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Supplementary Information for

Abortive herpes simplex virus infection of non-neuronal cells results in quiescent viral genomes that can reactivate

Efrat M Cohen, Nir Avital, Meir Shamay and Oren Kobiler

Corresponding author: Oren Kobiler
e-mail:okobiler@tauex.tau.ac.il

This PDF file includes:

Supplementary text
Tables S1

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Supplementary Information Text

Extended Material and Methods

Cells

HeLa cells derived from human cervical cancer cells were infected with a lentivirus carrying the GFP gene under the mouse PGK promoter, derived from the pMSCVpuro plasmid (clonetech). These HeLa cells were a kind gift from the Eran Bachrach lab. HB2 cells expressing mCherry were derived from a human mammary epithelial cell line. These cells were a kind gift from Joyce Taylor-Papadimitriou. H1299 cells, which originated from the non-small cell lung carcinoma cell-line containing the YFP gene fused to moesin gene as part of LARC (library of annotated reporter cell-clones (1), were a kind gift from Uri Alon. The HFF cells were immortalized by hTERT transfection. The immortalized HFF cells were a kind gift from Sara Selig. U2OS cells originated from osteosarcoma cells (ATCC HTB-96) and the Vero monkey kidney epithelial cells (ATCC CCL-81) were obtained from ATCC.

Vero, HeLa, U2OS, HFF and HB2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM X1; Gibco), supplemented with 10% Fetal bovine serum (FBS; Gibco) and 1% penicillin (10,000 units/ml) and streptomycin (10mg/ml) (Biological Industries Israel). The GFP-expressing HeLa cells' medium was supplemented with G418 (0.5mg/ml Gibco) to maintain the fluorescence expression. HB2 cells' medium was supplemented with Insulin (Sigma-Aldrich) and hydrocortisone (Sigma-Aldrich). The moesin-annotated LARC cells were grown in RPMI supplemented with 10% FBS (Gibco) and 1% penicillin (10,000 units/ml) and streptomycin (10mg/ml) (Biological Industries Israel).

Viruses

All viruses are derivatives of HSV-1 strain17+. Viral recombinant OK22 carries a single fluorescent protein mTurq2 with a nuclear localization tag under the CMV promoter between UL37 and UL38 genes as described previously (2). Viral recombinants carrying the mCherry fluorescent protein under the CMV promoter and genetic barcodes between UL37 and UL38 genes were described previously (3). Viral recombinant OK12 carries a single fluorescent protein EYFP with a nuclear localization tag under the CMV promoter between UL37 and UL38 genes as described previously (4).

Sorted single cell assay

This experiment was described previously (3). Briefly, HeLa cells were infected with an even mix of 14 different mCherry barcode viruses at MOI 10 and 100. At 3 HPI cells were trypsinzed and kept on ice until sorting. Single infected cells were sorted onto one well of a 96-wells plate according to their red fluorescence level with Astrios (Beckman-Coulter) sorter. Each well contained a monolayer of uninfected Vero cell before sorting. A fluorescence microscope (Nikon Eclipse Ti-E) was used to scan the 96-wells plate at 1, 2 and 7 DPI to observe a single infectious center per well. These single infectious centers were considered as productive cells. Infected cells that did not develop an infectious center after 6 days were considered as abortive cells. Overall, four experiments were performed in MOI 10 and 100, yielding 124 productive cells, 61 abortive cells.

Single cell assay

HeLa, A1299, U2OS, HFF and HB2 cells were infected with OK22 virus in multiplicity of infection (MOI) 1, 10 or 100. At 3 HPI, the infected cells were trypsinized and counted. One to five cells were placed per well on a 96-wells plate, pre-seeded with uninfected Vero cells. The 96-wells plate was scanned under a fluorescence microscope (Nikon Eclipse Ti-E) at 1DPI to find candidate abortive cells that expressed immediate-early CFP, but did not develop a single-focus plaque. These cells were monitored at 2 and 7DPI. A single cell was considered an abortive cell if it was detected at all time points and did not develop a plaque till the last time point. The ratio of abortive cells was calculated as the number of abortive cells at 7DPI divided by the total number of plaques that were developed at 1DPI.

Recovered infected cells population assay

HeLa and HB2 cells were infected with OK22 virus in MOI 10 and 100. At 3 HPI, 10 HPI or 7 DPI, cells were trypsinized, diluted in medium to a final volume of 6ml, and spread into six wells of a 12-wells plate, 1ml per well (G1 wells, Figure 2A). At the 7DPI experiments, medium was replaced at 10, 24 and 48 HPI, to remove access of newly formed viruses. At two and three WPI, the medium was changed into a fresh medium. At three or four WPI, each well from the 12-wells plate was trypsinized and divided into three wells of a 12-wells plate, or into one well of a 6-wells plate, depending on the experiment. The 2nd generation wells (G2) were scanned one to three times under a fluorescence microscope (Nikon Eclipse Ti-E). In cases where active viral replication was not observed in all three G2 wells (originating from one G1 well), these wells were taken for further experiments: one for DNA, one for RNA and one for either reactivation, ChIP or FISH experiments (see below).

