85 mM KCl, 0,5 % NP40). Cell nuclei were collected and resuspend in lysis buffer II (10 mM Tris, pH 7,5, 150 mM NaCl, 1% NP-40, 1% sodium-desoxycholate, 0,1 % SDS, 1 mM EDTA). Cell lysates were sonicated to achieve the majority of DNA fragments with 100-300 bp by using Bioruptor (Diagenode). 15µl Protein A-Dynabeads (Invitrogen) were mixed with 15µl Protein G-Dynabeads (Invitrogen), and then DNMT antibodies or IgG control (Supplementary Table S4) were added to the beads and incubated at 4 °C overnight. Sonicated cell lysates were incubated with the antibody-coated beads at 4 °C for 6 hours. After washing, genomic DNA was purified by phenol/chloroform extraction and ethanol precipitation, analyzed by qPCR with the primers listed in Table S4.

Statistics Statistical significance was determined by two tailed Student's t-test or Mann-Whitney U Test. The significance among multiple groups were determined by One-way or Two-way ANOVA with Bonferroni posttest using GraphPad Prism Version 6. The P-values of GSEA analysis were calculated by using Fisher's exact test. Pearson's correlation test on log10-transformed data was performed by using GraphPad Prism Version 6. P-value < 0.05 was considered to be statistically significant.

Supplemental references

1. Joung J*, et al.* (2017) Genome-scale activation screen identifies a lncRNA locus regulating a gene neighbourhood. *Nature* 548(7667):343-346.
2. Subramanian A*, et al.* (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 102(43):15545-15550.
3. Keene JD, Komisarow JM, & Friedersdorf MB (2006) RIP-Chip: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts. *Nat Protoc* 1(1):302-307.

Supplementary figures and legends:

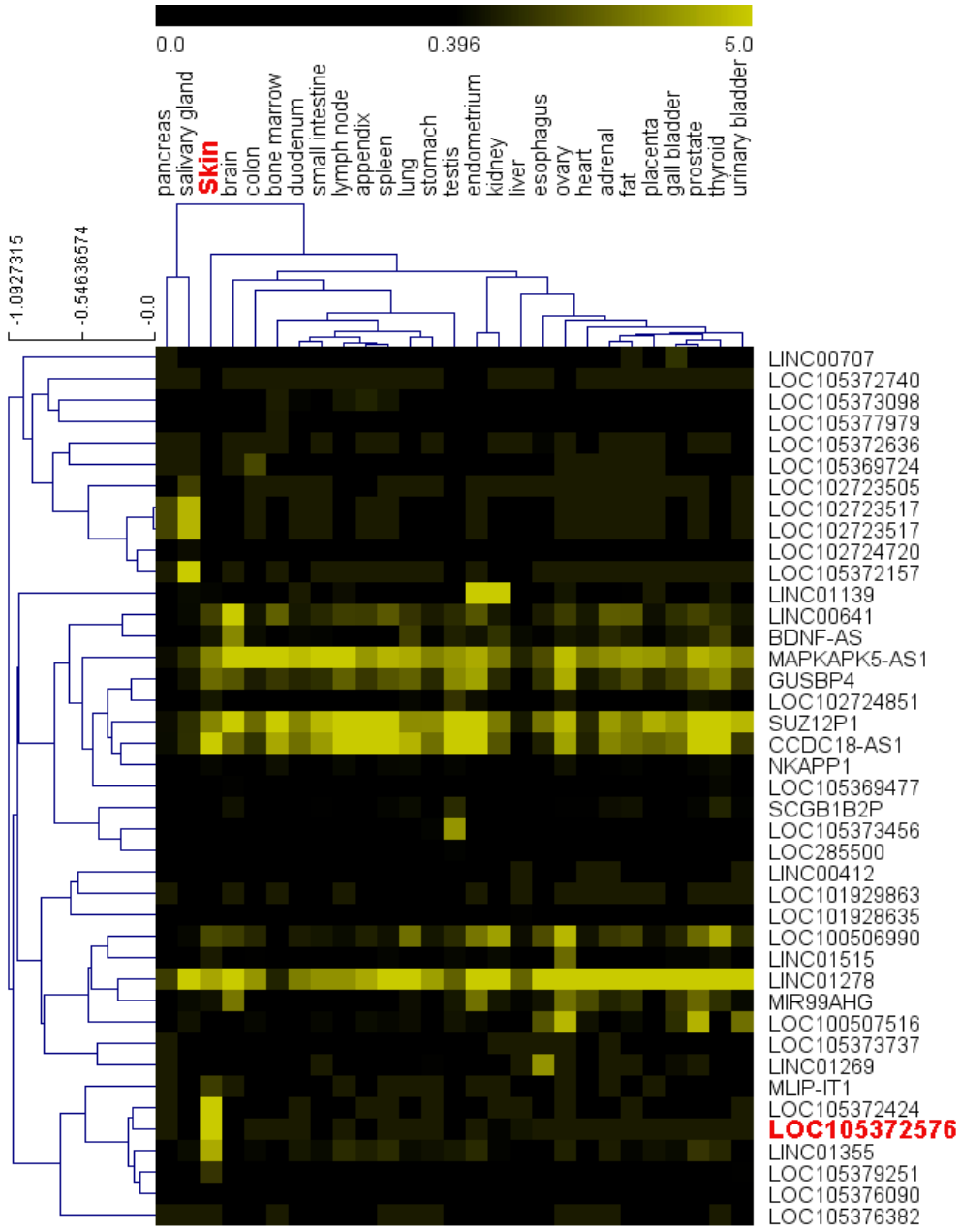

Fig. S1. WAKMAR1 / LOC105372576 is a skin specific lncRNA. Heatmap illustrates average expression levels of lncRNAs, which are differentially expressed (fold change ≥ 3, FDR <0.01) between diabetic foot ulcer and foot skin (GEO: GSE80178), in 27 different human tissues from 95 individuals. The data were extracted from the HPA RNA-sequencing dataset and presented as reads per kilobase per million reads (RPKM).

