Novel Role of the Innate Immune DNA Sensor IFI16 (Interferon Gamma Inducible Protein 16) as a Major Epigenetic Modulator During KSHV Infection and Lytic Reactivation

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Abstract

BACKGROUND: Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with AIDS related Kaposi's sarcoma and primary effusion lymphoma (PEL). KSHV hijacks multiple cellular proteins and pathways to establish lifelong latency in infected hosts, and latency is associated with KSHV malignancies. It is well known that KSHV uses the repertoire of host epigenetic mechanisms to orchestrate its gene regulations. We have previously shown that Interferon-γ inducible protein 16 (IFI16), a host nuclear innate immune DNA sensor, plays an important role in the maintenance of KSHV latency [*J Virol. 2016 Sep 12;90(19):8822-41*]. In addition, studies from our laboratory and others have suggested that IFI16 acts as an antiviral restriction factor against lytic replication of a number of DNA viruses, by inhibiting either viral-DNA replication (HCMV and HPV) or transcription (HSV-1, HCMV and HPV) through epigenetic modifications of the viral epigenome. However, till date, no specific epigenetic function of IFI16 has been identified to support this claim. Thus, the primary objective of this study is to identify possible epigenetic functions of IFI16.

METHODS: We thus hypothesized that IFI16 mediates epigenetic modifications of the KSHV episome in some way that leads to its heterochromatinization and/or maintenance of its heterochromatic form. To this end, we first attempted to decipher if IFI16 is associated with any histone methyltransferases (MTase) activity that leads to its observed transcriptional silencing function. We used both *de novo* infection and latency models of KSHV to validate our findings.

RESULTS: Co-immunoprecipitation and His-tag pulldown experiments revealed that IFI16 is able to pull-down an MTase that can specifically transfer methyl groups from S-adenosylmethionine (SAM) to histone H3. Knockdown of IFI16 followed by ChIP analysis in latently infected B cells confirmed that IFI16 plays an important role in recruiting a H3-MTase that specifically methylates at H3K9 leading to heterochromatinization of the KSHV genome. During *de novo* infection of endothelial cells, CRISPR mediated knockout of IFI16 limited the recruitment of H3K9-me3 and RNA polymerase II on both latent and lytic KSHV promoters leading to a dysregulation in the latency establishment process. Subsequently, we screened all known human H3K9-MTase and identified two proteins that interact with IFI16 and is recruited by IFI16 at KSHV promoters.

CONCLUSIONS: Thus, we have identified a previously unknown function of IFI16 that leads to epigenetic silencing of the KSHV genome via recruitment of the silencing mark, H3K9-me3 on the KSHV genome. Presently studies are underway to decipher further mechanistic details of this novel role of IFI16 in herpes viral life cycle and the possibility of using established epigenetic drugs to control KSHV infection and latency.

Introduction

- Lytic reactivation of the latent KSHV genome is one of the major contributors of KS, PEL and MCD pathogenesis, all of which are well known AIDS related malignancies.
- Studies by us and others have reported IFI16's role as a DNA sensor that detects nuclear replicating herpes viral genomes leading to IFI16-ASC-procaspase-1 inflammasome formation that results in the production of the inflammatory cytokine Interleukin 1β (IL-1β). Cell Host Microbe. 2011 May 19;9(5):363-75; PLoS Pathog. 2015 Jun 29;11(6):e1005030
- Recently, IFI16 has also been shown to be involved in the induction of IFN- β during KSHV and HSV-1 *de novo* infection of target cells via the IFI16-STING-TBK-IRF3 axis.
- Apart from its role in immune surveillance, IFI16 has also been shown to function as a viral restriction factor against DNA viruses like HCMV and HSV-1, neither of which can establish a successful latency *in vitro*. PLoS Pathog. 2014 Nov 6;10(11):e1004503