Burst size assay

HeLa and HB2 cells were infected with OK22 recombinant in MOI 100. At 7 DPI, cells were trypsinized, diluted, re-plated and incubated for 4.5WPI. These cells and naïve cells were infected with OK12 recombinant at MOI 10 in triplicates. At 24HPI, the lysate was collected and progeny viruses were tittered. For each sample, the titter was divided by the original number of cells in the plate to obtain the burst size per cell.

qPCR

G2 wells were incubated in 37°C and 5% CO_2 for 24 to 72 hours with lysis buffer (5) containing 10mM Tris-HCl, pH = 8.0, 1mM EDTA (Merck), 1% Tween 20 (Sigma-Aldrich), 0.04% Proteinase K (BIO-LAB, Israel). Proteinase K was deactivated by exposure to 95°C for 10 minutes. The lysate was kept in room temperature prior to analysis with qPCR. The samples were analyzed with qPCR (QuantStudio 12K Flex, Applied Biosystems), using SYBR master mix (Applied Biosystems). A list of primers used for the qPCR assay is specified in Supplementary Table 1.

RT-qPCR

200µl Bio-Tri RNA Reagent (BIO-LAB, Israel) was added to G2 wells and incubated in room temperature for 10 minutes. Then the samples were stored in -80°C until RNA extraction. The RNA extraction was performed according to manufacturer's protocol, with materials adjusted to the volume of Bio-Tri RNA Reagent. The RNA concentration was quantified with NanoDrop (Thermo Scientific), and approximately 1.2 to 1.5µg were taken to produce cDNA. cDNA was produced with qScript kit (Quanta, qScript). The resulting cDNA was used for qPCR analysis (QuantStudio 12K Flex, Applied Biosystems), using SYBR master mix (Applied Biosystems). Each cDNA sample was analyzed with viral immediate-early, early, late genes, LAT and a cellular housekeeping gene (HMBS). The sequences for primers for viral genes and cellular housekeeping gene appear in Supplementary Table 1.

Fluorescent in situ hybridization

For the FISH experiment, G1 wells were split into three G2 wells, one of them with a glass coverslip. FISH protocol was adapted from (6). Shortly, at 5 WPI, recovered cells (as well as cells that were not exposed to virus) were fixed with 1ml 4% Paraformaldehyde (PFA) in PBS for 10 minutes. Cells were permeabilized with 0.5% Triton X-100 in PBS for 15 minutes. Coverslips were incubated at 20% glycerol (Sigma-Aldrich) one to three days. Next, freeze and thaw cycles were carried out by dipping coverslips in liquid nitrogen four times. After several washing steps, coverslips were transferred to 50% formamide (Merck) in Saline Sodium Citrate buffer X2 (SSCX2; 0.3M Sodium Chloride (Bio-Lab), 30mM

Sodium Citrate (Merck) adjusted to pH 7.0 with HCl and filtrated) and kept in 4°C. Hybridization was done with hybridization solution (10% Dextran Sulfate (Calibochem) in SSCX2) containing the fluorescent probes final volume of 0.3 μM for each probe. The fluorescent probes labeled with Alexa flor 488 at the 5' end (IDT), each of a repeat sequence from the viral genome, are listed at Supplementary Table 1. Denaturation of cellular and probe DNA was carried out for 2 minutes at 95°C. Incubation was done at 37^{0}C humid environment for 3 days. After hybridization, each coverslip was washed 3 times with SSCX2 at 37°C and 3 times with SSCX0.1 at 63°C. Each coverslip was covered with Fluoroshield mounting medium containing DAPI (Abcam, Cambridge, MA, USA). Slides were visualized with a Nikon Eclipse Ti microscope equipped with Yokogawa CSU X-1 spinning disc confocal system.

Chromatin immunoprecipitation assays

For the ChIP experiment, G1 wells were split into one well from 6-wells plate. Cells were trypsinzed and diluted with medium into a final volume of 20ml. Number of cells was then determined and the cells were prepared for ChIP analysis (according to (7) protocol). Briefly, Formaldehyde (Sigma-Aldrich) was added to achieve a final concentration of 1%. The tube was shaken every 2 minutes, for 10 minutes. Then, Glycine (Sigma-Aldrich) was added to achieve final concentration of 125mM. Cells were washed twice in 3ml of ice cold PBS supplemented with 1:1000 HALT Protease Inhibitor (Thermo Scientific). After the second wash, cells were added with SDS-lysis buffer (Tris-HCl pH 8.1 50mM, EDTA 10mM, SDS 1%), 1ml per 10 million cells, supplemented with 1:1000 HALT Protease Inhibitor (Thermo Scientific,) and held in ice for 10 minutes. The cells were stored in -80°C until further ChIP analysis. Cells were defrosted on ice, and sonicated at 4°C. Sonicated samples in PBS/T 0.5% BSA buffer (PBS, Tween20 0.02%, BSA 0.5%) were diluted 1:10 in dilution buffer (Tris-HCl pH 8.1 16.7mM, NaCl 167mM, EDTA 1.2mM, SDS 0.01%, Triton X100 1.1%, complete volume with DDW) supplemented with 1:1000 of a mix of PMSF 0.5M (Sigma-Aldrich), pepsin A 10 mg/ml (Sigma-Aldrich), Leupeptin 10mM (Sigma-Aldrich) and Aprotinin (Sigma-Aldrich). Sonicated samples were centrifuged and 40μl from the supernatant was saved in (-20°C) as input. 2ml (2 million cells) of the supernatant were mixed with Dynabeads Protein A and Protein G (Invitrogen) pre-bound to one of three antibodies: Rabbit Anti-Histone H3 (abcam, ab1791), Mouse Anti-Histone H3 (tri methyl K27) (abcam, ab6002), Rabbit Anti-Histone H3 (tri methyl K4) (abcam, ab8580) and Normal Mouse IgG (santa-cruz, sc2025). Then, the Dynabeads-antibody chromatin tubes were rotated on a roller in 4°C overnight. On the next day, the tubes were