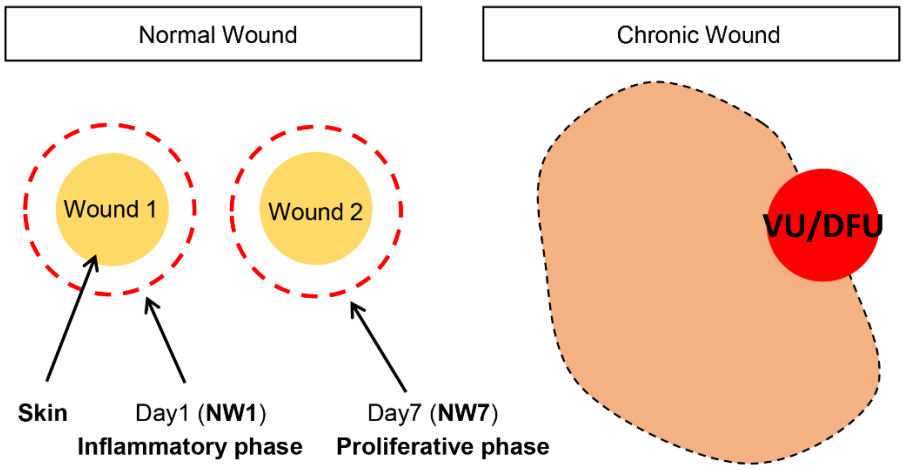

Fig. S2. Collection of human wound biopsies. On the skin of each healthy volunteer, one or two 4 mm-excisional wounds were created and the excised skin was saved as a baseline control. The wound-edges were collected with a 6 mm biopsy punch one (NW1) or seven days later (NW7), which time points were chosen to represent the inflammatory phase and the proliferative phase of wound healing, respectively. Chronic wound biopsies were collected using a 4 mm biopsy punch at non-healing wound-edges of venous ulcers (VU) or diabetic foot ulcers (DFU).

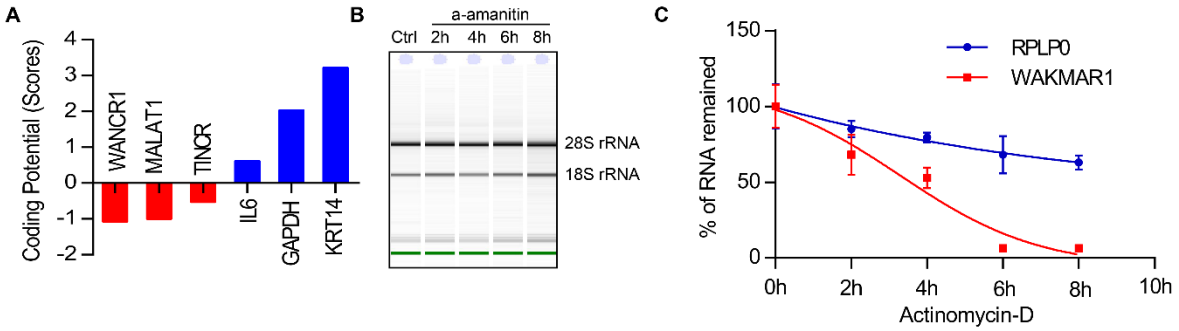

Fig. S3. Characterization of lncRNA WAKMAR1. (**A**) The coding potential of WAKMAR1, MALAT1, TINCR, IL6, GAPDH and KRT14 is analyzed by using Coding Potential Calculator (http://cpc2.cbi.pku.edu.cn/). (**B**) Agilent bioanalyzer analysis shows equal expression levels of 18S and 28S rRNA in keratinocytes treated without (Ctrl) or with α-amanitin (5 μg/ml) for 2-8 hours. (**C**) qPCR analysis of WAKMAR1 and RPLP0 mRNA in keratinocytes treated without or with Actinomycin-D (5 μg/ml) for 2-8 hours (n = 3). Data are presented as mean ± s.d.

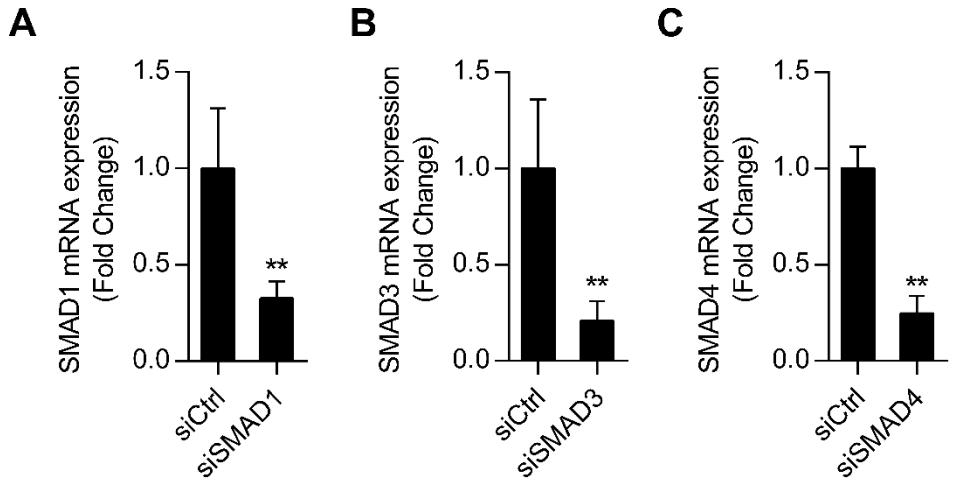

Fig. S4. Silencing SMAD expression in human primary keratinocytes. qPCR analysis of SMAD1 (**A**), SMAD3 (**B**) and SMAD4 (**C**) mRNA expression in keratinocytes transfected with specific siRNAs for 24 hours. ***P*<0.01, unpaired two-tailed Student's t-test. Data are presented as mean + s.d.

