Supplementary information

Manuscript title: circRNA Mediates Silica-induced Macrophage Activation via HECTD1/ZC3H12A-dependent Ubiquitination

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Methods

Microarray and quantitative analyses

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed with the R software package. Mouse lung tissues were immediately flash-frozen in liquid nitrogen and then homogenized with TRIzol reagent (Invitrogen). The amount of total RNA in each sample was quantified using a NanoDrop ND-1000 spectrophotometer. Sample preparation and microarray hybridization were performed based on standard Arraystar protocols.

Fluorescent *in situ* hybridization (FISH)

Cellular circHECTD1 expression was detected via fluorescent *in situ* hybridization (FISH) using a mixture of biotin-labeled DNA oligo probes that were specific for either endogenous or ectopically expressed circHECTD1. Briefly, cells were freshly fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature, washed twice with PBS, immersed in 70% ethanol overnight at 4 °C, permeabilized with 0.25% Triton X-100 for 15 min, and subjected to two 15-min washes with saline-sodium citrate (SSC) buffer. *In situ* hybridization was performed overnight at 37 °C using 10 pM biotin-labeled DNA oligo probes in hybridization buffer (HB), and this step was followed by serial washes with SSC buffer. The samples were then incubated in blocking buffer (1% BSA and 3% normal goat serum in PBS) for 1 h at room temperature and then with an anti-

biotin HRP antibody (1:200) in blocking buffer overnight at 4 °C. The samples were subsequently subjected to 2-min washes with PBS. Finally, DNA was stained with DAPI, and cell images were captured using a fluorescence microscope (Olympus BX53, Olympus America, Inc., Center Valley, PA, USA).

RNA-binding protein immunoprecipitation (RIP)

The RIP experiments were performed according to the manufacturer's recommended protocol (Millipore). Briefly, 1-3x10^7 cells were washed twice with ice-cold PBS and lysed in 200 μL of RIP lysis buffer. Then, 50 μL of a magnetic bead suspension was transferred to each tube. The samples were subsequently washed twice with RIP wash buffer and resuspended in 100 μL of RIP wash buffer, and 5 μg of the antibody of interest was then added to each tube. After incubation for 30 min at room temperature, the obtained pellets were washed three times with RIP wash buffer and resuspended in 900 μL of RIP immunoprecipitation buffer. Next, 100 μL of the supernatant of the RIP lysate was added to each tube to a final volume of 1000 μL, and after overnight incubation at 4 °C, the pellets were washed six times with RIP Wash Buffer and resuspended in 150 μL of Proteinase K buffer at 55 °C for 30 min. The purified co-precipitated RNA was subjected to qRT-PCR to analyze the presence of binding using the respective primers.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the TRIzol reagent (Takara, Japan), and the RNA was reverse transcribed with the HiScript®Q Select RT SuperMix for

qPCR (+gDNA wiper) Kit (Vazyme, Nanjing, China). Real-time PCR was subsequently performed with the AceQ® qPCR SYBR Green Master Mix (High ROX Premixed) Kit (Vazyme, Nanjing, China). The results were standardized to control values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Supplementary Figure S1