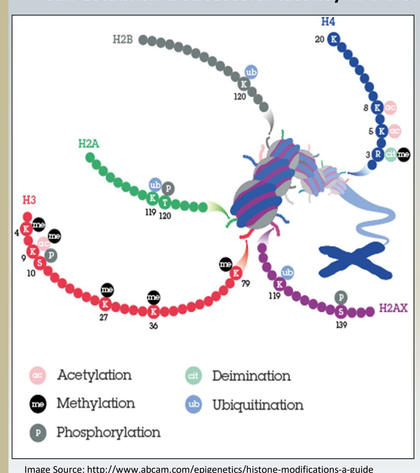


Image Source: http://www.abcam.com/epigenetics/histone-modifications-a-guide

- We also found that IFI16 co-localizes with latent KSHV genome in latently infected B-lymphoma cells. J Virol. 2013 Apr;87(8):4417-31
- We have previously shown that IFI16 plays an important role in the maintenance of KSHV latency. J Virol. 2016 Sep 12;90(19):8822-41
- We and others have shown that IFI16 regulates herpes viral transcription, possibly through modulation of epigenetic factors. PLoS Pathog. 2014 Nov 6;10(11):e1004503
- Here, we asked the question - "What is the potential mechanism of IFI16 mediated regulation of herpes viral transcription – role in epigenetic modulation?"

Results

IFI16 knockdown in PEL cells results in a global increase of KSHV lytic gene expression and viral DNA replication