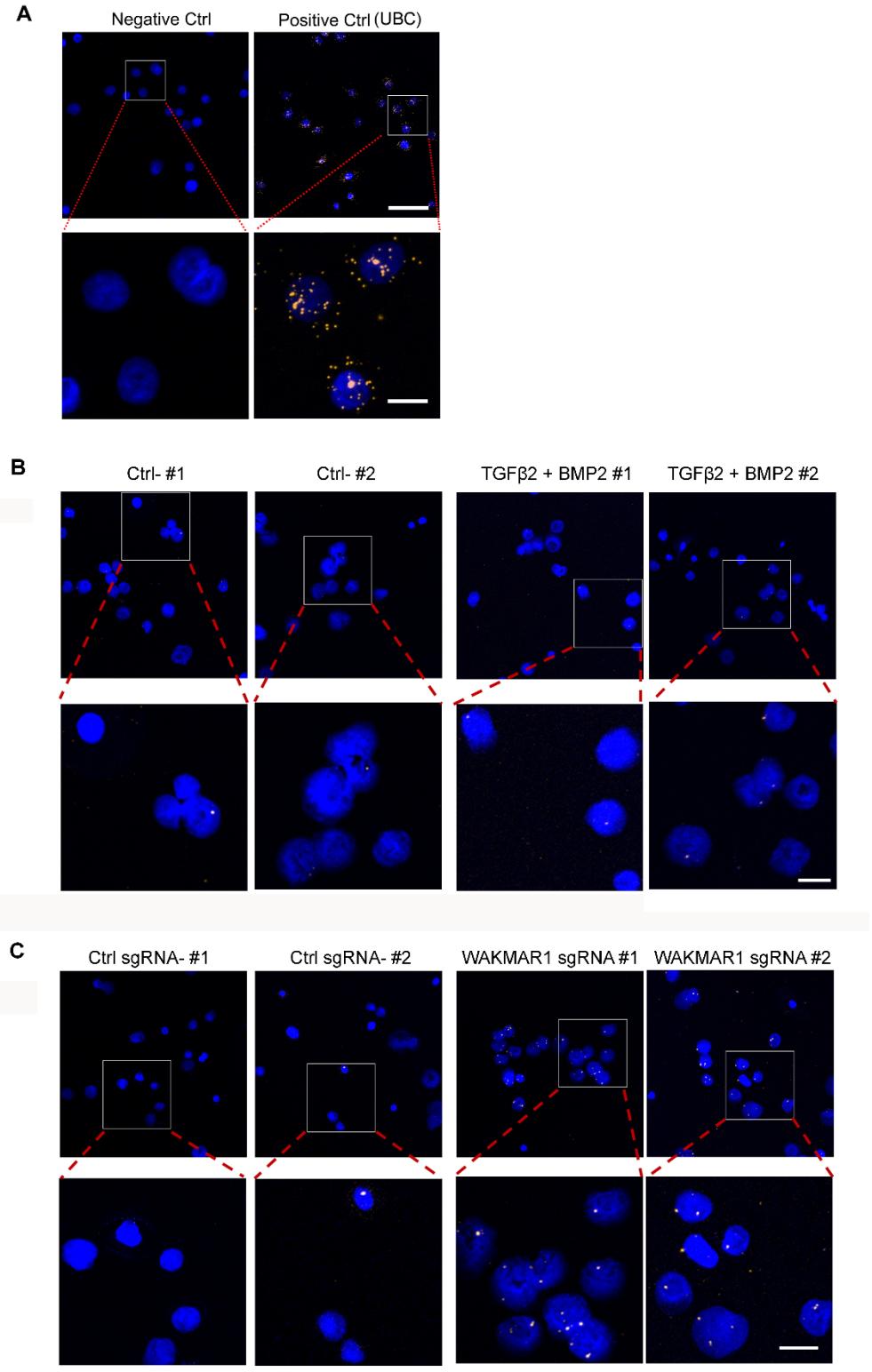

Fig. S5. In situ hybridization of WAKMAR1 in keratinocytes. (**A**) In situ hybridization analysis in human primary keratinocytes using a universal negative control probe, which targets the DapB gene from the Bacillus subtilis strain SMY, and a positive control probe targeting ubiquitin C (UBC). In situ hybridization of WAKMAR1 in keratinocytes treated with TGF-β2 and BMP2 for 24 hours (**B**), and in keratinocytes transfected with CRISPR/Cas9-SAM plasmids for 48 hours (**C**). Cell nuclei were co-stained with 4',6-diamidino-2-phenylindole (DAPI). Scale bar = 50 μm.

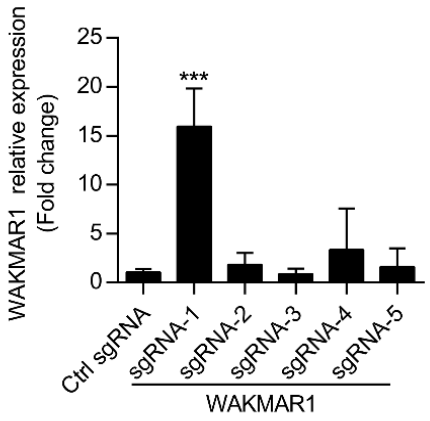

Fig. S6. Activation of endogenous WAKMAR1 expression in human primary keratinocytes with CRISPR-SAM system. qPCR analysis of WAKMAR1 expression in keratinocytes co-transfected with MS2-P65-HSF1_GFP plasmid, dCAS9-VP64_GFP plasmid and one of the five different sgRNA plasmids for 48 hours. *** $P<0.001$, one-way ANOVA test. Data are presented as mean + s.d..

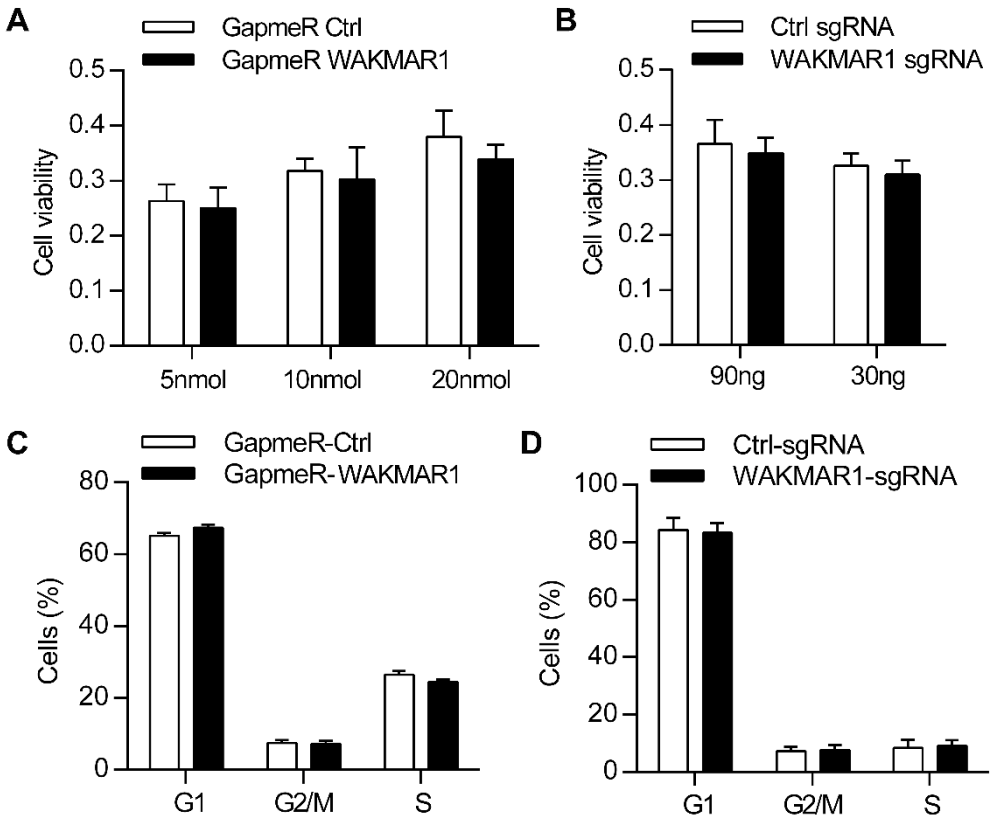

Fig. S7. Analysis of cell proliferation and viability in keratinocytes with WAKMAR1 inhibition or overexpression. MTS assay was performed in keratinocytes transfected with WAKMAR1 or control GapmeRs (**A**), or with CRISPR/Cas9-SAM plasmids (**B**) to evaluate cell viability. 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay was performed in keratinocytes transfected with WAKMAR1 GapmeRs (**C**), or with CRISPR/Cas9-SAM plasmids (**D**) to analyze cell proliferation.

