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Impact of ATP-dependent RNA Helicase DDX3X on Herpes Simplex Type 1 (HSV-1) Replication

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Résumé

Le criblage par siRNA de 49 protéines de l'hôte qui sont incorporées dans les particules matures du virus herpès simplex de type 1 (VHS-1) a révélé l'importance d'au moins 15 d'entre elle pour infectivité du virus (Stegen, C et al. 2013). Parmi celle-ci figure la protéine humaine DDX3X, qui est une ARN hélicase ATP-dépendante. Cette protéine multifonctionnelle participe à différents stages de l'expression génique, tels que la transcription, la maturation et le transport d'ARNm ainsi que la traduction. DDX3X est impliquée dans la réplication de plusieurs virus tels que le Virus de l'immunodéficience humaine de type 1 (VIH-1), l'hépatite B (VHB), le virus de la vaccine (VACV) et le virus de l'hépatite C (VHC). Le rôle exact de DDX3X dans le cycle de réplication du VHS-1 est toutefois inconnu. Ce mémoire consiste en l'étude détaillée de l'interaction de DDX3X avec le virus. De manière surprenante, tant l'inhibition que la surexpression de DDX3X réduit de manière significative l'infectivité du VHS-1. Fait intéressant, lorsque nous avons restauré la déplétion de DDX3X par une construction résistante aux ARNi utilisés, le virus pouvait de nouveau infecter les cellules efficacement, indiquant que le virus est sensible aux quantités de cette protéine de son hôte. Nos résultats indiquent de plus que le virus modifie la localisation de DDX3X et cause son agrégation tôt dès les premiers temps de l'infection. Cependant, le virus ne modifie pas les niveaux cellulaires de DDX3X dans deux des trois lignées cellulaires examinées. Nous avons également pu établir que cette protéine n'a pas d'effet sur l'entrée du VHS-1, suggérant qu'elle agit à un stade ultérieure de l'infection. En examinant cette relation plus en détail, nos résultats ont démontré que l'inhibition ou la surexpression de DDX3X inhibent toutes deux la production de nouvelles particules virales en réduisant l'expression des diverses classes cinétiques des protéines virales et ce au niveau de leur transcription. Malgré le rôle connu DDX3X dans la stimulation de la réponse immunitaire innée et la production d'interférons de type I, l'impact de DDX3X sur la réplication du VHS-1 est ici indépendante de cette fonction. Ces travaux démontrent donc une nouvelle voie d'action de DDX3X sur les virus en agissant directement sur la transcription de gènes viraux et la réplication du génome d'un virus à ADN. En comprenant mieux cette interactions hôte-pathogène, il est maintenant envisageable de concevoir des nouvelles approches thérapeutiques contre ce virus.

Mots-clés : Virus herpès simplex de type 1, DDX3X, expression génique virale, ARNi.

Abstract

siRNA screening of 49 host proteins that are known to be incorporated in the mature virions of herpes simplex virus type 1 (HSV-1) revealed the importance of at least 15 cellular proteins for viral infectivity (Stegen, C et al. 2013). Among these, was the human protein DDX3X, a DEAD-box ATP-dependent RNA helicase. This multifunctional protein participates in different stages of gene expression such as mRNA transcription, maturation, mRNA export and translation. DDX3X has been shown to be involved in the replication of several viruses such as human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV) vaccinia virus (VACV) and hepatitis C virus (HCV). The exact role of DDX3X in HSV-1 replication cycle is not known. Here we sought to find the detailed interaction between DDX3X with HSV-1. Surprisingly, the down-regulation as well as overexpression of DDX3X, significantly reduced the infectivity of HSV-1, indicating that the virus is sensitive to the precise levels of DDX3X. Accordingly, when we rescued DDX3X back to its normal cellular levels by sequential transfection of DDX3X siRNA and siRNA resistant DDX3X plasmid, the virus was able to infect cells efficiently compare to wild-type conditions. Furthermore, the virus changes the localization of DDX3X and causes its aggregation at early times in the infection. However, the virus does not change the cellular levels of DDX3X in at least two of three different cell lines tested. Using a luciferase assay we were able to establish that this protein has no effect on the entry of HSV-1. In fact, depleting or overexpressing DDX3X impaired the production on newly assembled viral particles by blocking the expression of all classes of viral proteins at the transcription level. Despite the known role of DDX3X in the stimulation of innate immune response and interferon type I production, DDX3X appears to act on HSV-1 replication independently of this pathway. This highlights a novel route of action of DDX3X by acting at the transcription level and the consequent genome replication of a DNA virus. By better understanding such pathogen interactions, it might now be possible to design novel therapeutic approaches.

Keywords: Herpes simplex virus type 1, DDX3X, viral gene expression, siRNA, protein overexpression and depletion

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ATP	Adenosine triphosphate
BGS	Bovine growth serum
cccDNA	Closed circular DNA
cIAP-1	Apoptosis protein-1
CNS	Central nervous system
CRM1	Chromosomal maintenance 1
CSPG	Chondroitin sulfate proteoglycan
DENV	Dengue virus
DMEM	Dulbecco's Modified Eagle Medium
D N A	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
ds	Double stranded (DNA or RNA)
E	Early (genes)
EBV	Epstein–Barr virus
eIF	Eukaryotic translation initiation factor
EJC	Exon junction complex
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase-3
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCF-1	Host cell factor 1
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HIF-1	Hypoxia inducible factor-1

HIV-1	Human immunodeficiency virus type 1
hpi	Hours post infection
HRE:	HIF-1 responsive elements
HS	Heparan sulfate
HSPG	Heparin sulfate proteoglycan
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HVEM	Herpesvirus entry mediator
ICP	Infected Cell Protein
IE	Immediate early (genes)
IFN	Interferon
IKK-ε	IκB kinase ε
IR	Inverted repeats
IRF3	Interferon Regulatory Factor 3
IR_L	Inverted repeats long
IR_S	Inverted repeats short
JEV	Japanese encephalitis virus
KHSV	Kaposi's sarcoma-associated herpesvirus
L	Late (genes)
LAT	Latency-associated transcript
LMB	Leptomycin B
miRNA	Micro RNA
MOI	Multiplicity of infection
mRNA	Messenger RNA
mRNP	Messenger ribonulceoprotein particles
ND10	Nuclear domain 10
NLS	Nuclear localization signal
NP	Nucleoprotein

NPC	Nuclear pore complex
NS	Non-structural (proteins)
NXF1	Nuclear RNA export factor 1
MAVS	Mitochondrial antiviral signaling protein
OCT-1	Octamer-binding protein 1
ORF	Open reading frames
Ori	Origin of replication
PACT	Protein Activator of the interferon-induced protein kinase
PFU	Plaque-forming units
PKD	Protein kinase D
PNS	Peripheral nervous system
rcDNA	Relaxed circular partially double stranded DNA
RNA	Rribonucleic acid
RNAi	RNA interference
RNAPII	RNA polymerase II
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG	Stress granules
siRNA	Small interfering RNA
ss	Single-stranded (DNA or RNA)
TANK	TRAF family member-associated NF-kappa-B activator
TBK1	TANK-binding kinase 1
TFII	Transcription Factor II
TGN	Trans-Golgi network
TNF	Tumor necrosis factor
TRAIL-R2	TNF-related apoptosis-inducing ligand receptor 2
U_L	Unique long
Us	Unique short

UTR	Untranslated region
UV	Ultraviolet
VHS	Virion host shutoff (protein)
VHS-1	Virus herpès simplex de type 1
VP	Viral protein
VZV	Varicella-zoster virus
WNV	West Nile Virus
wt	Wild-type

برای پدرم و برای مادرم

هر آنچه که دارم از شماست

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Chapter 1. Introduction

1.1. General

1.1.1. Definition and Discovery of HSV-1

In the ancient Greece, the term "herpes" was used to define lesions that appeared to "crawl" along the skin (1). Herpes characteristic sores have been prevalent enough in history to find their way in Shakespeare's play, Romeo and Juliet where he writes *"O'er ladies lips, who straight on kisses dream, which oft the angry Mab with blisters plague"* (Act I, Scene IV) (2). However, it was not until the early 20th century, that the viral etiology of the lesions was described by Lowenstein, when he showed the virus to be transferred from human skin to rabbit corneas (3).