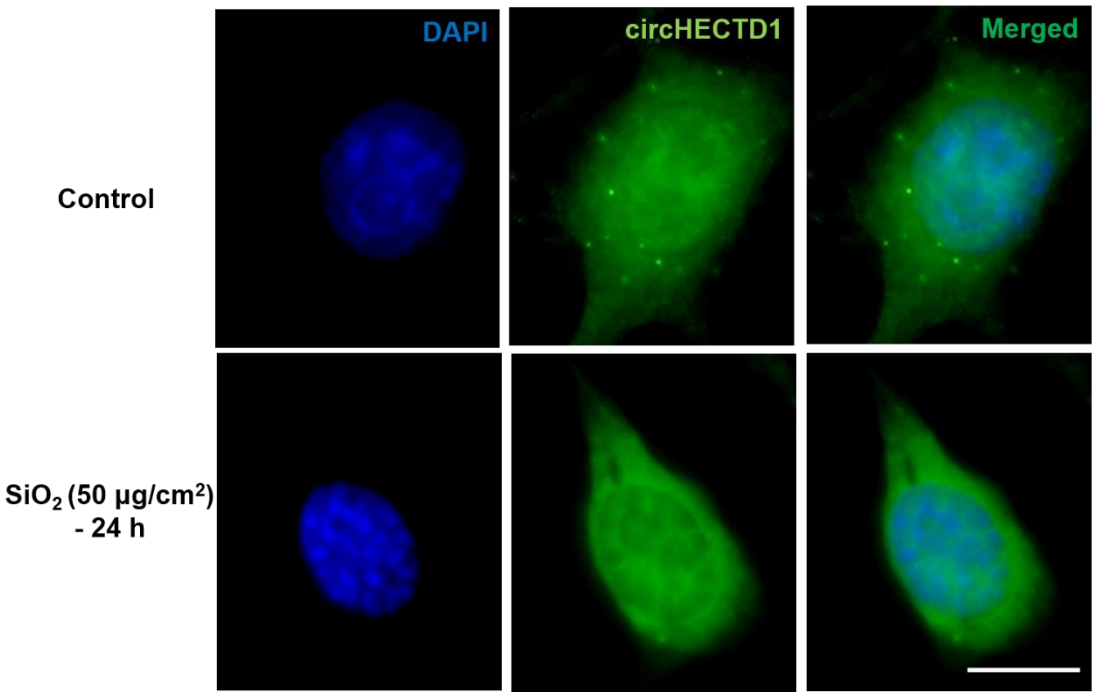

Figure S1 Expression of circHECTD1 in macrophages after exposure to SiO_2.

circHECTD1 was mainly detected in the cytoplasm of RAW264.7 cells in FISH assays.