placed on the magnet and the supernatant was discarded. Then the chromatin was washed twice in Low Salt buffer (NaCl 150mM in Tris-HCl pH 8.1 20mM, EDTA 2mM, SDS 0.1%, Triton X100 1% supplemented with 1:1000 of a mix of PMSF 0.5M, pepsin A 10 mg/ml, Leupeptin 10mM and Aprotinin. Next, the chromatin was washed twice in High Salt buffer (NaCl 500mM in the same buffer). Next, the chromatin was washed once in LiCl buffer (LiCl 250mM in Tris-HCl pH 8.1 20mM, EDTA 1mM, NP-40 0.5%, Ma-deoxycholate 0.5% supplemented with 1:1000 of a mix of PMSF 0.5M, pepsin A 10 mg/ml, Leupeptin 10mM and Aprotinin. The last two washes were carried out with TE buffer (Tris-HCl pH 8.1 10mM, EDTA 1mM) supplemented with 1:1000 of PMSF 0.5M. Finally, 150µl of Elution buffer (100mM Sodium bicarbonate, SDS 1%) were added to the IP tubes and they were rotated at room temperature for 15 minutes, then centrifuged. The 150µl supernatant was saved in new PCR tubes and 8µl of 5M NaCl was added to each of the IP tubes. The 40µl of input tubes from the day before were added with 110µl Elution buffer and 8µl of 5M NaCl was added to each of the input tubes. All tubes were incubated at 65°C overnight. The next day, 50µl TE buffer supplemented with 1:20 ProteinaseK (NEB) were added to each sample and incubated at 50°C for four hours. The output DNA was purified with DNA PCR purification kit (Machery-Nagel), according to manufacturer's protocol. The resulting DNA was used for qPCR analysis (QuantStudio 12K Flex, Applied Biosystems), using SYBR master mix (Applied Biosystems) and the products were run on 2% agarose gel. The sequences for primers for viral DNA appear in Supplementary Table 1.

Table S1. List of primer and probes.

Viral DNA + ChIP			
Gene	**Name**	**Sequence**	**Source**
UL3	UL3 fwd	GCTTCGGGTCGTTATTCAG	
	UL3 rev	GCCAGCATGACGTTATCTC	
US11	US11 fwd	ACCCAAACGCACCAAAC	
	US11 rev	GCATCGACGTGTTCGTAC	
ICP0 promoter	ICP0 promoter fwd	AGGCGTGGGGTATAAGTT	
	ICP0 promoter rev	TGTGGTGATGCGGAGAG	
ICP8 promoter	ICP8 promoter fwd	TCCCCTTCACCGACAAC	
	ICP8 promoter rev	ACCTGACCGTAAGCATCT	
Host SPATA	SPATA fwd	GCTAAGGCTGATTCCTCTTTG	
	SPATA rev	CCGACGTTAATAGCAAGGC	
Viral RNA			
Gene	**Name**	**Sequence**	**Source**
LAT	LAT enhancer F	GGCGTCGGCGACATCC	(8)
	LAT enhancer R	CCCGAGTGTTCATCTCAGGC	(8)
ICP4	ICP4 fwd	TCAGGTTGTTGCCGTTTATTG	(3)
	ICP4 rev	AAGTTGTGGACTGGGAAGG	(3)
UL29 (ICP8)	UL29 fwd	GAAGGTGCATAGGTTACAGGG	(3)
	UL29 rev	GCCAAGATGCTGTTTTACCTG	(3)
UL19	UL19 fwd	GACCGCTTTGTGACTGAGAA	(3)
	UL19 rev	CTGGGTGAGCGTGAAGTTTA	(3)
HMBS	HMBS fwd	GGCCTGCAGTTTGAAATCAT	(3)
	HMBS rev	CATTCTTCTCCAGGGCATGT	(3)
FISH Probes			
Gene	**Name**	**Sequence**	**Source**
Non coding	a’ Repeat	5Alex488N-CCCCCCGCTCCTCCCCCCGCT	(9)
Non coding	RS reiteration	5Alex488N-CGCCCCTCGCCCCCTCCCGCC	

Supplementary Table 1. List of primer and probes. The primers and probes used in this work are listed according to the assays.

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