SUPPLEMENTAL DATA

Attenuation of Intestinal Epithelial Cell Migration During *Cryptosporidium parvum* Infection involves Parasite Cdg7_FLc_1030 RNA-Mediated Induction and Release of DKK1 from Infected Host Cells

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SUPPLEMENTARY MATERIALS AND METHODS

Infection Models and Infection Assays

Models of intestinal cryptosporidiosis using cultured cell lines were employed as previously described; infection was done in serum free culture medium for 4 hours with a 1:1 ratio between *C. parvum* oocysts and host cells [1,2]. An *ex vivo* infection model employing enteroids from neonatal mice recently developed by us [3] was used for *ex vivo* experiments. Intestinal villus/crypt components from neonatal mice were isolated as previously reported [3]. For *ex vivo* infection of cultured enteroids, oocysts were treated with 1% sodium hypochlorite on ice for 20 min and subjected to an excystation solution consisting of 0.75% taurodeoxycholate and 0.25% trypsin for 30 min at 37°C. The excystation rate was calculated as previously described by others [4] and was determined for each new batch of oocysts. These freshly excysted infective sporozoites were collected and added to the cultures for *ex vivo* infection. A well-developed infection model of cryptosporidiosis in neonatal mice was used for *in vivo* experiments [5,6]. Mice at the age of 6 days after birth received *C. parvum* oocysts by oral gavage (10^5 oocysts per mice). Mice receiving the vehicle (PBS) by oral gavage were used as control. At 24, 48 and 72 hours after *Cryptosporidium* or vehicle administration, animals were sacrificed and ileum intestinal tissues were collected. At least five animals from each group were sacrificed and ileum tissues were obtained for immunohistochemistry and biochemical analyses. The animal study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under the Assurance of Compliance Number A3348-01. All animal experiments were done in accordance with procedures (protocol number #0959) approved by the Institutional Animal Care and Use Committee of Creighton University.

Real-time PCR, immunofluorescence microscopy, and immunohistochemistry were used to assay *C. parvum* infection as previously reported [7,8]. The intestinal (ileum) tissues were

stained with H&E and villus heights were measured under microscope, as previously reported [7,9]. For immunohistochemistry, tissue slides were first incubated with the primary antibodies, followed by the secondary antibody (Molecular Probes) and visualized as previously reported [1,8].

siRNAs and Plasmids

Custom-designed siRNA oligos against Cdg7_FLc_1030 and a scrambled siRNA were synthesized by Integrated DNA Technologies (Coralville, Iowa) and transfected into cells with Lipofectamine RNAimax (Invitrogen). The plasmids expressing parasite RNAs were generated by real-time PCR amplification of cDNA, using RNA from *C. parvum* sporozoites (Iowa strain) and cloned into the pcDNA3.1(+) vector, according to the manufacturer's protocol (Invitrogen). The human interleukin 8 luciferase reporter construct was a gift from LaRusso NF (Mayo Clinic). Cells were transfected with these plasmids with lipofectamine 2000 (Invitrogen) and pcDNA3.1(+) Empty vector transfected as control. The primer sequences for plasmid generation are listed in the Table S1.

Whole Cell Extracts, Western Blot, and Immunofluorescent staining

Whole cell extracts were prepared using the M-PER Mammalian Protein Extraction Reagent (Fisher) supplemented with cocktail protease inhibitors, according to manufacturer's instructions. Cell pellet was incubated in the M-PER Mammalian Protein Extraction Reagent on ice for 30 min, centrifuged at 16,100 rpm for 20 min and the supernatants were saved as the whole cell extracts. Protein concentration of each fraction or whole cell lysate was determined and subsequently analyzed by Western blot. The following antibodies were used for blotting: anti-Dkk1 (Santa Cruz), anti-Dkk2 (Abcam), anti-Dkk3 (Abcam), anti-Dkk4 (Millipore), and anti-GAPDH (Santa Cruz). Immunofluorescent staining was performed as previously reported [7,8]

and the flowing antibodies were used: anti-Dkk1 (Santa Cruz), anti-Cdc42-GTP (Cdc42-active, NewEast Biosciences) and anti-Par6 (Fisher).

RNA Immunoprecipitation (RIP), Chromatin IP (ChIP), and Chromatin Isolation by R**NA Purification (ChIRP) Analyses**

The formaldehyde crosslinking RIP was performed as described [10]. Cells were collected and washed twice with PBS, and resuspended cells in PBS to 1×10^7/10 mL cell density. Formaldehyde (37% stock solution) was then added to a final concentration of 0.3% (v/v) and incubated at room temperature for 10 min. Crosslinking reactions were quenched by the addition of glycine (pH 7.0) to a final concentration of 0.25 M followed by incubation at room temperature for 5 min. The cells were then harvested by centrifugation at 3000 rpm (237g) for 4 min followed by two washes with ice-cold PBS. Nuclear extracts were obtained as described above and solubilization of crosslinked complexes was done by mechanical sonication by three rounds of sonication, 20 s each, using a Branson Sonifier 150 with a microprobe at an amplitude setting of 5 (output, 10 W). Insoluble materials were removed by microcentrifugation at 14,000 rpm (16,000g) for 20 min at 4°C. Preclearing lysates with 20 µL of PBS washed Magna Protein A+G Magnetic Beads (Millipore, Massachusetts). The precleared lysate (1000 µg protein) was then diluted with WCE buffer to a total volume of 1 mL, mixed with the specific antibody-coated beads, and incubated with rotation at 4°C for 4 hours, followed by 4 times washing with WCE buffer containing protease and RNase inhibitors. Anti-Pol II (Millipore, CA) was used for the immunoprecipitation. The collected immunoprecipitated RNA-protein complexes and inputs were digested in the digestion buffer (100 mM NaCl, 10 mM TrisCl pH 7.0, 1 mM EDTA, 0.5% SDS) with addition of 100 µg Proteinase K and incubated at 50°C for 45 min with end-to-end shaking at 400 rpm. Formaldehyde cross-links were reversed by incubation at 65°C with rotation for 4 h. RNA was extracted from these samples using TRI-reagent (Invitrogen) and treated with DNA-free DNase Treatment & Removal I kit according to the