Supplementary Figure S2

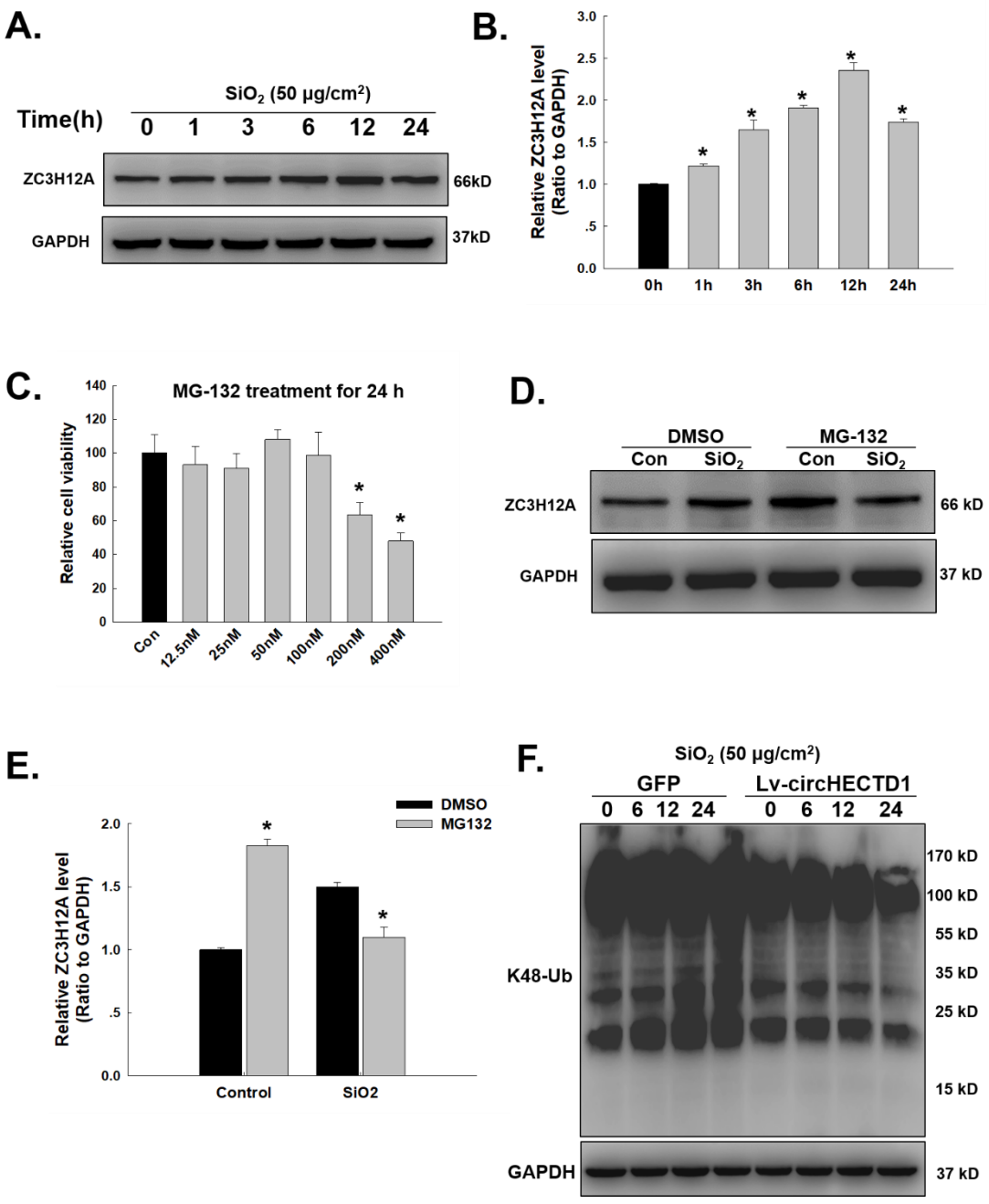

Figure S2 ZC3H12A expression is mediated by the ubiquitin-proteasome system and its own feedback regulation.

(A) Representative western blot showing the effects of SiO_2 (50 μg/cm^2) on ZC3H12A expression in RAW264.7 cells. **(B)** Densitometric analyses of five separate experiments suggested that SiO_2 induced ZC3H12A expression in a

time-dependent manner. *P<0.05 vs. 0 h. **(C)** According to the results of the MTT assay, MG-132, a proteasome inhibitor, decreased the viability of RAW264.7 cells (n=5); *P<0.05 vs. the control group. **(D)** Representative western blot showing the effects of MG-132 (50 nM) and SiO_2 (50 μg/cm^2) on ZC3H12A expression in RAW264.7 cells. **(E)** Densitometric analyses of five separate experiments suggested that MG-132 could enhance ZC3H12A expression, but not when combined with SiO_2. *P<0.05 vs. the corresponding control group. **(F)** Transfection of the circHECTD1 lentivirus in RAW264.7 cells showed that K48-ubiqutin was downregulated by circHECTD1.

