ADDITIONAL FILE

HDAC inhibitor Givinostat targets DNA-binding of human CGGBP1

Manthan Patel, Divyesh Patel, Subhamoy Datta, Umashankar Singh

Email: usingh@iitgn.ac.in

HoMeCell Lab, Biological Engineering, Indian Institute of Technology Gandhinagar, Gandhinagar, Gujarat. 382355, India.

-----------------------------------------------------------------------------------------------------------------------------

Material and Methods

Cell Culture and Cell lysate preparation

HEK293T cells were grown in DMEM (AL007A, Himedia) supplemented with 10% FBS (RM1112, Himedia) and antibiotic-antimycotic agent (15240062, Gibco). Cells were collected and washed with PBS (TL1006, Himedia). Washed cell pellet was lysed in RIPA buffer (150mM NaCl, 5mM EDTA, 50mM Tris, 1% NP40 (IGEPAL), 0.1 Na-deoxycholate and 0.1 % SDS) containing Halt protease and phosphatase protein inhibitor cocktails (PI78441, Invitrogen).

Crosslinking of DNA with membrane disc

Genomic DNA was isolated from HEK293T cells. DNA was sonicated using a Diagenode Bioruptor sonicator for 21 cycles at 30 second on followed by 30 second off. Sonication was standardised to get ~ 1kb fragment size DNA. Sonicated genomic DNA was dissolved in 1x TE buffer at 200 ng/µl. Nylon-membrane (GX222020NN, Genetix) discs (5 mm diameter) were soaked with the sonicated DNA overnight. DNA was crosslinked by vacuum heating the wet membranes discs at 800C for 90 minutes. DNA crosslinked nylon-membrane discs (called dot blots) were transferred to 96 well plates and incubated with blocking solution (PBST with 10% FBS). For the primary screen one dot blot was used for each of the 1685 compounds (LL100, Selleckchem) distributed across multiple 96 well plates. In addition, the control samples were run on two dot blots for each 96 well plate.

Dot-blot assay

The dot blots were pre-incubated with blocking solution for 90 minutes. Cell lysate (15 μl of a 25 ml lysate stock obtained from approximately 500 million cells) was incubated with compounds (final concentration 100 uM) or compound diluent (0.05% DMSO in 1xPBS) for 30 minutes. Pre-incubated compound-lysate mix was transferred to the dot blots and incubated for 90 minutes at 40 C. Cross linked dot blots were washed three times with PBS and protein-DNA complexes were crosslinked with 4% formaldehyde in 1x PBS. The dot blots were then incubated with 50 μl of rabbit polyclonal anti-CGGBP1 antibody mix (a mix of 1:120 dilutions of SC-292517, SCBT and 10716-1-AP, Proteintech) overnight with gentle rocking. Dot blots were washed with PBST three times and incubated with 30 μl of biotinylated anti-rabbit secondary antibody for 90 minutes (1:10 dilution, 865002, R&D Systems). After three PBST washes the dot blots were incubated with high sensitivity streptavidin conjugated to horseradish peroxidase (1:10 dilution, 865006, R&D Systems) for 90 minutes. All antibody dilutions and streptavidin-HRP conjugate dilutions were done in blocking solution. Dot blots were washed three times with PBST and incubated with 15 μl of DAB (3,3’-Diaminobenzidine) chromogen (2 ml of DAB chromogen (860001, R&D Systems) diluted in DAB chromogen buffer (860005, R&D Systems)). Positive and negative controls are described below.

|  |  |  |  |
| --- | --- | --- | --- |
|  | Cell lysate | Primary antibody | Secondary antibody (Biotinylated anti-Rabbit) |
| Positive Control | Yes | Anti-CGGBP1 Rabbit Polyclonal IgG | Yes |
| Negative Control 1 | No | Anti-CGGBP1 Rabbit Polyclonal IgG | Yes |
| Negative Control 2 | Yes | No | Yes |
| Negative Control 3 | Yes | IgG | Yes |
| Negative Control 4 | Yes | Anti-CGGBP1 Rabbit Polyclonal IgG | No |