1.1.2. Herpesvirus Classification

Herpesviruses are a family of ubiquitous, enveloped, large DNA viruses that concertedly cause infection in a wide host range. Three families comprise the herpesvirales order: Herpesviridae, the herpesviruses of reptiles, birds, and mammals; Alloherpesviridae, the

Herpesviruses of amphibians and fishes and Malacoherpesvirdae, the herpesviruses of bivalves (4, 5). The Herpesviridae family, based on host/cell tropism, multiplication speed and clinical aspects of disease, is further classified into three subfamilies, alpha-, beta- and gamma-. Nine herpesviruses have been recognized to primarily infect humans up to now, with members in each of the subfamilies: the alphaherpesviruses include the genera Simplexvirus (HSV-1 and HSV-2) and Varicellovirus (VZV); betahepesviruses, the genera Roseolovirus (HHV-6 and HHV-7) and Cytomegalovirus (HCMV); and gammaherpesviruses, the genera Rhadinovirus (KSHV) and Lymphocryptovirus (EBV). Herpes simplex virus 1, or **HSV-1**, is the prototypical alphaherpesvirus with a broad host range, rapid life cycle and the ability to establish latency in neurons of the peripheral nervous system (1, 6).

1.2. Herpes Simplex Virus Properties

1.2.1. Transmission and Epidemiology

Herpes simplex viruses can be readily transmitted through close contact, establishing a lifelong infection throughout which infected carriers, even in the absence of clinical symptoms are capable of shedding transmissible virus (7). HSV-1 is typically acquired early during childhood through direct contact of mucosal membranes or abraded skin with oral lesions. Virus stays dormant in the sensory ganglion's nucleus and may occasionally reactivate where newly produced virions travel back to mucosal sites or skin, ending in an active epithelial infection and disease reoccurrence (8).

HSV-1 infections are very common as 45% to 98% of the human population is estimated to be seropositive. Several demographic factors determines HSV-1 prevalence including geographic location, age and socioeconomic status, seroprevalance increasing with age and lower socioeconomic status (9). Interestingly, some studies estimate over 100% seroprevalence for herpes simplex viruses in the developing countries (10). This could raise serious problems in areas with a high prevalence of endemic human immunodeficiency virus (HIV), since the likelihood of HIV transmission and infection could increase in the presence of genital herpes (HSV- 1 and 2) infection (11-13).

1.2.2. Pathology and impact on human health

HSV-1 infections range in the severity from mostly mild or asymptomatic to life threatening (14). It is of note that albeit the common association of HSV-1 with orofacial lesions (herpes labialis) and HSV-2 with genital ulcers (herpes genitalis), both can invade either mucosal surfaces (1). Infections can also be manifested on widespread regions of the skin (herpes gladiatorum). These episodes of infection can be frequent, disfiguring and painful in the immunocompromised individuals, such as those with underlying diseases or undergoing immunosuppressive therapy (15). Several factors have been documented that might reactivate the virus from latency, resulting in the mentioned recurring episodes. These include exposure to stimuli, such as psychological stress (16), menstruation (17), ultraviolent light, and fever (18).

Organ transplant recipients often experience a progressive disease in which lesions are spread out to the esophagus, respiratory tract or gastrointestinal tract and these patients are typically resistant to antiviral therapies like acyclovir (19, 20).

Additional fatal and severe conditions are observed in HSV-1 infections despite the availability of antiviral medications. Nearly 10% of total acute encephalitis cases are due to Herpes simplex infections, HSV-1 being the recurrent cause of sporadic fatal encephalitis (21-23). For many years the origin of these infections within the central nervous system (CNS) has been a topic of interest, and it seems that primary infections and recurring infections equally contribute to encephalitis cases (24). The mortality rate, despite the availability of antiviral treatments such as acyclovir, remains at 20-30%. Furthermore, the majority of surviving patients experience a reduced quality of life, due to the fact that only in 2.5% of all patients' neurological function return to normal after recovery (25). Despite the rare intrusion of the virus in the CNS, the cost of encephalitis-associated hospitalization in the U.S. was about $2.0 billion in 2010 (26). Additionally, herpetic eye infection can lead to recurrent corneal infections known as herpetic keratitis or keratoconjunctivitis, which is the major cause of infectious corneal blindness in the developed countries (27). Corneal transplant is the only available treatment for this complication, yet due to the persistence of virus in the sensory ganglia, patients remain susceptible to recurrent infection (28). Lastly, neonatal HSV-1 transmission, which happens mostly when a pregnant woman acquires a primary infection in her third trimester, is frequently lethal in neonates (29, 30). This type of infection can be either intrapartum or postnatal, and cause diseases associated with the eyes, mouth and skin. Neonates are susceptible to a disseminated infection as well, which targets several organs like, lungs, liver central nervous system or adrenal glands. In the 60-75% cases of disseminated infections infants can also develop encephalitis (31). Where there is no antiviral therapy, 50% of surviving neonates with disseminated infection or encephalitis have lasting effects including learning disabilities, psychomotor retardation or vision impairment (32).

1.3. Herpes Simplex Virion

1.3.1. Viral Composition

The enveloped HSV-1 virion has an average diameter of 186 nm and is amongst the largest and most complex human viruses that have been identified (33, 34). The envelope of HSV-1 is derived from host cellular membranes, such as the trans-Golgi network (TGN), and it is decorated with spikes of embedded viral glycoproteins (1, 35-37). HSV-1 virions contain 13 distinct viral glycoproteins which help the attachment and entry of the virus. The tegument layer underneath the envelope is an asymmetrical, non-structured protein layer that carries at least 23 viral proteins, including transactivator proteins such VP16, ICP0 and ICP4 and the virion host shutoff (VHS) protein, as well as a number of other proteins that facilitate productive infection of permissive cells (38). Surprisingly, 49 cellular proteins are also found in the tegument layer, however their importance for the virus is still unknown (39). The well-structured, icosahedral (T=16 symmetry) viral capsid that lies within the tegument, is approximately 125 nm in diameter and contains 162 capsomeres (40, 41). Finally, HSV-1's genomic material is encompassed by the capsid and consists of a single molecule of approximately 150 kbp of double stranded (ds) DNA (Fig 1.1). Furthermore, contrary to other DNA viruses, like papillomaviruses or polyomaviruses, herpesvirus genomes are not associated with nucleosome proteins or significant amounts of any other DNA-binding proteins during latency (42-44). Instead, HSV-1 recruits the anionic polyamine spermine to balance the negative charge of the DNA phosphate backbone within the virion (45, 46).

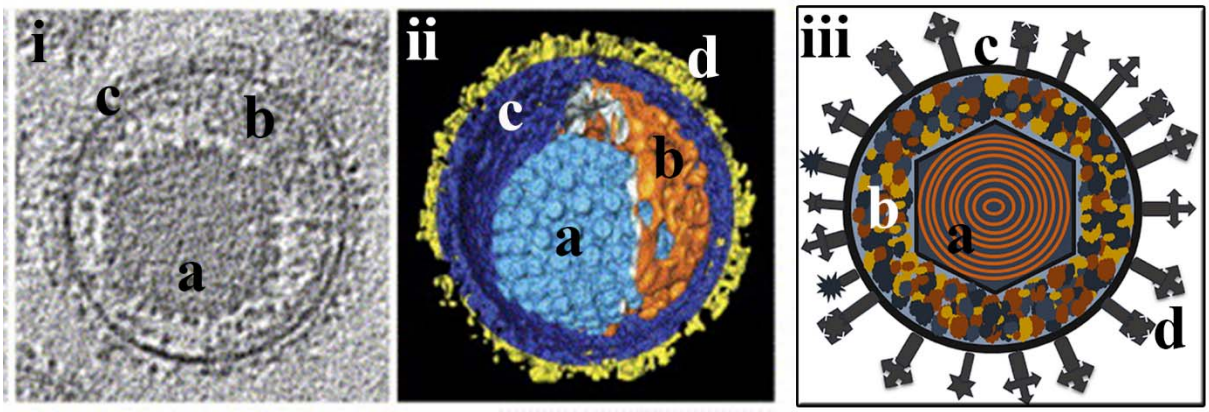

Figure 1.1. HSV-1 virion composition.

Electron cryo-tomographic visualization of virions; i) Central slice through each reconstructed volume (the tomogram), ii) cut-away views of surface renderings where distinct structural entities are represented by different colours (243). iii) Schematic representation. The virus is surrounded by an envelope membrane (c; dark blue in section ii) decorated with a number of different glycoprotein spikes (d; yellow in section ii), encompassing the proteinaceous tegument layer (b; orange in section ii) and the off-centre icosahedral capsid (a; in section ii) containing the genomic. Electron micrographs (panels i and ii) adapted from: *Grünewald K, and Cyrklaff M; Curr Opin Microbiol. 2006 Aug;9(4):437-42.* (47).