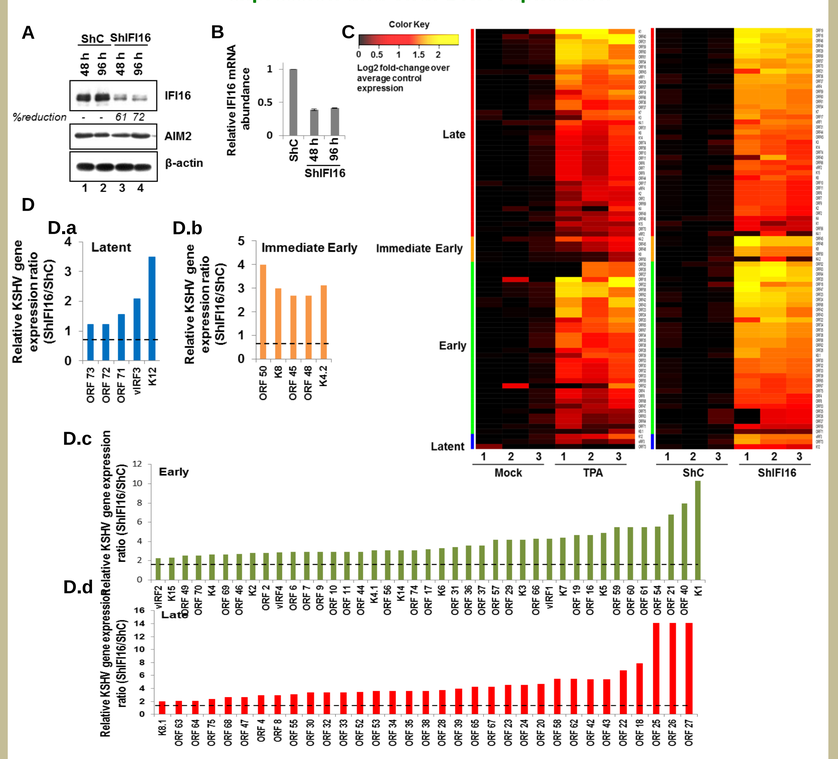


Figure 1. RNA-Seq analysis of KSHV gene expression after IFI16 knockdown. (A) Immunoblot showing efficient knockdown of IFI16 in **BCBL-1 cells (KSHV latently infected cells)** 48 h and 96 h post-transduction with lentivirus carrying ShIFI16. AIM2 immunoblot showing the absence of an off-target effect of the ShRNA used. **(B)** Real time RT-PCR reactions showing IFI16 mRNA levels after 2 and 4 days of IFI16 knockdown. **(C)** Transcriptome analysis of KSHV after either induction with 12-O-Tetradecanoyl-phorbol-13-acetate (TPA) or knockdown of IFI16 in BCBL-1 cells. Total RNAs were extracted 4 days after the indicated treatment and were subjected to cDNA library preparation using a QuantSeq 3'mRNA-Seq Library Prep kit for Illumina sequencing. The libraries were sequenced using HiSeq, and the sequences were mapped to the reference KSHV genome to determine the relative abundance of viral transcripts. Absolute values, calculated based upon the number of reads for each gene, were used for analyzing relative expression of KSHV genes as heat maps. **(D)** Next generation RNA sequencing results from the three independent IFI16 knockdown experiments shown in Fig.1C were compared to their respective control knockdown (ShC) and the results presented as relative fold change of KSHV latent **(a)**, immediate early **(b)**, early **(c)**, and late **(d)** genes expression ratios (ShIFI16/ShC). The dashed horizontal line indicated 1.5 fold mark, set as the threshold. Values presented are the average of three independent experiments.

IFI16 restricts KSHV lytic transcripts and proteins in the PEL cell line BCBL-1

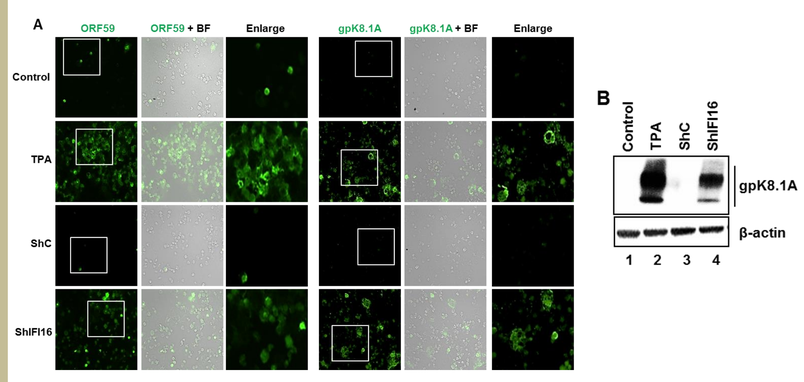


Figure 2. Analysis of the effect of IFI16 knockdown and TPA induction on KSHV lytic gene expression. (A) Immunofluorescence analysis of **BCBL-1 cells** after either TPA induction (4 days) or IFI16 knockdown (4 days). Anti ORF59 antibody was used as a marker of early lytic gene expression, while gpK8.1A antibody was used as a marker of late lytic genes. **(B)** Immunoblot of gpK8.1A after either IFI16 knockdown or TPA treatment (4 days).

IFI16 knockdown results in KSHV lytic reactivation, while overexpression leads to suppression of lytic reactivation