Supplementary Figure S3

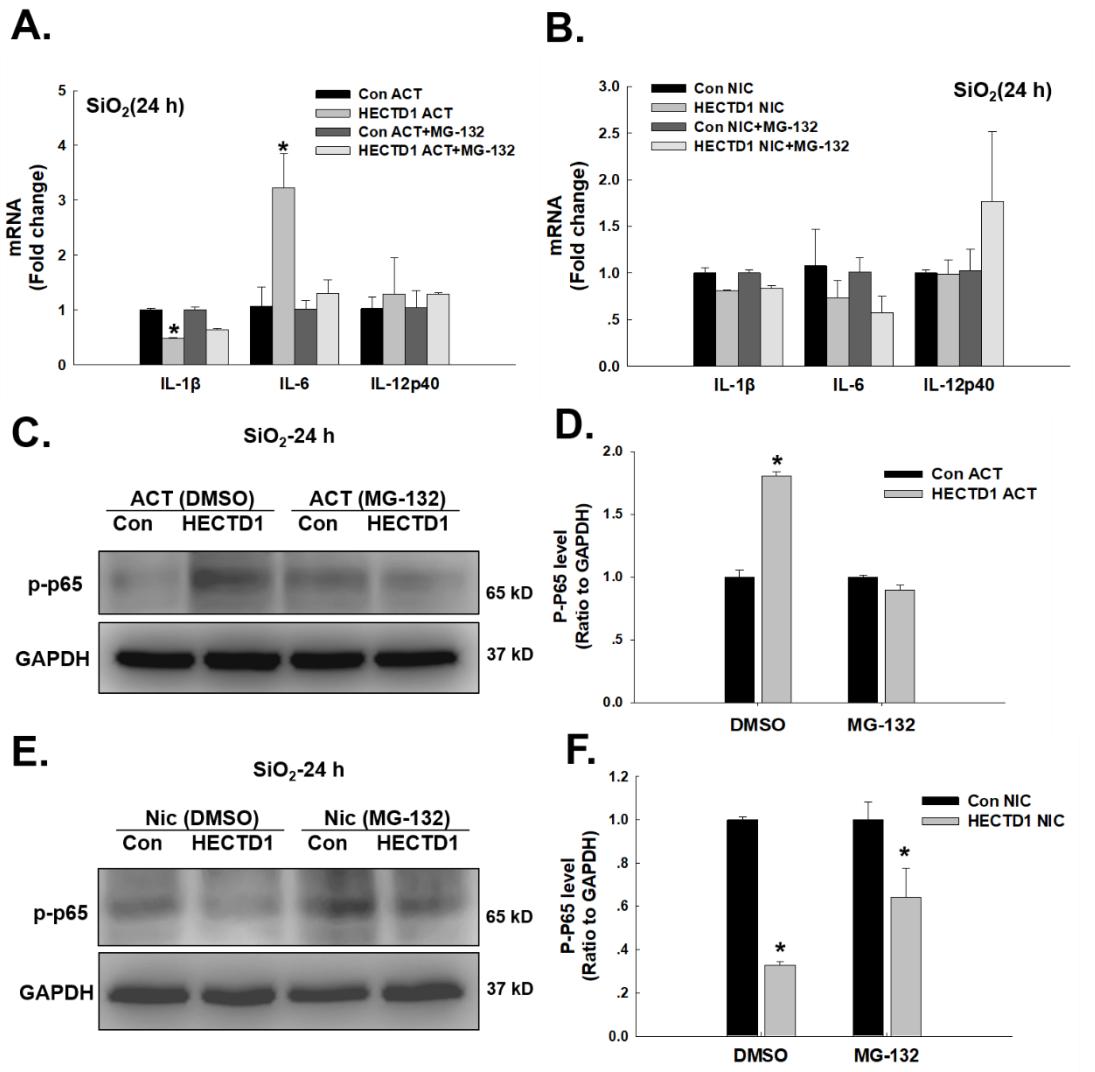

Figure S3 Transfection of the HECTD1 CRISPR activation plasmid (ACT) and CRISPR double nickase plasmid (NIC) with or without MG-132 in RAW264.7 cells.

(A) As shown in the qRT-PCR analysis, transfection with HECTD1 ACT with or without MG-132 could regulate the IL-1β and IL-6 mRNA levels in RAW264.7 cells but not that of IL-12p40 mRNA, and **(B)** transfection with HECTD1 NIC with or without MG-132 had no effect on these mRNAs. (n=5); **P*<0.05 vs. the corresponding control group**.** Representative western blot showing the effects

of **(C)** HECTD1 ACT or **(E)** NIC transfection with or without MG-132 on p-p65 expression in RAW264.7 cells. Densitometric analyses of five separate experiments suggested that **(D)** HECTD1 ACT or **(F)** NIC transfection with or without MG-132 affected p-p65 expression in RAW264.7 cells (n=5); *P<0.05 vs. the corresponding control group.