1.3.2. Genome Organization

The double stranded genomic DNA of HSV-1 is formed of two covalently linked regions called the unique long (U_L) and unique short (Us) segments which are flanked by inverted long (R_L) and short repeat (R_S) regions, respectively. These segments are joined with a variable number of repeated *a* sequences (48). A number of genes, including ICP34.5, ICP0 and the LAT, are located within these repeat regions and are thus carried as two copies within the genome (Fig 1.2). Although, methylation of the HSV-1 DNA has not been detected and likely does not affect viral replication, its genome have about 68% GC content, with many potential CpG motifs for DNA methylation (49-51).

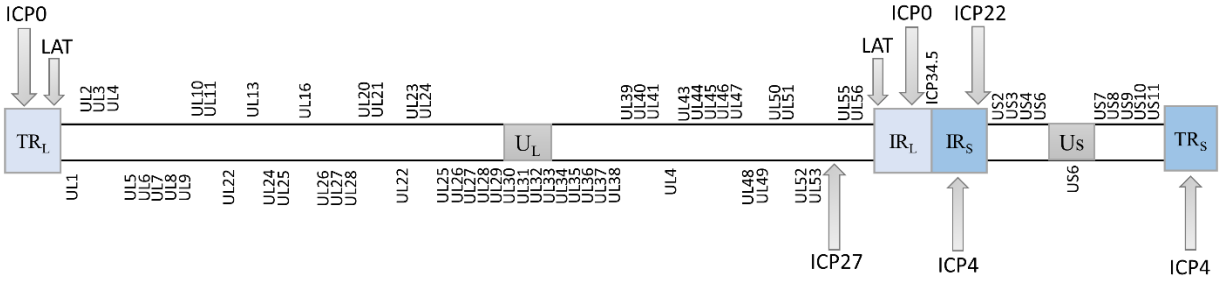

Figure 1.2. HSV-1 genome organization.

Linear dsDNA of HSV-1 is composed of two unique sequences, the unique long (UL) and unique short (Us) segments, each coding diverse viral proteins (UL1-UL56, US1-US11) which are flanked by inverted repeat elements (TR_L and IR_L; IR_S and TR_S), some of which also code for additional viral proteins. Adapted form: *Simonato M, et al; Trends Neurosci. 2000 May;23(5):183-90.* (52).

HSV-1's genomic DNA contains a total of 94 open reading frames (ORF), from which a pool of at least 75 distinct genes are known to be expressed. Among these genes are a set of 40 genes called "core genes" which are commonly present in all herpesviruses. Four capsid proteins, six DNA replication proteins, seven DNA packaging proteins, four envelope glycoproteins along many other proteins are translated from core genes. Other unconserved HSV-1 genes can be further categorized in essential and accessory genes (Table 1.1). Despite the necessity of all the HSV-1 accessory genes for replication and propagation *in vivo*, almost half of them are not required for replication in cell cultures (53, 54). Table 1.1 summarizes all identified HSV-1 genes, their regulation, products and proposed functions.

Table 1.1. HSV-1 genes, their protein products, expression kinetics (three classes of HSV-1 genes, i.e.; immediate early (α), eraly (β), true late (γ1) or leaky late (γ2)) and suggested functions. .

CORE GENES			
Capsid Assembly			
Gene	**Protein**	**Regulation**	**Proposed Function**
UL18	VP23	γ1	Forms triplex with VP19c
UL19	VP5	γ1	Major capsid protein
UL26	VP24 & VP21	γ	Serine protease (UL26 self-cleavage results in VP24 and VP21)
UL26.5	VP22a	γ	Scaffolding protein, cleaved from the capsid following the packaging of DNA
UL35*	VP26	γ2	Placed on the tips of the capsid hexons
UL38	VP19c	γ2	Member of the intercapsomeric triplex
DNA replication			
UL5	DNA helicase (HP1)	β	Member of DNA primase/helicase complex
UL8	HP3	β	Member of DNA primase/helicase complex
UL29	SSB (ICP8)	β	ssDNA-binding protein
UL30	POL	β	Catalytic subunit of DNA polymerase
UL42	PPS	β	Accessory subunit of DNA polymerase
UL52	HP2	β	Member of DNA primase/helicase complex
DNA cleavage/packaging			
UL6	PORT	γ	Capsid-associated, a subunit of the portal complex
UL15	TER1	γ	DNA terminase activity
UL17	CTTP	γ	Associated with B and C capsids(coded in the intron of UL15)
UL25	PCP	γ2	Seals capsids after DNA packaging?
UL28	TER2	γ	pac-motif-specific DNA-binding

UL32	CTNP	γ2	Essential for correct localisation of capsids?
Nucleic acid metabolism			
UL2*	UNG	β	Uracil DNA glycosylase
UL12*	NUC	β	Alkaline nuclease, takes part in in viral DNA processing
UL39*	RR1	β	Ribonucleotide reductase large subunit, protein kinase activity
UL50*	dUTPase	β	Deoxyuridine triphosphatase
Envelope glycoprotein			
UL1	gL	γ	In complex with gH, involved in entry, egress and cell-to-cell spread
UL10*	gM,	γ2	cell-to-cell spread
UL22	gH	γ2	In complex with gL, involved in entry, egress and cell-to-cell spread
UL27	gB	γ1	Forms a dimer, essential for entry
Others			
UL7*	EEP	γ1	Intracellular capsid- associated with, DNA packaging?
UL11*	CETP	γ1	Myristoylated virion protein, takes part in egress
UL13*	VPK	γ1	Tegument protein with protein kinase activity
UL14*	ECP	γ 2	Tegument protein with molecular chaperone function
UL16*	CETPbp	γ 1	Tegument protein, coded in the intron of UL15, DNA packaging?
UL24*	pUL24	γ1	Virion protein, nonglycosylated membrane-associated protein
UL31*	pUL31 (NELP)	γ 2	Interacts with UL34,nuclear matrix-binding protein
UL34*	pUL34 (NEMP)	γ1	Type II membrane protein associated with the inner nuclear membrane
UL36	ICP1/2 (LTP)	γ2	largest tegument protein, involved in both uncoating and egress
UL37	LTPbp	γ1	UL37 Tegument protein with nuclear export signal, involved in egress
UL49.5*	gN	γ2	Small membrane-associated protein, in complex with gM stimulates membrane fusion (55)

UL51*	CEF1	γ1	Palmitoylated virion protein, associated with the Golgi
UL54	ICP27 (MRE)	α	Regulation of gene expression at posttranscriptional level
NON-CORE GENES			
None-core essential genes			
UL9	ORI-B	β	Replication origin-binding protein
UL48	VP16	γ1	UL48 VP16, tegument protein stimulating immediate early gene expression
RS1	ICP4	α	Major regulatory protein
US6	gD	γ1	Required for entry, binds to herpesvirus entry mediator (HveA)
Non-core accessory genes			
RL1*	ICP34.5	γ 1	Modulates protein synthesis by binding to protein phosphatase 1α
RL2*	ICP0	α	Promiscuous transactivator with E3 ubiquitin ligase domains
UL3*	pUL3	γ2	Nuclear phosphoprotein, nucleolar localisation when expressed alone
UL4*	pUL4	γ2	Nuclear protein, colocalised with UL3 and ICP22
UL20*	pUL20	γ1	Involved in egress
UL21*	CEF2	γ1	Tegument protein, associated with microtubules
UL23*	TK	β	thymidine kinase, selective activation of acyclovir and ganciclovir
UL40*	RR2	β	Ribonucleotide reductase small subunit
UL41*	VHS	γ1	Virion host shutoff protein, involved in mRNA degradation
UL43*	NEMP	γ1	Membrane-associated protein
UL44	gC	γ 2	Takes part in adsorption, C3b-binding activity
UL45*	pUL45	γ 2	Type II membrane protein
UL46*	VP11/12	γ 1	Interacts with UL48 (VP16)
UL47*	VP13/14,	γ 1	Tegument protein that enhances immediate early gene expression
UL49*	VP22	γ 1	Tegument protein involved in intercellular trafficking