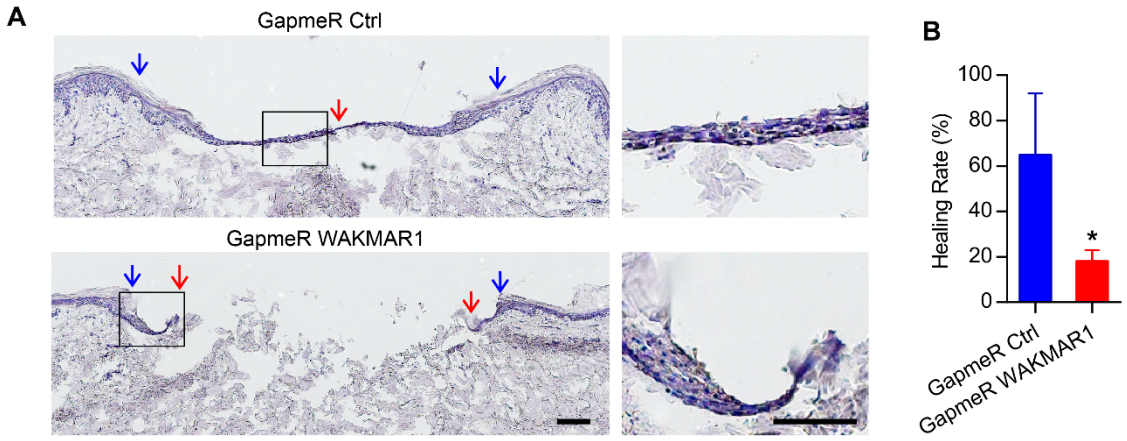

Fig. S8. WAKMAR1 regulates re-epithelialization of human ex vivo wounds. (**A**) Representative photographs of hematoxylin and eosin staining of day-5 human ex vivo wounds with topical treatment of WAKMAR1 GapmeRs. Blue arrows demarcate the initial wound edges, while the red arrows indicate a newly formed epidermis. Scale bar = 200 μm. (**B**) Re-epithelialization was quantified as healing rate = 100% – the percentage of the initial wound size. (n = 4 donors) *P<0.05, unpaired two-tailed Student's t-test. Data are presented as mean + s.d.

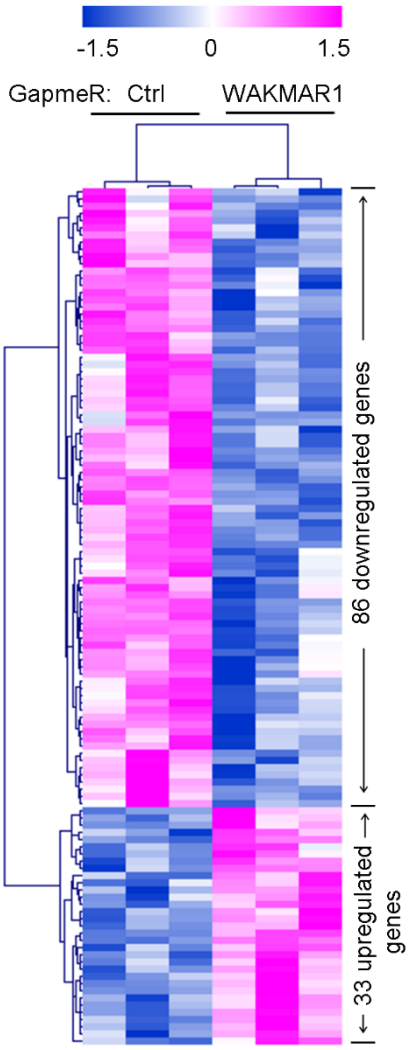

Fig. S9. Microarray analysis of human keratinocytes with WAKMAR1 knockdown. Microarray was performed in human primary keratinocytes transfected with WAKMAR1 or control (Ctrl) GapmeRs (n = 3). Heatmap illustrates the differentially expressed genes (absolute fold change ≥ 1.3, P value < 0.05). Z-score transformation was applied for visualization.

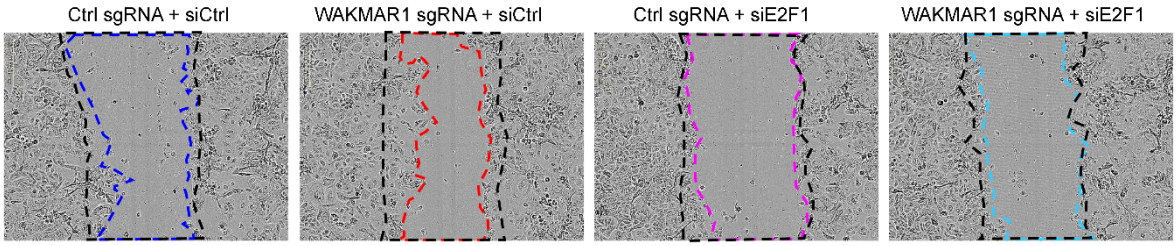

Fig. S10. Scratch wound assay of keratinocytes after WAKMAR1 knockdown and/or E2F1 silencing. Keratinocytes were co-transfected with WAKMAR1 CRISPR/Cas9-SAM plasmids with E2F1 or control siRNAs for 24 hours. Representative photographs of wounds 20 hours after scratch are shown. Black dot line indiactes the wound boundaries immediately after scratch, while the colorful dot lines demarcate the wound boundaries 20 hours later.