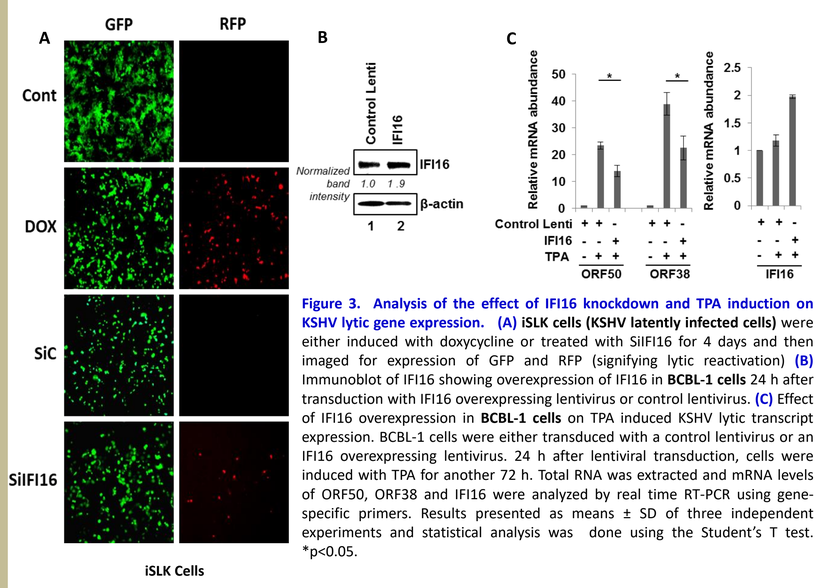


Figure 3. Analysis of the effect of IFI16 knockdown and TPA induction on KSHV lytic gene expression. (A) iSLK cells (KSHV latently infected cells) were either induced with doxycycline or treated with SilFI16 for 4 days and then imaged for expression of GFP and RFP (signifying lytic reactivation) **(B)** Immunoblot of IFI16 showing overexpression of IFI16 in **BCBL-1 cells** 24 h after transduction with IFI16 overexpressing lentivirus or control lentivirus. **(C)** Effect of IFI16 overexpression in **BCBL-1 cells** on TPA induced KSHV lytic transcript expression. BCBL-1 cells were either transduced with a control lentivirus or an IFI16 overexpressing lentivirus. 24 h after lentiviral transduction, cells were induced with TPA for another 72 h. Total RNA was extracted and mRNA levels of ORF50, ORF38 and IFI16 were analyzed by real time RT-PCR using gene-specific primers. Results presented as means ± SD of three independent experiments and statistical analysis was done using the Student's T test. *p<0.05.

IFI16 binds to the latent KSHV genome as well as to the incoming KSHV genome during *de novo* infection

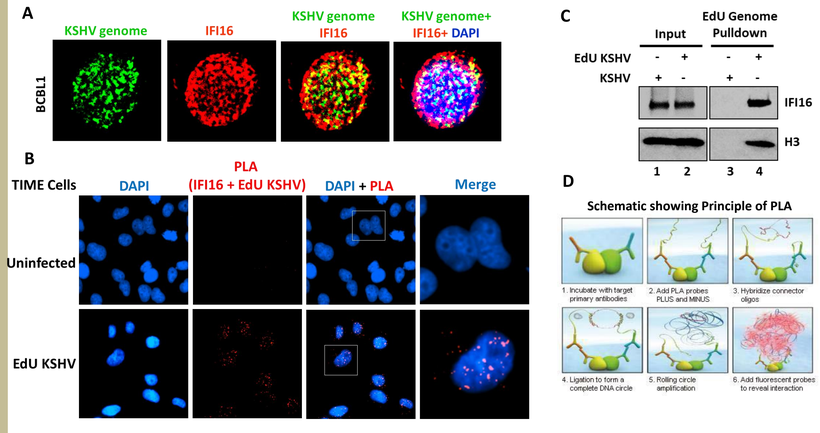


Figure 4. KSHV genome FISH, PLA and KSHV EdU genome pulldown experiments show that IFI16 binds to the KSHV genome. (A) FISH was performed in **BCBL1 cells** using KSHV specific genomic probes and co-localization with IFI16 was evaluated using IFA **(B) TIME cells** were infected with EdU labeled KSHV for 6 hours and then Proximity Ligation Assay (PLA) was performed using EdU antibody and IFI16 antibody. **(C) TIME cells** were infected with EdU labeled KSHV for 6 hours and then the EdU labeled genome was pulled down using CLICK chemistry. Binding of IFI16 and H3 was evaluated. **(D)** Schematic showing Principle of PLA reaction.

