

# Inhibition of H3K27me<sub>3</sub>-Specific Histone Demethylases JMJD3 and UTX Blocks Reactivation of Herpes Simplex Virus 1 in Trigeminal Ganglion Neurons

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**Herpes simplex virus 1 (HSV-1) genomes are associated with the repressive heterochromatic marks H3K9me<sub>2</sub>/me<sub>3</sub> and H3K27me<sub>3</sub> during latency. Previous studies have demonstrated that inhibitors of H3K9me<sub>2</sub>/me<sub>3</sub> histone demethylases reduce the ability of HSV-1 to reactivate from latency. Here we demonstrate that GSK-J4, a specific inhibitor of the H3K27me<sub>3</sub> histone demethylases UTX and JMJD3, inhibits HSV-1 reactivation from sensory neurons *in vitro*. These results indicate that removal of the H3K27me<sub>3</sub> mark plays a key role in HSV-1 reactivation.**

Histone posttranslational modifications, both euchromatic and heterochromatic, are found in association with herpes simplex virus 1 (HSV-1) genes as the genome associates with nucleosomes during latency. The presence of permissive euchromatic marks (H3KAc, H9KAc, and H18K18Ac) in association with the latency-associated transcript (LAT) promoter and 5' exon during latency is consistent with its transcriptional activity during latency (1–4). Intrinsic to the latent state, however, are several repressive epigenetic marks that are also found in association with lytic genes of the latent HSV-1 genomes, notably, H3K9me<sub>2</sub>/me<sub>3</sub> and H3K27me<sub>3</sub>, enriched along lytic genes. These marks have been implicated in helping to maintain lytic genes in a repressed state (3, 5, 6).

Presumably, to reactivate from latency, the H3K9me<sub>2</sub>/me<sub>3</sub> and H3K27me<sub>3</sub> repressive marks need to be removed by chromatin remodeling enzymes. H3K27me<sub>3</sub> is reversibly removed through the action of specific histone demethylases KDM6B/JMJD3 and KDM6A/UTX (7). H3K9me<sub>3</sub> can be removed by all members of the JMJD2 family (8), while H3K9me<sub>2</sub>/me<sub>3</sub>/1 can be removed by LSD1, KIAA1718, JHDM1F, and JMJD1A (9–12). In this study, we sought to determine whether blocking the removal of H3K27me<sub>3</sub> would also block HSV-1 reactivation. This issue was addressed using GSK-J4, a specific inhibitor of both JMJD3 and UTX (13).

**Inhibition of JMJD3 and UTX blocks reactivation-induced H3K27me<sub>3</sub> demethylation of HSV genomes.** To understand the roles of UTX and JMJD3 in viral reactivation, we used an *in vitro* model of HSV-1 latency in primary adult murine trigeminal ganglion (TG) neurons (14), where reactivation is stimulated by nerve growth factor (NGF) depletion (15). TGs from 6-week-old outbred Swiss Webster mice were incubated in papain (Worthington, Lakewood, NJ) reconstituted with neurobasal A medium (Gibco; catalog no. 10888-022) followed by Hanks balanced salt solution containing dispase (4 mg/ml) and collagenase (4.7 mg/ml) (Sigma). Mechanically dissociated TGs were purified on an iodixanol gradient (OptiPrep; Sigma). Neuronal cells were counted and plated onto plates precoated with poly-D-lysine and laminin. Cells were maintained in neuralbasal media containing NGF, glial cell line-derived neurotrophic factor, neurturin, L-glutamine, 2% (vol/vol) B-27 supplement minus AO (Gibco; catalog no. 10889-038), and 1% (vol/vol) penicillin-streptomycin. To suppress actively divid-

ing nonneuronal cells, the media were supplemented with fluoro-deoxyuridine for 72 h prior to infection with virus. Three-day-old TG cultures were infected with HSV-1 17syn+ at a multiplicity of infection of 10. The media were replaced after 1 h and cultures maintained for 7 days. Latency was verified by the presence of the LAT and the lack of detectable infectious virus.

It has been demonstrated that the disruption of NGF signaling can induce HSV-1 reactivation and that addition of anti-NGF antibody to explants or cultures can produce robust reactivation (15–17). Therefore, in order to determine the ability of GSK-J4 to block H3K27me<sub>3</sub> following NGF-induced reactivation, GSK-J4 (Xcess Biosciences Inc.) was added to latently infected TG neurons, and, 24 h following treatment with the inhibitor, anti-NGF antibody (Abcam; catalog no. ab6198) was added at a working concentration of 2 µg/ml to induce HSV-1 reactivation. Twenty-four hours following α-NGF induction, HSV-1 genomes were analyzed for H3K27me<sub>3</sub> by chromatin immunoprecipitation (ChIP). ChIP assays were performed as previously described (1, 3) using anti-H3K27me<sub>3</sub> antibody (Millipore; catalog no. 07-449).