UL53*	gK	γ1	Viral egress, many syncytial mutations
UL55*	pUL55	γ 2	Nuclear protein, nuclear matrix-binding protein
UL56*	pUL56	γ 2	Type II membrane protein, associated with the Golgi and early endosome
US1*	ICP22	α	Stimulates the expression of late genes
US2*	pUS2	γ 2	Virion protein, interacts with cytokeratin
US3*	US3 PK	γ 1	protein kinase with antiapoptotic activity
US4*	gG,	γ 1	Envelope protein, involved in entry and egress
US5*	gJ	γ	Envelope protein, involved in protection from Fas-mediated apoptosis
US7*	gI	γ 1	Forms a complex with gE, cell-to-cell spread
US8*	gE	γ 1	Forms a complex with gI, Fc receptor activity, cell-to-cell spread
US8.5*	pUS8.5	γ 1	Localised in the nucleoli of infected cells
US9*	pUS9	γ	Type II membrane protein, anterograde transport of envelope glycoprotein?
US10*	pUS10	γ 1	Tegument protein, tightly associated with capsids
US11*	pUS11	γ 2	Tegument protein, RNA-binding activity, intercellular trafficking activity
US12*	TAPbp	α	TAP-binding protein, involved in MHC class I downregulation

Stars show the genes that are dispensable for replication *in vitro*. The table has been adapted from (*Nishiyama. Rev. Med. Virol. 2004; 14: 33–46.*) (53) and (http://people.virginia.edu/~jcb2g/background_genome.html)

1.4. HSV-1 Life Cycle

1.4.1. Lytic Replication

1.4.1.1. Viral Entry

The viral life cycle starts through attachment and entry of an infectious virion into a permissive host cell. Since, infection with HSV-1 begins at the mucosal epithelium or through a break in the skin, entry happens at these sites (1); However, HSV-1 penetrates most vertebrate's cell lines in vitro (54). Depending on the cell type, HSV-1 enters cells at a neutral pH through fusing with the plasma membrane or by endocytosis in either a pH-dependent or a pH independent manner. For example, in Vero cells the entry happens at a neutral pH; however, in HeLa and CHO-K1 cells, viral entry occurs through a pH-dependent endocytosis (55) while in C10 murine melanoma cells through pH independent endocytosis (56). This flexibility might ensure the productive infection in various cell lines (57). Interestingly low pH has no impact on the function of any HSV-1 glycoproteins except for minor changes in gB's conformation (58, 59). The complex entry process of herpesviruses comprises distinct steps: 1) attachment to the cell surface (tethering), 2) interaction with a unique entry receptor (Fig 1.3A), 3) internalization, and 4) membrane fusion (Fig 1.3B). Several viral envelope glycoproteins with specific roles in one or several steps are required for successful entry. Viral entry begins with an initial binding of the virus to ubiquitous heparan sulfate present on most cells (Fig 1.3A; dashed arrows). Although the viral glycoproteins mediating this process are dispensable for entry, this initial interaction tethers the virus to the cell membrane, thus favoring an encounter with a less accessible entry receptor (Fig 1.3A; solid arrows). Tethering of HSV-1 to the cell surface is mediated by gC binding to heparin sulfate and chondroitin sulfate proteoglycans (HSPG and CSPG) and, where gC is absent, gB can play the same role (60, 61). Following tethering, gD interacts with a cellular receptor, nectin-1 and activates the viral fusion machinery (62). Two other receptors are also capable of binding to gD and mediate entry: HVEM (herpesvirus entry mediator), a member of the tumor necrosis factor (TNF) receptor family (63) and a 3-O-sulfotransferases (3-O-STs) modified heparan sulfate (HS) (64). Activation of the fusion machinery that is constituted of gB and gH/gL (65) leads to fusion of viral envelope with plasma membrane or endocytosis to release the tegument and nucleocapsid into the cytoplasm (66, 67).

It is noteworthy that recruiting a definite entry receptor accomplishes two important goals: target-cell specificity and the activation of the fusion machinery only in close proximity of the virion to the cell membrane (56). After the virus has entered the cell, due to the high density of the cytoplasm and the distance between the port of entry and the nucleus, the virus actively transports its nucleocapsid on cellular microtubules (57) toward the nuclear pore complex (NPC) where it injects the naked viral genome inside the nucleus (58). Once in the nucleus, the linear viral genome circularizes rapidly (59, 60).

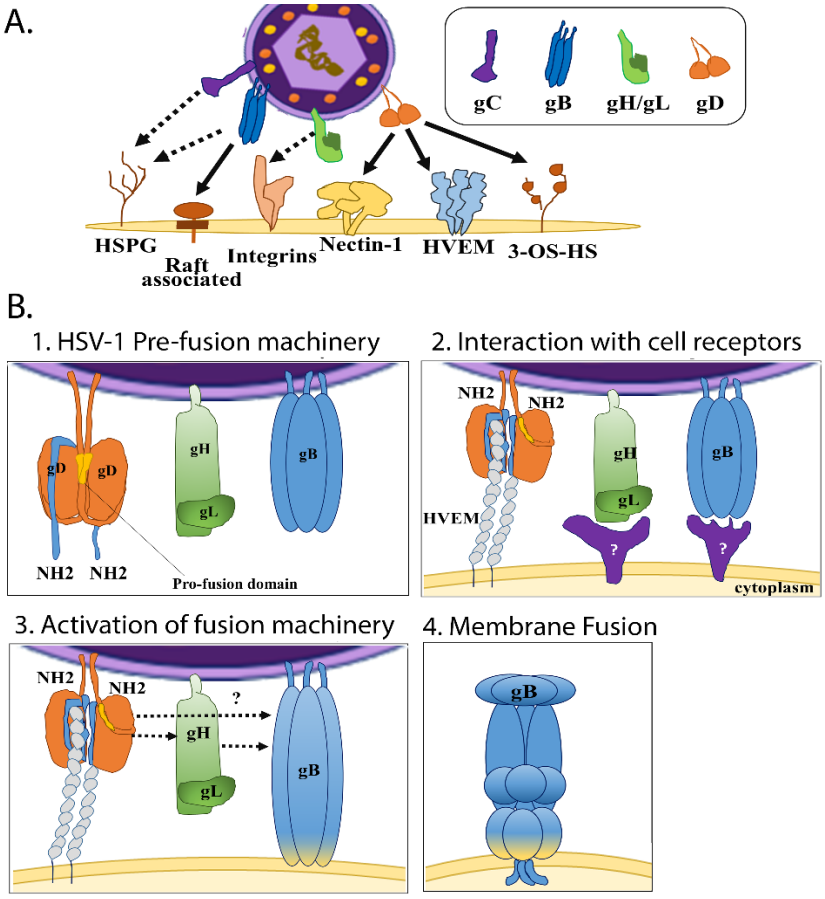

Figure 1.3. HSV-1 Entry.

A) Schematic illustration of HSV-1 glycoproteins and their known cellular receptors. Adapted from: *Krummenacher C, et al; Adv Exp Med Biol. 2013; 790:178-95*. (56). B) Fusion procedure : 1) Resting HSV-1 glycoproteins (pre-fusion form), gD is a dimeric molecule, spots close to its carboxyl termini (yellow) block the receptor-binding site. 2) After gD binds to

HVEM, the amino terminus of gD (blue) creates a loop and relocate the gD C terminus which contains the pro-fusion domain (yellow). The gB trimer also binds to a receptor and the gH–gL heterodimer may bind to a cellular receptor. 3) gD pro-fusion domains can interact with gH–gL and gB, and gH–gL and gB may interact with each other after gD receptor binding. 4) These interactions stimulate gB to insert its fusion loops (yellow shades) into the cell membrane and fold into its post-fusion form to mediate fusion. Adapted from: *Connolly SA, et al; Nat Rev Microbiol. 2011 May;9(5):369-81.*

1.4.1.2. Viral Gene Expression

The host cell RNA polymerase II (RNAPII) transcribes viral genes in an ordered cascade of viral gene products that is regulated by viral factors (61, 62). During productive infection at least three kinetic classes of genes are expressed, namely α- or immediate-early (IE) genes, β- or early (E) genes, and γ- or late (L) genes (63, 64) (Fig 1.4). The IE genes are those with the ability to be expressed prior to *de novo* viral protein synthesis. Yet, the activation of these genes is mediated by the late viral VP16 protein that enters the cells along with the incoming virions (65). The multiprotein activator complex composed of VP16, host cell factor 1 (HCF-1) and octamer-binding protein 1 (OCT1) binds target viral sequences within IE gene promoters to stimulate them through recruitment of additional cellular transcription factors, including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (66, 67). ICP0, ICP4, ICP22, ICP27, ICP47 and pUS1.5 proteins are encoded from IE genes (Table 1.1). Between 2 and 6 hours post infection (hpi), IE proteins reach their maximal levels (63). These proteins, particularly ICP4, are essential for the activation of E genes and progression of the viral lytic cycle (68, 69). Among early gene products are those required for viral DNA replication (70, 71). Late genes are transcribed once the DNA replication is initiated, and their products include proteins that are necessary for assembly of infectious progeny virions. The late genes are further grouped into true-late (γ1) and leaky-late (γ2). While, prior DNA replication is absolutely essential for the expression of the γ1 group, the γ2 genes are readily at detectable levels before DNA synthesis (1).