IFI16 binds to promoter sequences of all KSHV gene classes

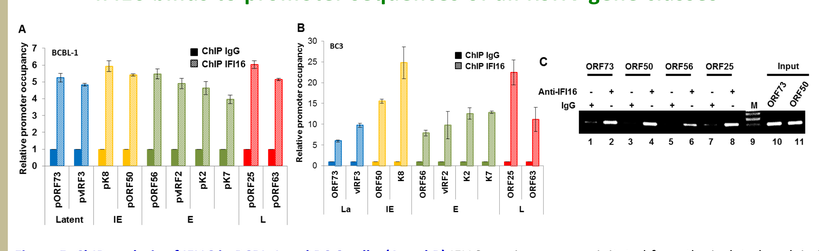


Figure 5. ChIP analysis of IFI16 in BCBL-1 and BC-3 cells. (A and B) IFI16 was immunoprecipitated from the isolated nuclei of **BCBL1** and **BC-3 cells** and bound DNA was analyzed by real-time RT-PCR with primers specific to regions (~100-200 bp) flanking the transcription start sites of the indicated genes. Relative promoter occupancy compared to IgG immunoprecipitation is represented. Results presented as means ± SD of three independent experiments. **(C)** Agarose gel showing the specific PCR amplification of the indicated KSHV promoter regions after IFI16 ChIP (BCBL-1 cells).

IFI16 functions as a repressor for KSHV gene transcription

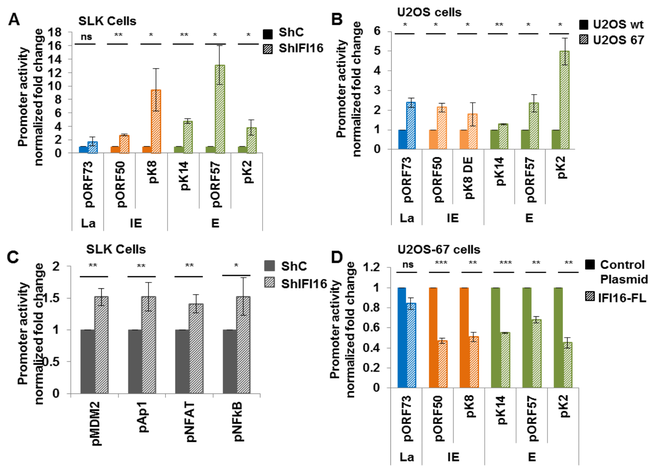


Figure 6. Luciferase reporter assay measuring IFI16 transcriptional repressor activity. (A) IFI16 was knocked down via lentivirus mediated ShRNA transduction in **SLK cells**. 24 h post-KD, luciferase constructs of different KSHV gene promoters were transfected using lipofectamine. 24 h post-transfection, luciferase signal was quantitated using a dual-luciferase assay and plotted. **(B)** Similar experiment as in A, using wt-U2OS cells and CRISPR-IFI16 KO U2OS clone 67 cells. **(C)** Similar experiment as in A, using SLK cells and luciferase constructs of different human gene promoters. **(D)** Luciferase assay after overexpression of IFI16 in U2OS 67 cells (IFI16 KO). U2OS-67 cells were transfected with either IFI16 overexpressing plasmid or control plasmid and after 24 h, luciferase constructs of different KSHV gene promoters were transfected. 24 h post-transfection, luciferase signal was quantitated using a dual-luciferase assay and plotted. Results presented as means ± SD of three independent experiments and statistical analysis was done using the Student's T test. *p<0.05, ** p<0.01, ***p<0.001.

Dynamics of different H3 modifications after reactivation of the latent KSHV genome

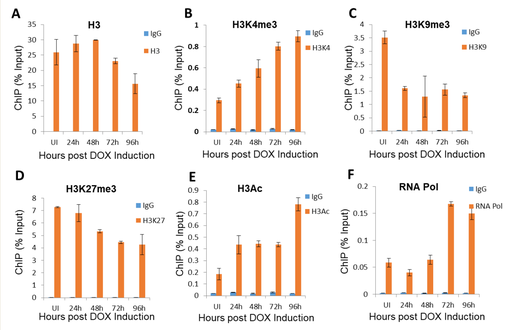


Figure 7. Chip was performed with the indicated antibodies and binding to the ORF63 promoter (late promoter) was tested. (A-F) TREX BCBL1 cells (KSHV latently infected cells) were induced for different lengths of time with Doxycycline (Dox) and ChIP was performed against total H3, H3K4me3, H3K9me3, H3K27me3, H3Ac (PAN acetylation) and RNA Polymerase to assess the change in H3 trimethylation with time post induction. IgG ChIP was performed as negative control and promoter occupancy is represented as % input pulldown.