Real-time PCRs were performed using Applied Biosystems TaqMan Fast Universal PCR master mix (catalog no. 4352042; Life Technologies, Grand Island, NY, USA) and target-specific primers for immediate-early (ICP4), early (thymidine kinase [TK]), and late (U<sub>L</sub>20) HSV-1 lytic genes (Table 1). In addition, TaqMan forward and reverse primers and probes were designed for an internal negative control and positive control corresponding to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and

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TABLE 1 Real-time PCR primer-probe sets

| Gene target      | Forward primer            | Reverse primer            | Probe           |
|------------------|---------------------------|---------------------------|-----------------|
| UL20             | CCATCGTCCGGCTACTACGTTAC   | CGATCCCTCTTGATGTTAACGTACA | CCCGCACC GCCCAC |
| TK               | CACGCTACTGCGGGTTTATATAGAC | GGCTCGGGTACGTAGACGATAT    | CACCACGCAACTGC  |
| Hox A5, upstream | AGCAGCAGGGCCAATTCT        | GCTGCCAAGCCAGCTT          | CCCGGATGCACCC   |
| ICP4 CDS         | CACGGGCCGCTTAC            | GCGATAGCGCGCTAGA          | CCGACGCGACCTCC  |

Hox A5 cellular genes, respectively (Table 1). For analysis of mRNA transcripts, samples were normalized to the mouse 18S rRNA gene.

H3K27me3 ChIP analysis revealed that 50  $\mu$ M GSK-J4 inhibited the decrease in H3K27me3 that occurred following anti-NGF-induced reactivation in the vehicle-treated group in latently infected neurons (Fig. 1A). In addition, transcripts representing the three classes of HSV-1 lytic genes were all significantly reduced in the presence of GSK-J4 relative to the vehicle-treated control results (Fig. 1B). There was no significant change in the levels of H3K27me3 on both cellular con-

trols examined in cells treated with GSK-J4 compared to vehicle (Fig. 1C). This suggests that the effect seen in Fig. 1A where GSK-J4 treatment caused an increased enrichment of H3K27me3 on the HSV-1 genomes occurred by virtue of the fact that it blocked the demethylation of H3K27me3 on the reactivating genomes. As shown by examination of 18S RNA levels, there was a significant decrease observed in GSK-J4-treated samples compared to the vehicle-treated sample (Fig. 1D), indicating that GSK-J4 treatment could have been affecting transcription of at least some cellular genes as well. Taken together, these results suggest that during induced reactivation, JMJD3