1.4.1.3. Viral Replication

Viral DNA synthesis, which begins as early proteins accumulate, typically happens around 6-10 hpi (72). Upon viral DNA synthesis, viral genome relocates to reorganized nuclear domain 10 (ND10) (73). ND10s are nuclear structures that comprise over 150 constituents (74). These compartments are involved in several pathways, including cell cycle arrest (75), gene regulation (76), DNA repair (77), apoptosis (78), oncogenesis (79) and anti-viral defense (80). While it is known that ICP0 degrades ND10 to inhibit the interferon response, it is not clear why viral DNA is found in the vicinity of ND10 (81). As DNA synthesis progresses, replication compartments are formed that ultimately fill the nucleus and marginalize cellular chromatin (82).

Seven early viral proteins are essential for DNA replication (83). DNA synthesis begins with binding of pUL9 and ICP8 to one of three origins of replication that result in dsDNA distortion. More precisely, pUL9 helps the DNA bending at the origin of replication so the viral polymerase can act on short sections of single-stranded DNA (ssDNA) and ICP8 along with pUL9 to stimulate helicase activity. ICP8 also binds to ssDNA at the replication fork to protect it. This event leads to the exposure of a small portion of ssDNA that can be further unwound by the helicase primase complex (84). The helicase-primase complex is composed of pUL5, pUL8 and pUL52 and unwinds double-stranded DNA, generates primers for lagging strand synthesis, and facilitates the interactions between additional viral proteins involved in the viral replication (72). The pUL30/pUL42 polymerase complex is the last to arrive at the site of replication, where new genomes are synthesized via theta replication resulting in a rolling circle strategy(85). pUL30, which has 3'-5' exonuclease activity and vast substrate affinity, is the catalytic subunit of DNA polymerase (86). pUL42, the processivity unit of DNA polymerase, "slides" on DNA and assures the association of pUL30 to the viral genome. This process eventually generates head-to-tail concatemers (87).

Since most of the adult human cells are not actively dividing and require low levels of deoxynucleotide triphosphates (dNTPs), HSV-1 has evolved to deal with this issue by encoding five metabolic enzymes that can increase nucleotide synthesis, including; thymidine kinase (pUL23), dUTPase (pUL50), ribonucleotide reductase (pUL39 and pUL40), uracil DNA glycosylase (pUL2) and alkaline nuclease (pUL12) (1).

1.4.1.4. Viral Assembly and Egress

As mentioned earlier, late proteins have structural functions and help the assembly of new capsids. Since capsids are assembled in the infected cell nucleus, all components need to be transported there whether by nuclear localization signal (NLS) or by piggy backing on proteins that contain such signals (88 126). The first step of capsid assembly is the interaction of VP5 and the pre-formed triplex proteins VP19C-VP23 along with the scaffolding protein, pre-VP22a to give rise to spherical procapsids (89, 90). Next, proteolytic degradation of the scaffold gives a stable, icosahedral, mature capsid (91) and viral DNA is concomitantly inserted to the capsid in an energy-dependent fashion (92).

Mature nucleocapsids need to exit the nucleus. The most widely-accepted model for capsid egress and maturation is envelopment/de-envelopment/re-envelopment pathway, through which capsid first acquires an envelope by budding across the inner nuclear membrane. Capsids then fuse with the outer nuclear membrane and lose this primary envelope and end up "naked" in the cytoplasm of the host cell. Tegument proteins associate with the viral capsid in different cellular compartments such as nucleus, the cytoplasm, and the site of final envelopment, however a clear picture of tegumentation sequence is still lacking (93-98). The capsids actively transit to the final assembly/envelopment site. Although under debate, most of the studies hint at the trans-Golgi network (TGN) as the final envelopment site (95). Once at this site, the tegument-coated capsids bind to glycoprotein-dense regions of the TGN, thus virus acquires its final envelope (98-100). This step is followed by the budding of enveloped particles into the secondary TGN-derived vesicles, a process that depends on several glycoproteins, including gD and the inner tegument proteins like pUL37. Afterwards, protein kinase D (PKD), a cellular protein, mediates fission at TGN (101-103). TGN derived vesicles containing mature virions then travel to the plasma membrane, a route that depends on cell protein, Myosin Va (104). Finally, the vesicles reach plasma membrane and release their cargo via exocytosis (104, 105) (Fig 1.4).

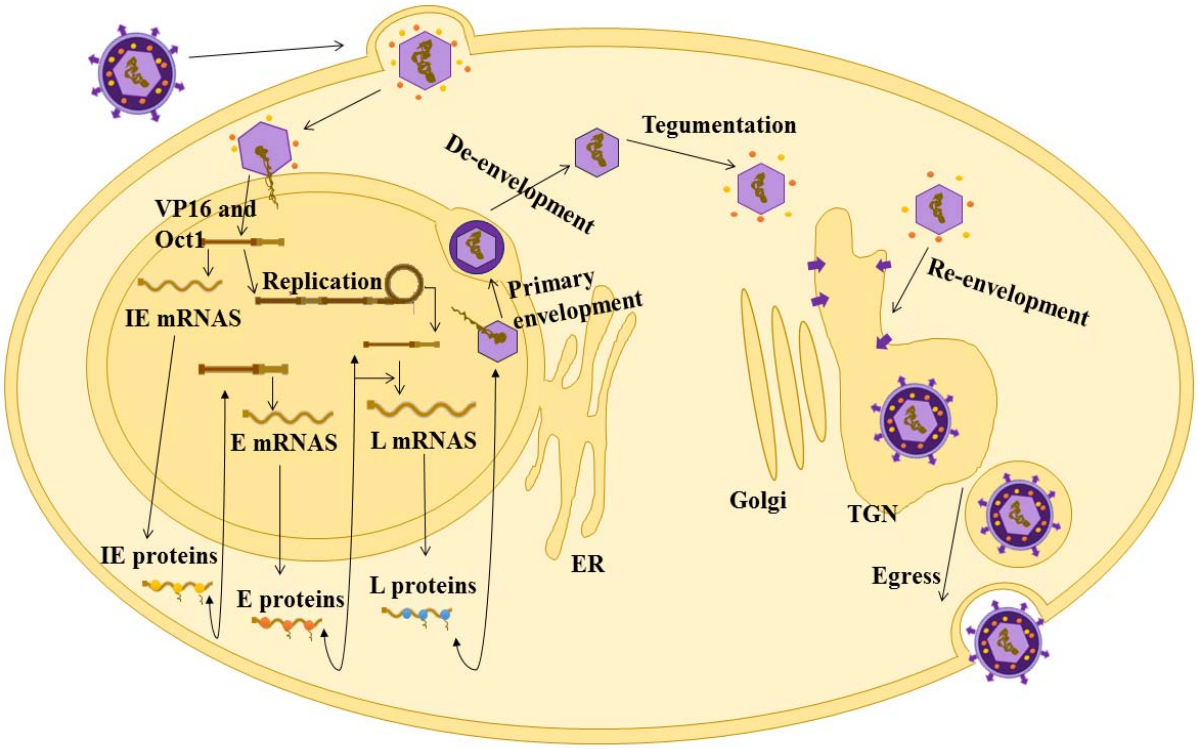

Figure 1.4. HSV-1 gene expression, assembly, transport and egress.

Adapted from: *Henaff D, et al; Traffic. 2012 Nov;13(11):1443-9.* (95) and *Simonato M, et al; Trends Neurosci. 2000 May;23(5):183-90.* (52).

1.4.2. Latent Infection

Lytic and latent cycles differ drastically in the pattern of viral gene expression and the outcome of infection. Latency is marked by the presence of the viral genomes in neurons of the peripheral nervous system (PNS) without the significant production of infectious progeny (1).

1.4.2.1. Establishment and Maintenance of Latency

After the primary infection of HSV-1 in epithelium, viral particles can spread to innervating sensory neurons of lips, gingiva and eyes that are in contact with the infected epithelium (106). Viral nucleocapsids reach the neuronal cell body/soma within a ganglion by fusing at the axonal termini and retrograde transport along the axons (107, 108). Establishment of latency in trigeminal ganglia can occur with or without lytic replication in the ganglia (109).