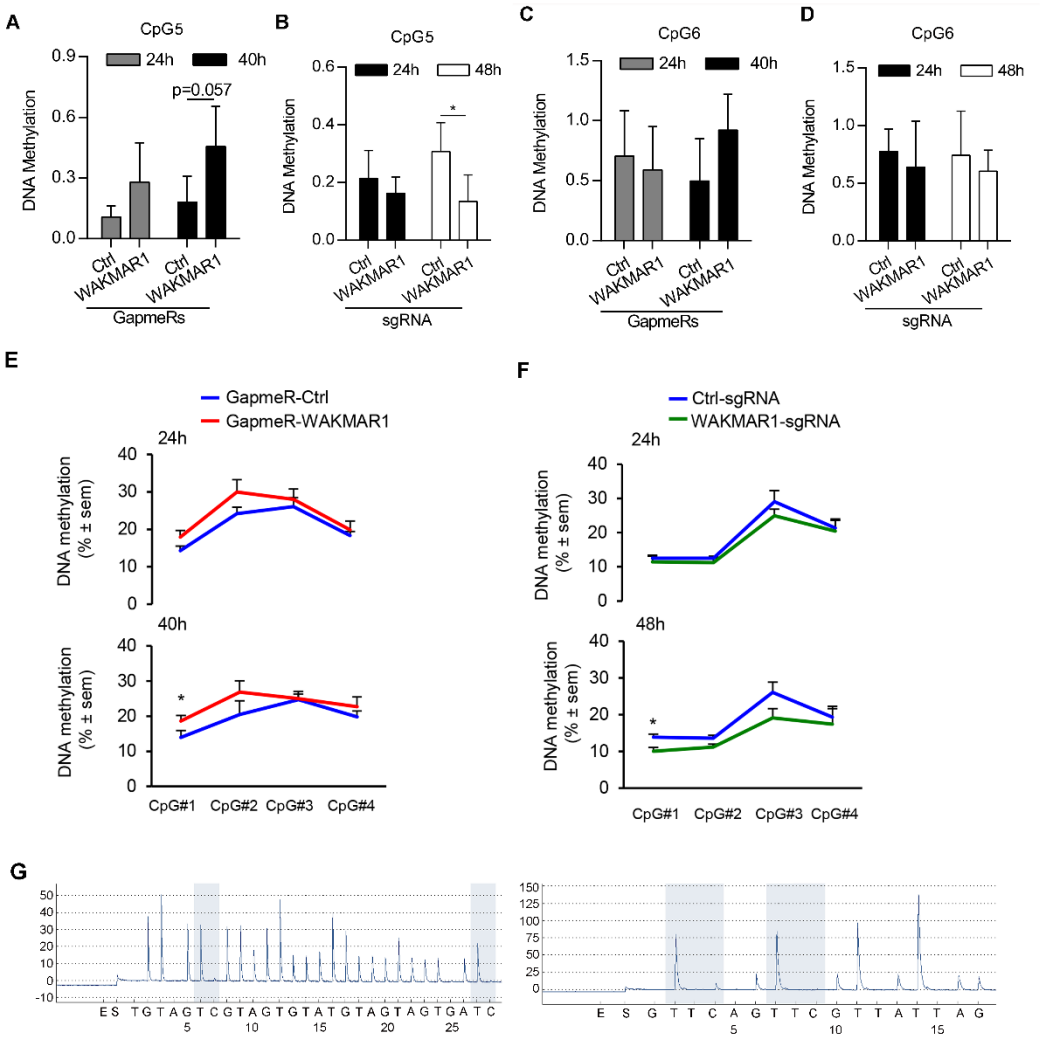

Fig. S11. WAKMAR1 negatively regulates E2F1 promoter methylation. MSRE-qPCR analysis of DNA methylation at CpG5 (**A, B**) and CpG6 (**C, D**) in keratinocytes transfected with WAKMAR1 GapmeR for 24 and 40 hours (n = 6) (**A, C**), or CRISPR/Cas9-SAM plasmids for 24 and 48 hours (n = 6) (**B, D**). Bisulfite-pyrosequencing of CpGs including MSRE-qPCR-CpG1 in keratinocytes transfected with WAKMAR1 GapmeR (**E**) or CRISPR/Cas9-SAM plasmids (**F**) for 24 and 48 hours (representative experiments with n = 5-6 replicates per condition). (**G**) Representative pyrograms for the pyrosequencing assays targeting CpG1-2 (left) and CpG3-4 (right). *P<0.05, unpaired two-tailed Student's t-test. Data are presented as mean + s.d (**A-D**), or mean + s.e.m. (**E, F**).

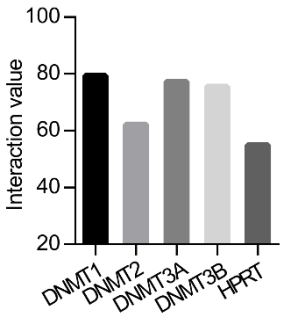

Fig. S12. LncPro prediction of the interaction of WAKMAR1 with DNMT1, DNMT2, DNMT3A, DNMT3B or HPRT.