Secondary screening for direct or indirect inhibition of the primary hits

The secondary screening was performed for eight inhibitors obtained as hits from the primary screen along with a positive control (no inhibitor) and a negative control (no primary antibody). The assay was done in 6-well plates with each inhibitor-rCGGBP1 combination assayed in 8-10 technical replicates. For each sample, dot blots were incubated with 250 μl of blocking solution (10% FBS in PBS) for 1 hour at room temperature in a moist chamber. Simultaneously, the rCGGBP1 (500 ng per compound) diluted in RIPA lysis buffer (containing protease phosphatase inhibitor cocktail) was incubated with inhibitors (at a final concentration of 100 μM for 30 minutes) at 4° C. As a positive control, the compound diluent (0.05% DMSO in 1x PBS). The protein-inhibitor mix was diluted in blocking solution such that the final volume was 250 μl for each sample. The blocking solution was removed and rCGGBP1-inhibitor mix was transferred onto the membranes in each well for 1 hour at 4° C in a moist chamber with gentle rocking. This was followed by fixation of the interactions using 1% PFA for 5 minutes and subsequent PBS washes three times. Membranes were incubated with Mouse anti-FLAG antibody (1:1000 of SC-166384, SCBT, diluted in blocking solution) for 1 hour at room temperature with gentle rocking, followed by washing three times with PBST. The membranes were then incubated with anti-Mouse HRP-conjugated secondary antibody (1:5000 of NA931, GE Healthcare) for 1 hour at room temperature followed by three washes with PBST. The signal was detected with ECL substrate (32106, Pierce). Further the membranes were stripped with 0.2 N NaOH for 5 minutes and incubated with anti-CGGBP1 antibody (1:1000 in blocking solution) overnight at 4° C followed by three PBST washes. The membranes were incubated with anti-Rabbit HRP-conjugated secondary antibody (1:5000 of NA934, GE Healthcare) for 1 hour at room temperature and subsequently washed with PBST thrice. The signal was captured detecting chemiluminescence as described above. The signal was quantified by densitometry analysis of images using ImageJ software. The statistical analysis and data presentation was performed using Open Office and GraphPad Prism8.

Synthesis of Alu DNA

For the generation of full-length Alu DNA, an established DNA-binding target of CGGBP1, the full length consensus sequence of Alu SINE was synthesized in five overlapping oligonucleotides. The sequences of the oligonucleotides are as follows: The 5’ end of the first fragment and the 3’ end of the last fragment contained the T7 and SP6 primers respectively. The Alu DNA product was obtained through overlapping PCR using an equimolar mix of overlapping oligonucleotides as template and T7 and SP6 sequences as primers. The PCR product was run on the agarose gel and purified using PCR purification kit (A1222, Promega). The Alu PCR product was cloned into pGEM-T Easy Vector (A1380, Promega). The clones obtained were subjected to Sanger sequencing for verification. This clone was used as a template for amplifying Alu DNA for *in vitro* DNA-rCGGBP1 immunoprecipitation assay. Two different lengths of Alu DNA were amplified due to two priming sites for T7 as well as SP6 in the clone (~320 bp (T7 and SP6 sites in the insert) and other at ~400 bp (T7 and SP6 sites in the vector backbone). The Alu DNA was amplified using either unmethylated cytosine or 5’-methylated dCTPs. The sequence of the Alu DNA is as follows:

5'-TAATACGACTCACTATAGGGGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGCGGGAGGATCGCTTGAGCCCAGGAGTTCGAGACCAGCCTGGGCAACATAGCGAGACCCCGTCTCTACAAAAAATACAAAAATTAGCCGGGCGTGGTGGCGCGCGCCTGTAGTCCCAGCTACTCGGGAGGCTGAGGCAGGAGGATCGCTTGAGCCCAGGAGTTCGAGGCTGCAGTGAGCTATGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACCCTGTCTCTTCTATAGTGTCACCTAAAT-3'

*In vitro* DNA-IP and qPCR

The *in vitro* DNA immunoprecipitation was performed with or without Givinostat. rCGGBP1 (0.5 μg) diluted in 1x PBS was incubated with inhibitor (100 μM final concentration or the compound diluent (0.05% DMSO in 1x PBS)) for 45 minutes at room temperature. The final volume was adjusted to 50 μl with PBS. The protein-inhibitor mix was incubated with 1μg of Alu DNA. Simultaneously 3 μg of the anti-FLAG antibody (described above) was subjected to incubation with protein-G sepharose beads (60 ul, 17061801, GE Healthcare) for 1 hour with tumbling. The DNA-protein-inhibitor mix was transferred to the tube containing the anti-FLAG antibody-bound protein-G sepharose and incubated for 60 minutes at room temperature with tumbling. The beads were allowed to settle down followed by gentle spin and the supernatant containing the unbound antibody and DNA was removed. The beads were gently washed three times with ice cold 1x PBS. For each sample the 1x TE buffer (40 μl per sample) was added and mixed followed by heating at 80° for 20 minutes to elute the bound DNA.

qPCR

The immunoprecipitated Alu DNA in presence and absence of Givinostat was used as template for the qPCR (1725124, Biorad) using T7 and SP6 primers. The PCR was performed for both the samples (Alu DNA immunoprecipitated with Givinostat-inhibited or and mock-inhibited rCGGBP1). The template was used at different dilutions of the immunoprecipitated DNA (1:100 and 1:200 diluted) for qPCR in multiple replicates. The input Alu DNA template was used as a control to calculate the first delta Ct (dCt). The second delta Ct values were calculated by subtracting the dCt values obtained for the mock-inhibited sample from those of the Givinostat-inhibited sample. Following are the PCR conditions for Alu PCR:

95° C-5 minutes, (95° C- 20 seconds, 55° C- 20 seconds, 72° C-30 seconds, 80° C-30 seconds) x50, melting curve (50° C to 95° C with an increase of 0.3° C per second.