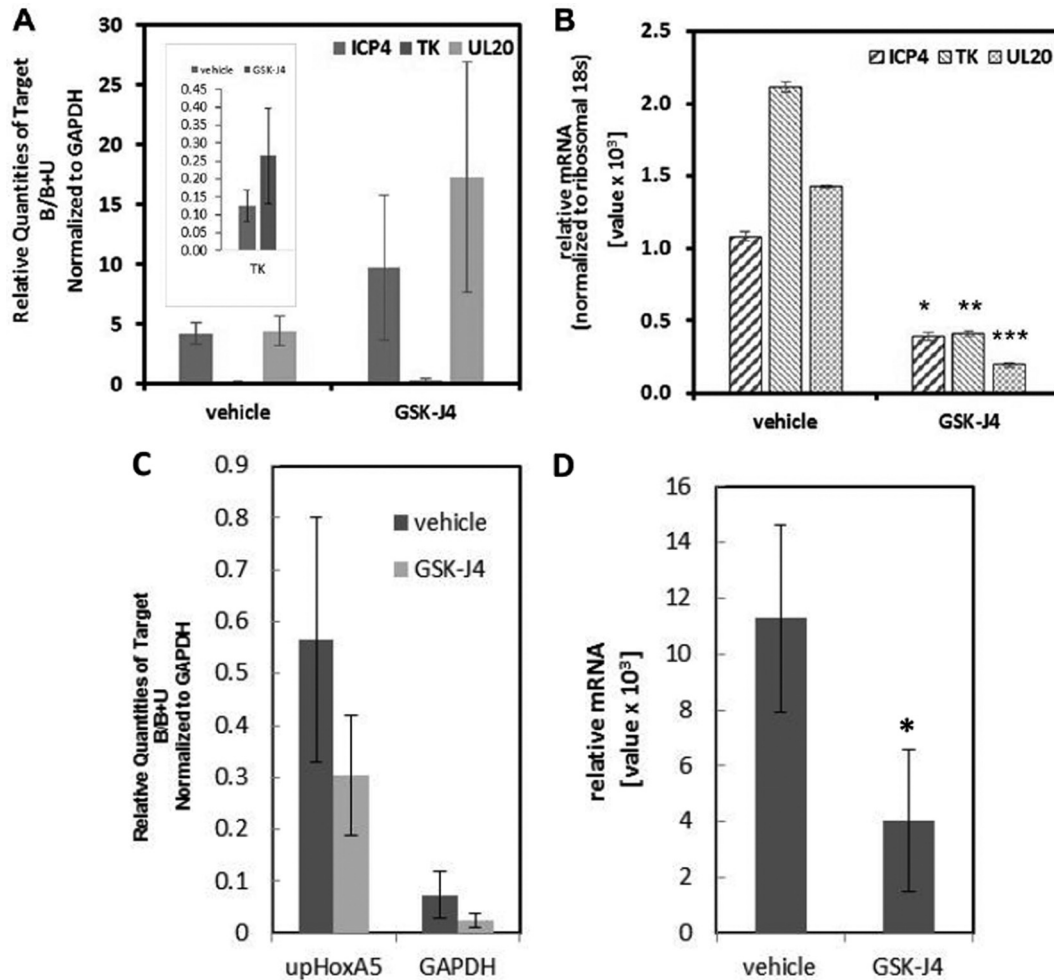


FIG 1 JMJD3/UTX-specific inhibitor GSK-J4 inhibits HSV-1 replication in reactivated TG neurons. (A) Enrichment of H3K27me3 via ChIP analysis of reactivated TG neurons treated with GSK-J4 for HSV-1 genes ICP4, TK, and UL20. Relative quantities of enrichment are represented as bound/bound + unbound. (B) HSV-1 ICP4, TK, and UL20 mRNA levels. Error bars are  $\pm$  standard deviations (SD). *P* values were determined using Student's *t* test (\*, *P* = 0.003; \*\*, *P* = 0.00001; \*\*\*, *P* < 0.000001). (C and D) Cellular controls for H3K27me3 ChIP (C) and mRNA 18S (D) assays of latently infected TGs stimulated with NGF antibody in the presence of GSK-J4. \*, *P* value < 0.06.

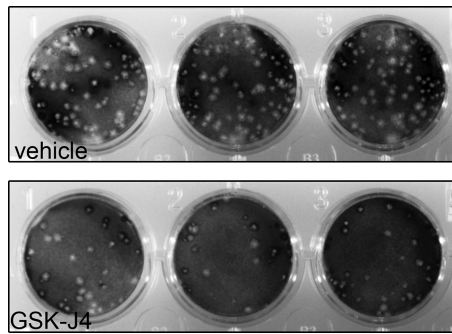


FIG 2 Plaque assay of infectious HSV-1 particles reactivated from latently infected TG neurons in the presence of JMJD3/UTX-selective inhibitor GSK-J4.

and UTX actively reduce levels of H3K27me3 demethylation, thereby allowing viral transcription.

**GSK-J4 treatment reduces the production of infectious virus following  $\alpha$ -NGF-induced reactivation.** To determine if the ability of GSK-J4 to block JMJD3 and UTX and maintain viral gene repression translated to a block in productive reactivation, we quantified infectious virus particles produced following induced reactivation. Latently infected TG neurons were analyzed 24 h following anti-NGF treatment in the presence or absence of GSK-J4. This analysis indicated that GSK-J4 treatment resulted in a greater than 5-fold reduction in viral yield during reactivation (Fig. 2 and Table 2).

Profiles of HSV-1 epigenomes in latently infected neurons demonstrate the existence of both constitutive and facultative heterochromatic marks (18, 19). It has been demonstrated that H3K9me2/3 demethylases (JMJD2s) and H3K9me1/2 demethylase LSD1 reduce HSV-1 reactivation both *in vitro* and *in vivo* (20–22). It is difficult to state why inhibitors of the H3K9me2/me3 demethylases did not completely inhibit reactivation fully given issues with penetrance in the tissue and the experimental half-life of the drug. Because it is known that at least as large a proportion of the latent genomes is associated with the H3K27me3-repressive mark, this left open the issue of whether inhibitors of H3K27me3 could also inhibit reactivation by blocking reactivation from HSV-1 genomes that were repressed by this other heterochromatic mark. In summary, the observations presented here indicate that removal of the H3K27me3 mark is required for efficient reactivation of HSV from latency. These results provide new insights into the regulation of the HSV-1 epigenome in latently infected neurons undergoing reactivation and suggest that separate but parallel pathways to reactivation exist based on the need to remove

TABLE 2 Summary of reactivated HSV-1 infectious particles following treatment of latently infected neurons in the presence of JMJD3/UTX-selective inhibitor GSK-J4

| Biological replicate | Vehicle (PFU/ml) | GSK-J4 (PFU/ml) <sup>a</sup> |
|----------------------|------------------|------------------------------|
| 1                    | 517              | 58                           |
| 2                    | 210              | 82                           |
| 3                    | 403              | 67                           |
| Avg                  | 377              | 69*                          |

<sup>a</sup> \*, the *P* value determined with a Student's *t* test using a two-tailed distribution of vehicle to GSK-J4 is 0.08.

both the H3K9me2/me3 and H3K27me3 heterochromatin marks. Finally, these results suggest that small-molecule inhibition of UTX and JMJD3 histone H3K27me3 demethylases could be a promising strategy for therapeutic intervention for recurrent HSV disease.

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