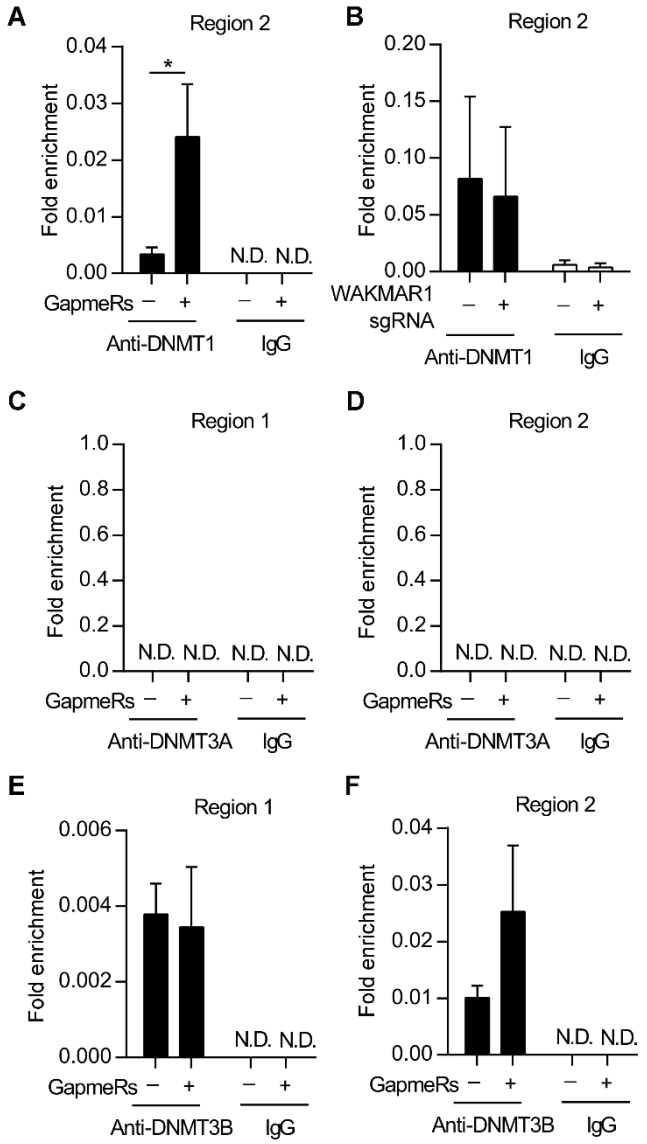

Fig. S13. WAKMAR1 suppresses DNMT binding to E2F1 promoter. ChIP-qPCR of E2F1 promoter region 2 was performed in keratinocytes transfected with WAKMAR1 GapmeRs (**A**) or CRISPR/Cas9-SAM plasmids (**B**), and immunoprecipitated using DNMT1 antibody or IgG (n = 3). ChIP-qPCR of E2F1 promoter region 1 (**C**) and 2 (**D**) was performed in keratinocytes with WAKMAR1 knockdown, and immunoprecipitated using DNMT3A antibody or IgG (n = 3). ChIP-qPCR of E2F1 promoter region 1 (**E**) and 2 (**F**) was performed in keratinocytes with WAKMAR1 knockdown, and immunoprecipitated using DNMT3B antibody or IgG (n = 3). N.D.: not detected. *P<0.05, unpaired two-tailed Student's t-test. Data are presented as mean + s.d.

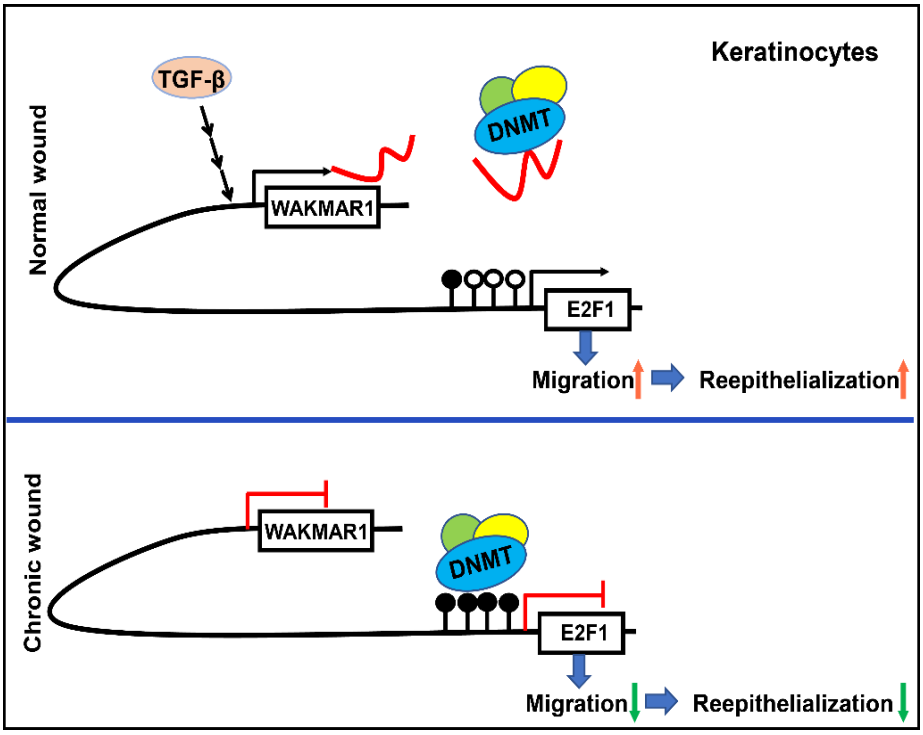

Fig. S14. Proposed model of WAKMAR1 regulation of E2F1 expression. The model depicts the role of the TGF-β -WAKMAR1-E2F1 regulatory axis in keratinocyte migration and re-epithelialization of human skin wounds. WAKMAR1 expression is induced by TGF-β signaling in human wound-edge keratinocytes during wound repair. WAKMAR1 activates the expression of E2F1, a key transcription factor upstream of a migratory gene network, by sequestering DNMTs and interfering with methylation of E2F1 promoter. In human chronic wounds, deficient TGF-β signaling may contribute to the low WAKMAR1 expression, which may further lead to hypermethylation of CpG sites at E2F1 promoter and E2F1 gene silencing.

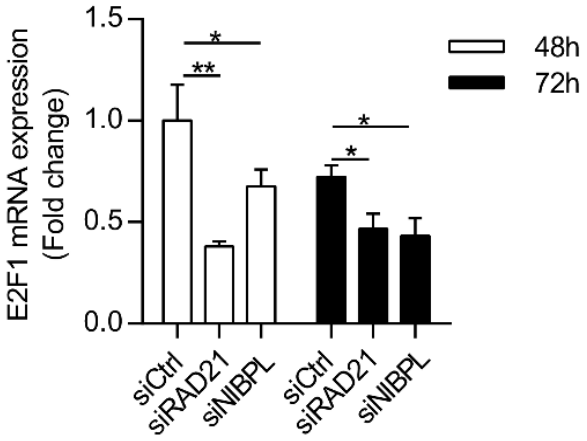

Fig. S15. **E2F1 expression in keratinocytes after silencing the expression of critical factors for chromatin occupancies of the cohesion complex.** qPCR analysis of E2F1 expression in keratinocytes transfected with siRNAs specific for RAD21 or NIBPL. * $P<0.05$, ** $P<0.01$, student t-test. Data are presented as means + s.d.

Captions for movies S1-3

Movie S1. Scratch wound assay of keratinocytes with WAKMAR1 knockdown. Confluent keratinocyte layers were scratched using the Essen wound maker to generate approximately 800 μm width wounds. The cell culture plates were placed into IncuCyte live cell analysis system and imaged every 2 hours. Video were generated using the IncuCyte software. 1: keratinocyte transfected with GapmeR Ctrl; 2: keratinocyte transfected with GapmeR WAKMAR1-1; 3: keratinocyte transfected with GapmeR WAKMAR1-2.

Movie S2. Scratch wound assay of keratinocytes with enhanced WAKMAR1 expression. 1: Keratinocytes co-transfected with CRISPR-SAM plasmids: MS2-P65-HSF1_GFP, dCAS9-VP64_GFP and MS2 plasmid expressing control sgRNA; 2: Keratinocytes co-transfected with CRISPR-SAM plasmids: MS2-P65-HSF1_GFP, dCAS9-VP64_GFP and MS2 plasmid expressing WAKMAR1 sgRNA-1.

Movie S3. Scratch wound assay of keratinocytes with silenced KIF11, E2F1, HMMR, CDK6 or FOS expression. Keratinocytes were transfected with gene specific siRNAs. 1: siControl; 2: siKIF11; 3: siE2F1; 4: siHMMR; 5: siCDK6; 6: siFOS.