IFI16 knockdown causes reduction in H3K9me3 recruitment on latent KSHV genomes

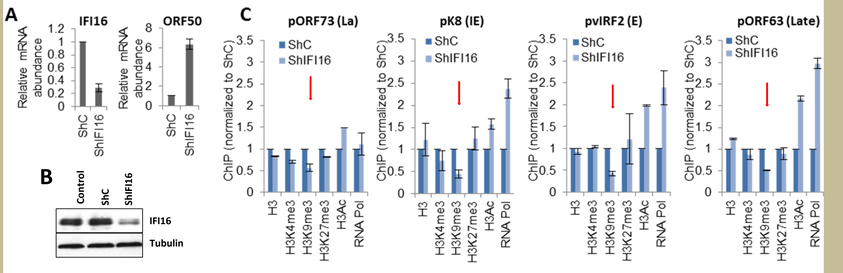


Figure 8. Chip was performed with the indicated antibodies after lentivirus mediated knockdown of IFI16 and binding to different promoters were tested. (A) IFI16 was knocked down using Lentivirus mediated ShRNA transduction in **BCBL1 cells** for 72 hours. Real time RT-PCR showing mRNA levels of IFI16 and KSHV ORF50 after KD. ShC was used as a control. **(B)** WB showing IFI16 KD. **(C)** ChIP was performed against the indicated antibodies. All 4 temporal KSHV gene classes were evaluated by real-time PCR following ChIP.

Knockdown of IFI16 also causes reduction in H3K9me3 recruitment during *de novo* infection of TIME cells with KSHV

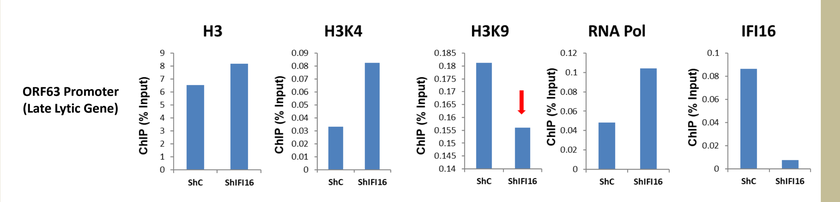


Figure 9. Chip was performed with the indicated antibodies after SiRNA mediated knockdown of IFI16 in TIME cells. IFI16 was KD using SiRNA in **TIME cells** and 72 hours after KD, infected with KSHV for 8 hrs. ChIP was performed against the indicated proteins. Abundance of different proteins and histone marks on the late lytic promoter, ORF63 was tested. Promoter occupancy is represented as % input pulldown.

Re-introduction of IFI16 into CRISPR-Cas knocked-out U2OS cells (U2OS 67) results in increased recruitment of H3K9me3 on infecting KSHV genome