Supplementary Figure S4

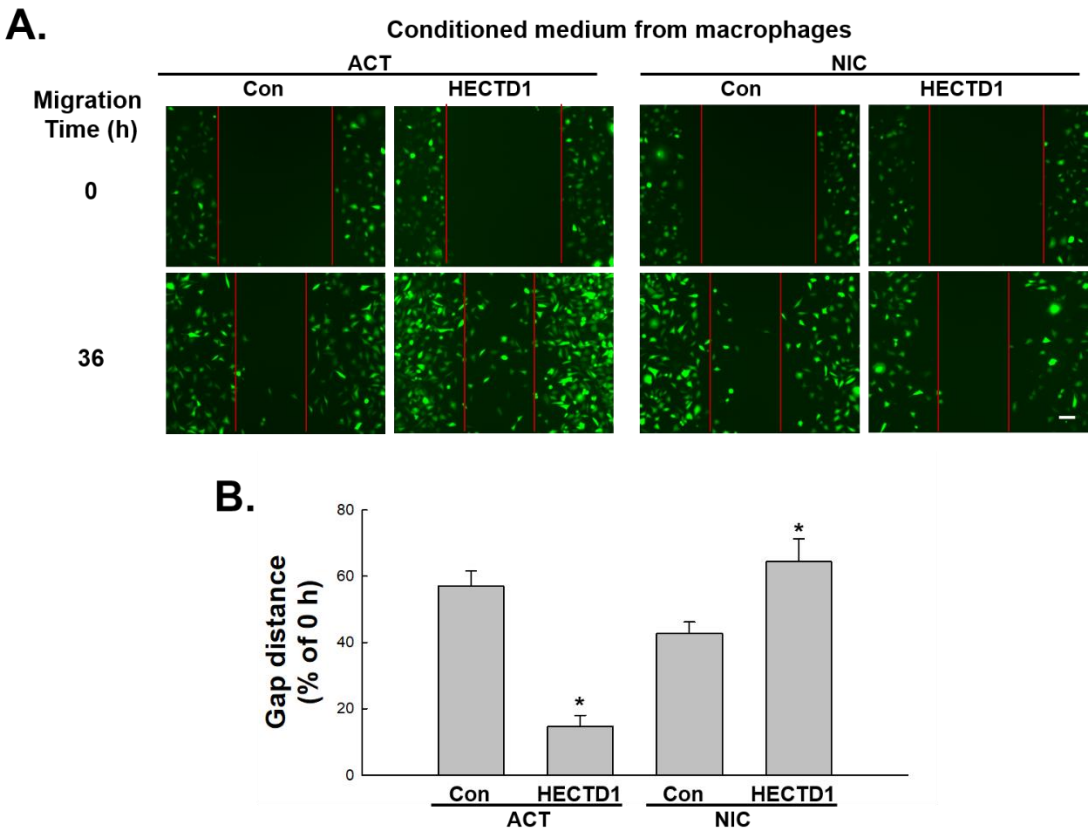

Figure S4 Regulatory effects of HECTD1 on the activation and migration of fibroblasts.

(A) Representative images showing the effects of conditioned media from RAW264.7 cells on the migration of GFP-labeled L929 cells. Scale bar=80 μm. (B) Quantification of scratch width in six separate experiments. **P*<0.05 vs. the corresponding control group.

Supplementary Table S1

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) primers and FISH probe

mRNA qPCR primers		
Gene	**Forward (5′-3′)**	**Reverse (5′-3′)**
GAPDH (mouse)	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
HECTD1 (mouse)	TTGAAACATGTCCACCTCGT	CACGGGCTGTCACCTCTAAG
IL-1β (mouse)	TGCCACCTTTTGACAGTGATG	ATGTGCTGCTGCGAGATTTG
IL-6 (mouse)	CCGGAGAGGAGACTTCACAG	ACAGTGCATCATCGCTGTTC
IL-12p40 (mouse)	CAGAAGCTAACCATCTCCTGGTTTG	CCGGAGTAATTTGGTGCTTCACAC

circRNA qPCR primers		
Name	**Forward (5′-3′)**	**Reverse (5′-3′)**
circHECTD1 (Divergent primer)	AACTTAGGCGTATTTGGGAGC	ACATAGTCGTCATCCCAGGC
circHECTD1 (Convergent primer)	GCCTGGGATGACGACTATGT	GCTCCCAAATACGCCTAAGTT

Fish probe	
circRNA	**Sequence**
mmu_circHECTD1 (Biotin-fish probe)	aaaCATACTCTTCTTCTTCGTGTAAGTGGGCTCCC