Table S1. Information of healthy donors

Healthy Donor	Gender	Age	Race	Figure
1	F	66	Caucasian	1C
2	F	60	Caucasian	1C
3	F	67	Caucasian	1C
4	F	65	Caucasian	1C
5	F	66	Caucasian	2A, 5E,H
6	F	68	Caucasian	2A, 5E,H
7	F	67	Caucasian	2A, 5E,H
8	M	69	Caucasian	2A, 5E,H
9	F	64	Caucasian	2A,C, 5E,H,G,J
10	F	60	Caucasian	2A,C, 5E,H,G,J
11	M	30	Caucasian	2C, 5G, J
12	M	33	Caucasian	2C, 5G, J
13	M	35	Caucasian	2C, 5G, J
14	M	50	Caucasian	2C, 5G, J
15	M	26	Asian	2B, 5F, I
16	F	27	Asian	2B, 5F, I
17	F	27	Asian	2B, 5F, I
18	F	25	Asian	2B, 5F, I
19	F	43	Asian	2B, 5F, I
20	M	30	Asian	2B, 5F, I
21	M	26	Asian	2B, 5F, I
22	M	29	Asian	2B, 5F, I
23	F	46	Caucasian	3I-K
24	M	40	Caucasian	3I-K
25	F	24	Caucasian	S8, 4E
26	M	56	Caucasian	S8, 4E
27	F	58	Caucasian	S8, 4E
28	M	31	Caucasian	S8, 4E

M: male; F: female

Table S2. List of patients with venous ulcer.

Venous Ulcer	Gender	Age	Duration (months)	Wound size (cm)	Diagnosis	Figure
1	M	86	48	6x5	VU, AU	2A, 5E,H
2	F	68	24	15x15	VU, DM	2A, 5E,H
3	M	70	4	3x0.5	VU, CVD, Spinal stenosis, Anemia.	2A, 5E,H
4	F	78	18	15x12	VU, CVD	2A, 5E,H
5	F	71	240	20x20	VU, CVD	2A, 5E,H
6	M	99	6	20x10	VU, AU, CVD	2A,C, 5E, H, G, J
7	F	77	54	2.5x3 + 15x15	VU, Skin defect after surgery	2A,C, 5E, H, G, J
8	M	51	36	2x1.5	VU	2A,C, 5E, H, G, J
9	M	69	24	12x15	VU	2A,C, 5E, H, G, J
10	F	81	24	7x2.5	VU, CVD	2C, 5G, J

AU: Arterial Ulcer; CVD: Cardiovascular Disease; DM: Diabetes; F: Female; M: Male; VU: Venous Ulcer.

Table 3. List of patients with diabetic foot ulcer.

Diabetic Foot Ulcer	Gender	Age	Duration (months)	Wound size (cm)	Diagnosis	Wagner Classification	Figure
1	M	66	2	2×2	T2DM, DFU, DPN	1	2B, 5F, I
2	F	64	2	3×3	T2DM, DFU, DPN	1	2B, 5F, I
3	M	66	6	4×3	T2DM, DFU	2	2B, 5F, I
4	F	78	6	4×0.5	T2DM, DFU, HTN grade 3	2	2B, 5F, I
5	M	64	2	8×2	T2DM, DFU, DPN, PAD,	2	2B, 5F, I
6	F	59	5	3×4	T2DM, DFU	2	2B, 5F, I
7	M	63	2	2×2	T2DM, DFU, PAD, CVD, HTN grade 3	3	2B, 5F, I
8	F	66	2	2×2	T2DM, DFU, Cerecral infarction	3	2B, 5F, I
9	M	60	4	1×2	T2DM, DFU, DPN	3	2B, 5F, I
10	F	61	5	3×3	T2DM, DFU, PAD, DPN	3	2B, 5F, I
11	M	70	3	3×3	T2DM, DFU, CVD, HTN	3	2B, 5F, I
12	M	41	2	7×5	T2DM, DFU, PAD	3	2B, 5F, I
13	M	41	2	4×5	T2DM, DFU, PAD	3	2B, 5F, I
14	M	60	2	1×2	T2DM, DFU, DPN	3	2B, 5F, I
15	M	54	24	2×1	T2DM, DFU, DPN	3	2B, 5F, I
16	M	91	9	10×2	T2DM, DFU, DPN, PAD, HTN grade 3	3	2B, 5F, I
17	M	69	4	2×1	T2DM, DFU, PAD	3	2B, 5F, I
18	M	90	8	20×2	T2DM, DFU, PAD, DPN, HTN grade 3	3	2B, 5F, I
19	M	52	2	3×3	T2DM, DFU, PAD	4	2B, 5F, I
20	M	81	6	6×1	T2DM, DFU, DPN, CVD, HTN grade 3	4	2B, 5F, I
21	M	52	2	3×3	T2DM, DFU, PAD	4	2B, 5F, I

22	M	55	48	3×3	T2DM, DFU, HTN grade 3	4	2B, 5F, I
23	M	52	3	3×3	T2DM, DFU, PAD	4	2B, 5F, I
24	M	64	5	4×3	T2DM, DFU, PAD, DPN, HTN grade 3	4	2B, 5F, I
25	M	51	8	3×3	T2DM, DFU, DN, CKD, HTN grade 3	4	2B, 5F, I
26	M	77	12	After toe amputation	T2DM, DFU, PAD, HTN grade 2	4	2B, 5F, I
27	M	63	6	4×3	T2DM, DFU, DN, CKD, DPN, CVD, HTN grade 2	4	2B, 5F, I
28	F	70	12	10×2	T2DM, DFU, PAD, DPN, CVD, HTN	4	2B, 5F, I
29	F	74	2	After toe amputation	T2DM, DFU, PAD, DPN, HTN grade 3	4	2B, 5F, I

CVD: Cardiovascular Disease; DFU: Diabetic Foot Ulcer; DN: Diabetic Nephropathy; DPN: Diabetic Peripheral Neuropathy; F: Female; HTN: Hypertension; M: Male; PAD: Peripheral Arterial Disease (lower limbs); T2DM: Type 2 Diabetes Mellitus. RNA samples from all these donors have been used in a previous studie: Li X, Li D, Wang A, Chu T, Lohcharoenkal W, Zheng X, et al. MicroRNA-132 with Therapeutic Potential in Chronic Wounds. J Invest

Table S4. List of the reagents and primers.