manufacturer's protocol (Ambion Inc., Austin, TX). The presence of RNA was measured by quantitative real-time PCR using the CFX Connect Real-Time system (BioRad). Gene-specific PCR primer pairs are same as for real-time PCR.

For ChIP analysis, a commercially available ChIP Assay Kit (Upstate Biotechnologies) was used in accordance with the manufacturer's instructions. In brief, cells were fixed with 1% formaldehyde for 10 min and the genomic DNA was then sheared to lengths ranging from 200 to 1000 bp by sonication, as previously described [10]. While one percent of the cell extracts was taken as input, the rest of the extracts were incubated with specific antibodies overnight at 4°C, followed by precipitation with protein G agarose beads. The DNA-protein complex was eluted; after reversal of cross-links with NaCl at 65°C overnight, proteins were digested with proteinase K, and the DNA was detected by real-time quantitative PCR analysis. The following antibodies were used for ChIP analysis: anti-H3K4me1 (Abcam), anti-H3K36me3 Abcam), anti-Pol II (Millipore).

ChIRP analysis was performed as previously reported [11]. Briefly, a pool of tiling oligonucleotide probes with affinity specific to the *C. parvum* Cdg7_FLc_1030 RNA sequences was used and glutaraldehyde cross-linked for chromatin isolation. The DNA sequences of the precipitates from chromatin isolation by RNA purification were confirmed by real-time PCR using the same primer sets covering the gene promoter regions of interest as for ChIP analysis.

Cell Migration and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

Wound-healing assay was used to analyze cell migration. Briefly, confluent cells grown on tissue culture 6-well plates were exposed to *C. parvum* infection and scraped 3 times vertically using a 20 µL plastic pipette tip. Wounded monolayers were washed once with culture medium to remove detached cells and debris, and then incubated in serum-reduced medium. For some experiments, cells were cultured with the medium in the presence or absence of the

recombinant mouse Dkk1 protein (R&D, MN) or neutralizing anti-Dkk1 (R&D). Cell migration distance was quantified by measuring the distance between two leading edges of wounds under a SOP-Nikon Microscope after cell wounded 0, 24, 48 and 72 hours. At least 50 wounded points were included and all experiments were repeated 3 times. Cell proliferation Assay was carried out using the CellTiter 96 AQueous One Solution Cell Proliferation (MTT) Assay Kit (Promega Corporation). Data were recorded as the absorbance at 490 nm with the VERSAmax microplate reader (Molecular Service). Standard curves were recorded at 0 hour.

Statistical Analysis

All values are given as mean ± SEs. Means of groups were from at least three independent experiments and compared with Student's *t* test (two-tailed unpaired) or the ANOVA test when appropriate. *p* values < 0.05 were considered statistically significant.

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. List of primers used for real-time quantitative PCR, ChIP analysis, and generating constructs (Excel file).

Supplementary Table 2. List of probe sequences for ChIRP analysis (Excel file).

Supplementary Figure 1. Proliferation of IEC4.1 cells after incubation with the supernatants from IEC4.1 cultures following *C. parvum* infection. IEC4.1 cells were incubated with the supernatants from infected IEC4.1 cultures. The wound-healing approach for cell migration assay was applied to the cell cultures. Cell proliferation of the cultures was assessed by using the MTT assay. Data represent means ± SEs from 3 independent experiments. (TIFF file).

Supplementary Figure 2. Expression of parasite RNA transcripts in IEC4.1 cells. IEC4.1 was transfected with each of the constructs expressing the full-lengths of selected parasite RNA transcripts for 48 hours. Cells transfected with the empty vector were used as the control. Whole cell extracts were collected and expression levels of the corresponding parasite RNA transcripts were measured with real-time PCR (TIFF file). Data represent means ± SEs from 3 independent experiments.

Supplementary Figure 3. Expression of *Dkk1* gene in IEC 4.1 cells expressing the selected parasite RNA transcripts. IEC4.1 was transfected with each of the constructs expressing the full-lengths of selected parasite RNA transcripts for 48 hours. Cells transfected with the empty vector were used as the control. Whole cell extracts were collected and

expression levels of Dkk1 RNA were measured with real-time PCR (TIFF file). Data represent means ± SEs from 3 independent experiments.

Supplementary Figure 4. Staining of Cdc42-active and Par6 in cells along the migrating edge upon wounding in the cell cultures after incubation with the culture medium in the presence or absence of the recombinant mouse Dkk1. IEC4.1 cells were incubated with the culture medium, in the presence or absence of the recombinant mouse Dkk1 (rmDkk1). Wound-healing was applied to the cell cultured as the cell migration assay, followed by immunostaining with anti-Par6 or anti-Cdc42-GTP (Cdc42-active), respectively. Representative images showing the staining of Par6 and Cdc42-GTP in cells along the migrating edge (indicated by arrowheads) after wounding are shown. Data represent means ± SEs from 3 independent experiments. Bar = 20 μm (TIFF file).

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