Inducing factors of particular gene transcription during latency are not well understood. However, it has been shown that VP16 and other outer tegument proteins are transported independently of viral nucleocapsids along the axons and is unable to reach the soma (67), thus viral gene transcription cannot be initiated and virus enters the latency programs. Based on these findings, a recent study by injecting viral particles containing VP16, either directly to the soma or axons, shows that virus direct entry to the soma favors the productive infection (110).

Upon the injection of viral genome into the neuron's nucleus, it rapidly becomes circularized and incorporates histones (111). During latency, viral transcription is limited to a number of sites, resulting in the generation of the latency-associated transcript (LAT) that can be processed into a stable intron and numerous viral micro RNAs (miRNAs) (112, 113). While LAT is not necessary for latency establishment, maintenance, or reactivation (114), it has a profound impact on these phases likely through interfering with other viral transcripts owing to the LAT-encoded miRNAs (115-117). miRNAs are short noncoding RNAs that are encoded by eukaryotic cells as well as various viral pathogens. (118, 119). Despite the extensive efforts, the function of the most of HSV-1 miRNAs have not yet been fully elucidated. However, they likely have a role in maintenance of latency and escaping the host immune response (119), as it has been shown that some HSV-1 miRNAs down-regulate the expression of IE genes such as ICP4, ICP0 or ICP34.5 (120, 121).

While lytic gene transcription during latency is repressed, recent advances in techniques detecting transcripts at the single-cell level propose that majority of infected neurons initially undergo a lytic gene transcription (122), and that lytic transcription happens in most of the latently infected LAT-expressing neurons (123). Although, there is no direct evidence indicating translation of lytic transcripts during latency, existence of stimulated, virus-specific CD8+ T cells within the latently infected sensory neurons supports the idea that at least some lytic proteins are expressed during this period (124-126).

1.4.2.2. Reactivation from Latency

A wide range of stimuli such as neuronal damage or stress can wake up the virus from latency. This periodical reactivation can occur throughout the host's life. Since LAT-negative

viruses reactivate less efficiently from neurons, it suggests that LATs mediate reactivation (127-130). Nonetheless, their role in reactivation is not known. The level of LATs and viral miRNAs declines during reactivation, at the same time lytic gene transcripts accumulate, ultimately leading to viral productive replication (131-133). Next, virions or virion components are anterogradely transported down neuronal axons, and the infectious particles are released at the axonal termini (134, 135). At this site, virus can access permissive peripheral tissue, typically proximate to the site of primary infection thus being able to resume lytic replication. Clinically, reactivation is important because it can result in painful recurrent lesions, blindness upon ocular reactivation, and transmission to new susceptible individuals (1).

1.5. HSV-1 incorporates host proteins

The majority of enveloped viruses tend to incorporate host cellular proteins. Although the role of all of these proteins has not been fully elucidated, it is clear that viruses highjack these molecules to favor their replication (136). HSV-1 as an enveloped virus is not an exception and a recent study from our lab has demonstrated that approximately 49 distinct cellular proteins with a wide range of functions are incorporated into mature HSV-1 virions (39). Later on, we evaluated whether these proteins have a significant impact on HSV-1 yields using small interfering RNAs (siRNA) technology. This screen revealed 15 that of these proteins are critical for viral propagation in cell culture. The ATP-dependent RNA helicase DDX3X is an interesting and top scoring candidate among them (137).

1.6. DEAD-Box proteins

The family of DEAD box proteins was first described in 1989 (138), and belongs to the RNA helicase superfamily 2 (139). DEAD-box proteins engage in the unwinding of double-stranded RNA molecules and the remodeling of RNA-protein interactions in an ATP-dependent fashion. These proteins are found in the majority of organisms and play central roles in almost all aspects of RNA metabolism including pre-mRNA splicing, transcription, RNA export, ribosome biogenesis, RNA decay and translation (140). DEAD-box RNA helicases comprise a

cluster of 9 conserved motifs placed in the center of the proteins that span a region of nearly 400 amino acids (Fig 1.5A). However, their N- and C termini are highly variable in both length and sequence, are likely involved in the interactions with substrate and cellular factors and dictate subcellular localization of the proteins (141). The majority of DEAD-box protein domains are involved in ATP metabolism: First, a Q motif takes part in modulation of ATP binding and hydrolysis as well as RNA substrate affinity (142, 143). Second, motif I, also known as the Walker A motif (Walker et al., 1982), is required for ATPase and helicase activities. Third, the term "DEAD-box" emerges from motif II, also known as the Walker B motif (144) is also involved in ATP-binding and/or ATP hydrolysis (144, 145). Forth, motif III is suggested to link ATPase and helicase activities (145). Fifth, motif IV is a poorly studied DEAD-box protein motif and no definite function has been assigned to this region (141). Finally, motif V is likely to be an RNA binding motif (146) and motif VI is essential for RNA binding and ATP hydrolysis (147).

1.6.1. DDX3

One of the members of the DEAD-box RNA helicase family is DDX3. Two functional genes of the DDX3 family exists in the human genome (148). Thus, there is a DDX3 gene located on the X chromosome, termed DDX3X (Uniprot.org O00571; DDX3X_HUMAN) (149) while its homolog (91 % amino-acid identity) DDX3Y is placed on the Y chromosome (Uniprot.org O15523; DDX3Y_HUMAN) (150). Despite the expression of DDX3X gene across a wide range of human tissues, DDX3Y is only expressed in the male germ line (151). Known DDX3 orthologues include mouse PL10 (152) and mDEAD3 (153), Xenopus An3 (154), yeast Ded1p (155) and *Drosophila* Belle (156). ATPase and helicase activities of DDX3 have been demonstrated experimentally since its labeling as a putative RNA helicase (Fig 1.5B). DDX3 has been defined as a helicase with "relaxed steroselectivity" as it can be activated by several ribo- and deoxynucleic acids (157, 158). Although, the exact roles of DDX3 remains ambiguous, it has been implicated in various cellular processes such as nucleo-cytoplasmic RNA shuttling, RNA transport, splicing, translation, tumorigenesis, IFN induction and apoptosis. Interestingly,

DDX3 has been shown to be involved in the replication of both RNA and DNA based viruses (159). The following sections will briefly go through the literature available on DDX3 to date.

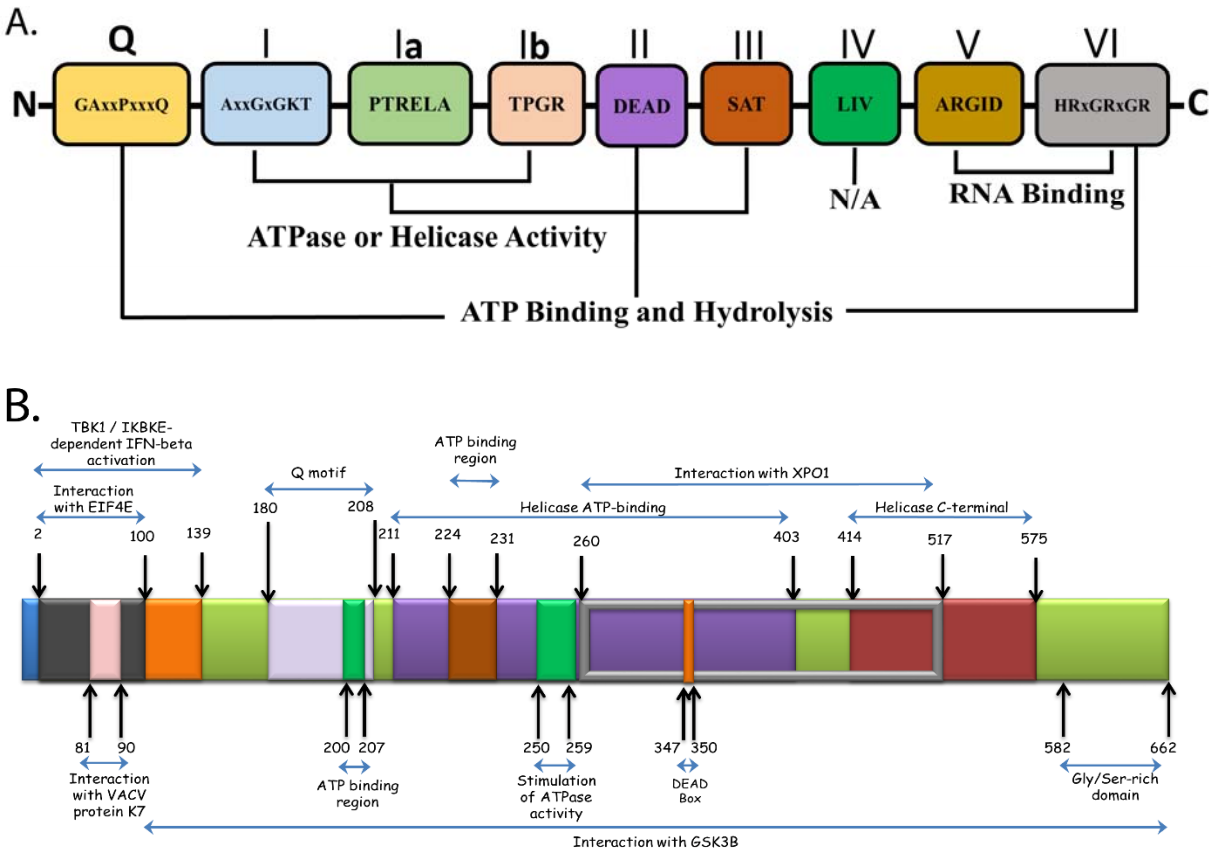

Figure 1.5. Dead-box proteins map.