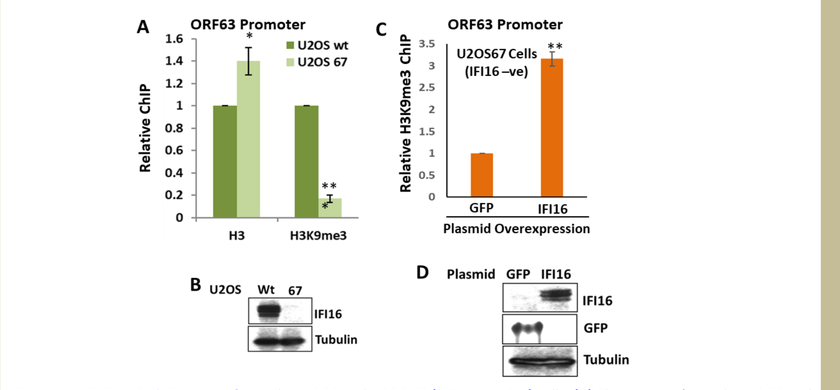


Figure 10. H3K9me3 ChIP was performed in U2OS and U2OS 67 (IFI16 negative) cells. (A) ChIP was performed in **U2OS and U2OS 67 (IFI16 negative) cells** infected with KSHV for 12 hours. Recruitment of H3K9me3 on the KSHV late ORF63 promoter was tested. **(B)** WB showing knockout of IFI16 in U2OS 67 cells. **(C)** U2OS 67 cells were either transfected with GFP plasmid or IFI16 overexpression plasmid followed by KSHV infection for 12 hours after 72 hours of transfection. ChIP was performed against H3K9me3 and recruitment of H3K9me3 on the KSHV late ORF63 promoter was tested. **(D)** WB showing overexpression of IFI16 and GFP in U2OS 67 cells.

IFI16 is associated with a cellular H3K9me3 methyltransferase

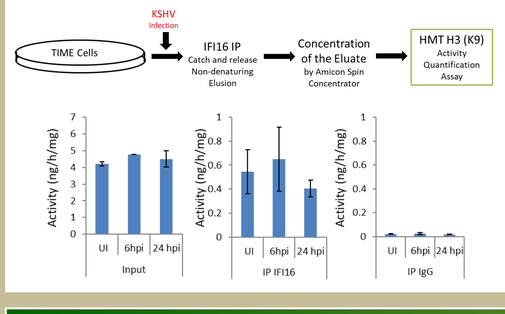
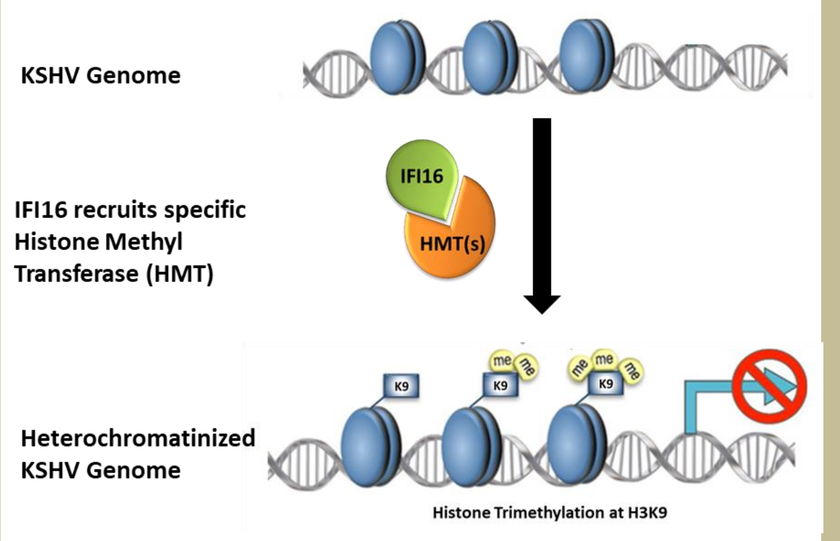


Figure 11. Immunoprecipitation of IFI16 results in pull-down of a H3K9me3 methyltransferase. TIME cells were infected with KSHV for 6 or 24 hours and subsequently, IFI16 was IP'ed with anti-IFI16 antibody. The IP was eluted under non-denaturing conditions and the eluate was concentrated using a spin concentrator. The concentrated eluate was assessed for H3K9me3 methyltransferase activity using an H3K9me3 specific capture antibody based assay (abcam). The H3K9me3 methyltransferase activity is represented as ng/h/mg. IgG IP was used as a negative control.

Conclusions



Acknowledgements

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