qPCR Primers	Sequence / Vendor
WAKMAR1	F: CCCCTGAGACATTTCTTCCA R: AGCCTTGGAGGGTTTCAAAT
MALAT1	F: GATCTAGCACAGACCCTTCAC R: CGACACCATCGTTACCTTGA
HPRT	F: GCTATAAATTCTTTGCTGACCTGCTG R: AATTACTTTTATGTCCCCTGTTGACTGG
RPLP0	F: GCTGATCCATCTGCCTTTGT R: AAGTTGGTTGCTTTTTGGTGA
HIST1H1D	F: AGCGTCACCGTAAGGTATTG R: CTCCGCGAGTTTCCTCATAAA
CDK6	F: AGTGCCCACTGAAACCATAAA R: GAGGTTAGAGCCATCTGGAAAC
HMMR	F: GCAGACCAAGTCAGCACTAA R: GAGGAGACGCCACTTGTTAAT
E2F1	F: GCTGGACCACCTGATGAATATC R: CGGGCCTTGTTTGCTCTTA
KIF11	F: GCTCGGGAAGCTGGAAATATAA R: CAAGATTGAGAGATGCAGGAGAA
SMAD1	IDT Predesigned qPCR Assays: Hs.PT.58.4596538
SMAD3	IDT Predesigned qPCR Assays: Hs.PT.58.2698328
SMAD4	IDT Predesigned qPCR Assays: Hs.PT.58.472774
FOS	F: GGACCTATCTGGGTCCTTCTAT R: AAGACGAAGGAAGACGTGTAAG
CHIP E2F1 promoter region 1	F: AGATGGAAGGACAGGCAGAG R: CTAACCGTTGGTGAGCCATT
CHIP-E2F1 promoter region 2	F: CAGTTGAATACACGTGGGCTA R: CCTGTTTGCTGCTCTGTGCT
E2F1 MSRE-qPCR-CpG1 (GRCh37/hg19, chr20:32273183)	F: AGATGGAAGGACAGGCAGAG R: CTAACCGTTGGTGAGCCATT
E2F1 MSRE-qPCR-CpG5 (GRCh37/hg19, chr20:32273576)	F: CAGTTGAATACACGTGGGCTA R: CCTGTTTGCTGCTCTGTGCT
E2F1 MSRE-qPCR-CpG6 (GRCh37/hg19, chr20:32273924)	F: CGAAGAGCAGCAGGTCAGG R: TCGCAGATCGTCATCATCTC
BS-pyrosequencing: CpG1 (GRCh37/hg19, chr20:32273183), CpG2 (GRCh37/hg19, chr20:32273212)	F: AGATGGAAGGATAGGTAGAGG 5'biot-R: TTAAAAAAACCCTCCTTCCTACC Seq: GGAGGGTTGTTTTTAGA
BS-pyrosequencing: CpG3 (GRCh37/hg19, chr20:32273273), CpG4 (GRCh37/hg19, chr20:32273278)	F: TGGTAGATGGAAGGATAGGTAGA 5'biot-R: AAACCTACTAACTTCAACTCAAAATTTACA Seq: GGTTAGGTTTTAGGATTTTT

GapmeR/siRNA	Sequence / Vendor	Cat.	Vendor
Ctrl GapmeR	AACACGTCTATACGC		Exiqon
WAKMAR1 GapmeR-1	GCTGCAATCGATCCTT		Exiqon
WAKMAR1 GapmeR-2	TGTAAGAGAGACATGG		Exiqon
siSMAD1	CAGUCUAUGAGCUUACAAA	S8394	Life Technologies
siSMAD3	AGGUCUGCGUGAAUCCCUA	S8401	Life Technologies
siSMAD4	GCCCUAUUGUUACUGUUGA	S8403	Life Technologies
siE2F1	SMARTpool	L-003259-00-0005	Dharmacon
siKIF11	SMARTpool	L-003317-00-0005	Dharmacon
siHMMR	SMARTpool	L-010409-00-0005	Dharmacon
siCDK6	SMARTpool	L-003240-00-0005	Dharmacon
siFOS	SMARTpool	L-003265-00-0005	Dharmacon
siRAD21	SMARTpool	L-006832-00-0005	Dharmacon
siNIPBL	SMARTpool	L-012980-00-0005	Dharmacon

WAKMAR1 sgRNA	Sequence / Vendor

Ctrl sgRNA	F:CACCGAAGATGAAAGGAAAGGCGTT R:AAACAACGCCTTTCCTTTCATCTTC
WAKMAR1 sgRNA-1	F:CACCGAACCAGCTTCCCCGCCAACA R:AAACTGTTGGCGGGGAAGCTGGTTC
WAKMAR1 sgRNA-2	F:CACCGGCCCTCAGTCACAAAGCGGC R:AAACGCCGCTTTGTGACTGAGGGCC
WAKMAR1 sgRNA-3	F:CACCGGGGATTAAGTCGAATCTCTC R:AAACGAGAGATTCGACTTAATCCCC
WAKMAR1 sgRNA-4	F:CACCGCCAGGGCAATTTCGAGGGTT R:AAACAACCCTCGAAATTGCCCTGGC
WAKMAR1 sgRNA-5	F:CACCGATCAAGCCCGGAGCATGTCC R:AAACGGACATGCTCCGGGCTTGATC

Chemical inhibitors	**Cat.**	**Concentration**	**Vendor**
α-amanitin	A2263	5ug/ml	Sigma
Actinomycin-D	A1410	5ug/ml	Sigma
SB431542	1614	15uM	Tocris
DMH1	D8946	5uM	Sigma
5-Aza-2′-deoxycytidine	A3656	5uM	Sigma

Cytokines/Growth Factors	**Cat.**	**Concentration**	**Vendor**
IL1a	11349013	20ng/mL	Immuno tools
IL1b	11340013	20ng/mL	Immuno tools
IL8	11349084	50ng/mL	Immuno tools
IL17a	11340174	30ng/mL	Immuno tools
IL17f	11349174	30ng/mL	Immuno tools
GM-CSF	11343123	50ng/mL	Immuno tools
KGF	11343653	20ng/mL	Immuno tools
IGF-1	11343314	20ng/mL	Immuno tools
VEGF-A	11343663	20ng/mL	Immuno tools
TGFb1	11343161	10ng/mL	Immuno tools
TGFb2	11344751	10ng/mL	Immuno tools
TGFb3	11344482	10ng/mL	Immuno tools
BMP2	11343273	100ng/mL	Immuno tools

Antibody	**Cat.**	**Concentration**	**Vendor**
DNMT1	ab13537	3ug/sample	Abcam
DNMT3a	ab13888	3ug/sample	Abcam
DNMT3b	ab13604	3ug/sample	Abcam
EZH2	5246	3ug/sample	Cell Signaling Technology

RNA scope probe	**Cat.**	**Concentration**	**Vendor**
WAKMAR1 probe	469651-C3	1:50	Advanced Cell Diagnostics
3-plex positive control probe-Hs	320861	1:1	Advanced Cell Diagnostics
3-plex negtive control probe-Hs	320871	1:1	Advanced Cell Diagnostics