A) Schematic illustration of conserved motifs of DEAD-box proteins, flanked by variable N- and C-termini. Boxes contain the amino acid sequence of each motif, x is representative of any amino acid. Suggested function of these motifs are shown in the image. Adapted from: *Rocak S & Linder P; Nat Rev Mol Cell Biol. 2004 Mar;5(3):232-41*. (140). B) Map of DDX3 protein, recognized motifs and their position on the protein are indicated (Uniprot.org O00571 (DDX3X_HUMAN)).

1.6.2. Cellular Localization of DDX3

The intracellular distribution of DDX3 has been reported to be predominantly in cytoplasmic granules by most studies (157, 158, 160-163). Furthermore, DDX3 has been demonstrated to constantly shuttle between the cytoplasm and nucleus through binding to the export shuttling protein CRM1 (Chromosome maintenance region 1). Treating the cells with the CRM1 inhibitor Leptomycin B (LMB) drives DDX3 accumulation in the nucleus (158, 163). This extensive nuclear export of DDX3 may be the very reason that most studies report DDX3 as a cytoplasmic protein. However, two studies have also showed partial nuclear localization of DDX3 (158, 164).

1.6.3. DDX3 in Splicing and RNA Transport

Based on sequence analyses, DDX3 bears a C-terminal (amino acids 582-632) arginine/serine rich RS-domain consisting of 7 serine-arginine or arginine-serine dipeptides, identical to that of other splicing factors (164). Nuclear-bound DDX3 has been found in spliceosomes in HeLa cells, in association with core proteins of exon junction complex (EJC) (165). This protein has also been spotted in messenger ribonucleoprotein particles (mRNP) along with spliced mRNAs (166). The minimal effect of DDX3 knockdown on splicing events (161), suggests that DDX3 rather partners with RNPs after splicing to exert functions such as RNA export and may not have an active role in splicing. In line with this hypothesis, DDX3 physically interacts with NXF1 as well as CRM1, proteins that are associated with mRNA export (158, 167). Although, the association of DDX3 to these export factors has been shown to be important for the export of some cellular and viral mRNAs (158, 168, 169), its depletion had no impact on the nuclear export of poly(A) + mRNA or a β-globin reporter gene (160, 161). This observation suggests that DDX3 is rather involved in the export of specific mRNAs and not the bulk mRNA (170). Furthermore, DDX3 has been characterized as a component of kinesin granules that help the transport of RNA in neurons. However, DDX3 knockdown showed very small effect on the RNA transport(171).

1.6.4. DDX3 and Apoptosis

Apoptosis can be either intrinsic (mitochondrial) or extrinsic (death receptors). However, there is a significant crosstalk between these two pathways, eventually resulting in the stimulation of effector molecules such as caspase 3, caspase 7, or PARP (159). DDX3 is among the TRAIL-R2 (death receptor) associated proteins, some treatments induce the dissociation of DDX3 from TRAIL-R2 signal transduction and this dissociation seems to modulates death signals (172). DDX3 also forms a death antagonizing signaling complex with Glycogen synthase kinase-3 (GSK3) and apoptosis protein-1 (cIAP-1) that are known to stall apoptosis (173, 174). Cleavage of DDX3 and cIAP-1, along with inactivation of GSK3, stimulates the apoptotic pathway (175). Finally, DDX3 has been linked to p53-dependent apoptosis, however there are some contradicting results regarding this interaction. The p53 protein responds differentially with respect to the type of tissue and stress agent (176) so this might be the reason for variable data available concerning the DDX3 and p53 (159).

1.6.5. DDX3 and Antiviral Immune Response

DDX3 is amongst the proteins that can sense viral RNA or DNA inside the cytoplasm and induce the expression of IFN-β (177). Two related kinases, IKK-ε and TBK1, act under the TLR-induced signaling pathway and induce the IFN-β gene expression through phosphorylation and activation of IRF-3 and-7 (178). DDX3 has been shown to act on this pathway at several levels: i) Upstream of IKKε/TBK1, i.e. DDX3, itself senses viral RNA or regulates the expression of a viral RNA sensor, interferon-induced protein kinase (PACT), resulting in the activation of mitochondrial antiviral-signaling protein (MAVS) which in turn causes IRF3/7 phosphorylation and IFN-β expression (167, 179). ii) DDX3 can partner with IKKε and TBK1 (71, 163, 180, 181). In this scenario, DDX3 binds to IKKε and causes its autophosphorylation, then IKKε phosphorylates DDX3 that successively causes the phosphorylation of IRF3 (180). iii) Downstream of IKKε/TBK1, i.e. DDX3 upon phosphorylation by TBK1, directly binds to the promoter enhancing region of IFN-β in an ATPase- and helicase-independent manner and activates the expression of IFN-β (181, 182).

1.6.6. DDX3 and Protein expression

Interaction of DDX3 with 80S ribosomes and eukaryotic initiation factors points at the likelihood of its participation in translation initiation (168, 181). Although the role of DDX3 in promoter regulation of proteins such as IFN-β, E-cadherin and p21 has been shown (170, 182, 183), its knockdown has no impact on general protein expression (167, 184, 185). These observations propose that RNA type and specific co-factors determine DDX3 functionality (166). Furthermore, DDX3 physically interacts (co-immunoprecipitation) with various components of cap-dependent translation initiation such as eIF4E, eIF3, PABP1 and Ezrin (185) and it has been suggested that DDX3 mediates the translation of specific mRNA's with complex 5'-UTR's through the destabilization of complex RNA structures (186).

1.6.7. DDX3 an Stress response

Stress granules (SGs) are cytoplasmic non-translating messenger ribonucleoproteins (mRNPs) that promote mRNA decay. These foci are observed upon a wide range of environmental factors such as oxidative stress, hyperosmolarity, UV irradiation, heat shock and viral infections (181). DDX3 has been recognized as one of the components of these aggregations and facilitates their assembly in an ATP-independent fashion (182, 183). In addition, DDX3 is able to rescue the effect of Gle1A knockdown which is a determinant part of stress granules (184).

1.6.8. DDX3 and Tumorigenesis

Depending on the cell type and the nature of manipulation of DDX3 in different studies, results regarding the contribution of DDX3 in tumorigenesis is somewhat contradictory and represent DDX3 as both oncogene and tumor suppressor (166). The present section will try to summarize the available data on the matter. Tumor hypoxia is one of the results of tumor progression (181). Hypoxia inducible factor-1 (HIF-1) is a transcription factor that help cells surviving hypoxia (182). DDX3 promoter contains several HIF-1 responsive elements (HRE).

Upon hypoxia HIF-1 binds to these sites and induces DDX3 expression (183). DDX3 also contribute to cell cycle progression likely through the regulation of cyclin D1 and cyclin E1 mRNA translation (166). However, some groups have reported that DDX3 actually arrests the cell cycle via the modulation of p53-DDX3-p21 (184-186). Furthermore, DDX3 has been identified as a regulator of Wnt/β-catenin transduction cascade (187) that is involved in the emergence of several complications such as colon cancer, melanoma and medulloblastoma (188).

1.6.9. DDX3 and Viral pathogens

1.6.9.1. RNA viruses

Two individual publications in 1999, showed the interaction of **hepatitis C virus (HCV)** core protein and DDX3 by yeast two-hybrid technology (164, 183). This provided the groundwork for future studies regarding the role of DDX3 in viral replication. They also noticed the aggregation of DDX3 in specific spots throughout the cytoplasm in the presence of core protein, pointing to the fact that virus likely targets a cytoplasmic function of DDX3 (183). These spots were later shown to be the so called stress granules (184). Unfortunately, the literature dealing with the role of DDX3 in HCV life cycle is somehow unclear, some proving DDX3 to be necessary for viral replication, others showing the protein to be dispensable for the replication of HCV. One study has shown that, association of core protein with DDX3, arrests the capped translation (cellular mRNA translation) but no the uncapped translation (HCV translation events), probably killing the competition for the resources in favor of the viral translation (162). In order to identify the extent of host-pathogen relationship, a group used a systematic RNA interference (RNAi) screen and targeted 62 cellular genes in cells infected with HCV. Interestingly, silencing DDX3 resulted in significant inhibition of viral release and replication (185). Later on, a study showed that the interaction of DDX3 and HCV core protein is essential for proper RNA replication when they observed that DDX3 knockdown ends in the impaired accumulation of HCV RNAs (186). In another study, it was shown that this DDX3-core interaction might be essential for the virus, since the core protein by binding DDX3, might prevent it from activating the interferon response (167). On the other hand, a mutation of the

HCV core protein that abrogates the DDX3-core interaction, indicated that the interaction of DDX3 and the viral core protein is not necessary for DDX3 function in viral RNA replication (187). However, use a different HCV genotype by another group, found the DDX3 and core protein interaction to be essential for viral replication (188).

Upon the entry of **human immunodeficiency virus type-1 (HIV-1)** into cells, viral RNA is converted to DNA in the cytoplasm, which is then translocated to the host's nucleus to be integrated in the cell genome. At this stage, viral mRNAs are transcribed using cellular factors. These mRNAs can be categorized in three distinct groups, including the ones that are multiply spliced, unspliced and partially spliced mRNAs. These mRNAs must reach the cytoplasm to be further processed. The intron-containing species (unspliced and partially spliced) require the viral protein Rev for their transport to the cytoplasm. HIV-1 Rev can shuttle between the nucleus and cytoplasm (189) and it interacts with cellular nuclear export protein CRM1 (chromosome region maintenance 1) to exert its function (190). DDX3 knockdown from cells aborts Rev/CRM1 dependent RNA export, thus defining it as a determinant factor in HIV-1 life cycle. Interestingly, DDX3 regulates HIV-1 RNA export in a helicase/ATPase-dependent manner (158).

Japanese encephalitis virus (JEV) is an ssRNA mosquito-borne flavivirus. This virus encodes 3 structural proteins and 7 non-structural (NS) proteins. Among the NS proteins are viral serine protease (NS2B-NS3) and methyltransferase/RNA polymerase (NS5) that is crucial for viral genome replication (191). DDX3 knockdown significantly impairs JEV replication by interacting with JEV NS3 and NS5 to regulate viral protein expression in a helicase-dependent manner (192). **Dengue virus (DENV)** and **west Nile virus (WNV)** are two other flaviviruses whose replication might be regulated by DDX3 in the same manner as JEV (193, 194).

Norovirus belongs to the Caliciviridae family and is a small plus-strand RNA virus (195). DDX3 has been found in association with the norovirus genomic RNA through riboproteomics (196). DDX3 appears in aggregated spots as mentioned in the case of some other viruses. Moreover, DDX3 knockdown results in drastic reduction of viral NS7 polymerase and genomic material (196).

Influenza A as a member of Orthomyxoviridae family is a segmented ssRNA virus (197). It has been speculated that the viral proteins NS1 and nucleoprotein (NP) are involved in the inhibition of SG formation (198, 199). As DDX3 physically interacts with both proteins, this interaction might explain the anti-SG behavior of these proteins (200).

1.6.9.2. DNA viruses

Hepatitis B virus (HBV), as a member of hepadnavirus family, contains a relaxed circular partially double stranded DNA (rcDNA). During the viral replication, HBV polymerase (pol) repairs the rcDNA into covalently closed circular DNA (cccDNA) within the host nucleus. The virus then utilizes this cccDNA as a template for its gene transcription by the host RNA polymerase II. Among the generated viral RNAs, pregenomic RNA (pgRNA) is packaged into newly formed capsids along with the HBV pol. The viral polymerase then reverse transcribes the pgRNA back to rcDNA (201). It has been shown that DDX3 is incorporated to HBV nucleocapsids and interacts with the viral pol to hamper reverse transcription in an ATPase-dependent fashion. Moreover, DDX3 overexpression was able to reduce viral yields in a dose-dependent manner (202). In some HBV-induced HCC (hepatocellular carcinoma) cases, DDX3 is down-regulated likely as a viral strategy to counteract DDX3 effects and promote its propagation (203). Interestingly, the HBV pol breaks the interaction between and DDX3 and IKKϵ to inhibit IFNβ induction (204, 205). This type of interaction makes it complicated to target DDX3, since pol-DDX3 blockage can result in both positive (release of HBV pol) and negative (IFN stimulation) effects on virus (206).

Vaccinia virus (VACV) is a dsDNA virus belonging to the family of poxvirus. VACV express numerous proteins such as K7 that can antagonize the host immune system (207). The K7 protein can form a complex with DDX3 by binding to its N-terminal region, more specifically amino acids 61 and 90, to evade innate immune responses (163, 208).

Human cytomegalovirus (HCMV), as mentioned earlier is a member of Herpesviridae family. A group conducted an unbiased proteomics approach to identify host proteins that might be involved in post-transcriptional regulation of HCMV mRNAs and they noticed that HCMV infection alters the level and activity of DDX3 (209). Although, this finding suggests a role for

DDX3 in HCMV RNA metabolism, further investigation is needed to elucidate this host-pathogen interaction.

Overall, it appears that DDX3 contributes in the life cycle of both RNA and DNA viruses. Although, the interplay between and viruses is not fully understood and sometimes even varies for a single virus from one study to another, there are number of findings that seem to follow one of two patterns. One is the necessity of DDX3 for the viral replication through its interaction with key viral factors. These interactions, depending on the virus, can impact viral RNA metabolism at the level of transcription, translation or RNA export. Another one, is the effort of several viruses in antagonizing DDX3 activity in the stimulation of interferon response.

Chapter 2. Results

2.1. Aims of project

As mentioned earlier, our findings indicated that 15 of the virus-incorporated host proteins are required for HSV-1 efficient replication. Among them, DDX3X particularly interested us, since it has been involved in the replication of many other viral pathogens and proved to be our strongest hit. Therefore, we sought to investigate the relationship between HSV-1 and this host protein and elucidate in more details on the role of DDX3X in HSV-1 life cycle by studying: **i)** The cellular distribution of DDX3X in the context of infection, to see whether virus drives any changes in the localization of this protein and if this localization is associated to a specific cellular compartment at different time points during the infection. **ii)** The effect of HSV-1 infection on the expression of DDX3X in different cell types. **iii)** The impact of DDX3X overexpression on viral yields using titration assay. **iv)** How does the overexpression or depletion of this protein affects viral gene expression at both RNA and protein levels. **v)** Whether DDX3X as a protein that modulates IFN-β response, controls the virus through the stimulation of IFN expression.

2.2. Comments on the text

Results section contains the full text of the article "The ATP-dependent RNA Helicase DDX3X modulates Herpes Simplex Virus Type 1 Gene Expression" by Bita Khadivjam that was submitted to *PLoS Pathogens* on 15 July, 2016. However, minor changes have been made to the manuscript to fulfil the requirements of this memoir.

2.3. Author's contributions

Bita Khadivjam: Contributed to the writing of the article as well as the design, analysis and mounting of most experiments (Figures 2.2-2.6, 2.10 and 2.14 - preparation of samples for RT-qPCR in Figures 2.11-2.13)

Camile Stegen: Designed and analysed some experiments (Figures 2.1 and 2.8-2.9)

Marc-Aurèle Hogue Racine: Designed and analysed the HSV-1 entry assay (Figure 2.7)

Nabil El Bilali: Designed and analysed the RT-qPCR assays (Figures 2.11-2.13)

Katinka Döhner and Beate Sodeik: Generated the HSV-1(17+)Lox-Luc viral strain and criticized the manuscript

Roger Lippé: Created the project and supervised, designed and analyzed experiments and wrote the article

2.4. Article

The ATP-dependent RNA Helicase DDX3X modulates Herpes Simplex Virus Type 1 Gene Expression

By

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Running Title: Cellular DDX3X regulates HSV-1 transcription

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Abstract

The human protein DDX3X is a DEAD-box ATP-dependent RNA helicase that regulates transcription, mRNA maturation, mRNA export and translation. DDX3X concomitantly modulates the replication of several RNA viruses and promotes innate immunity. We previously showed that herpes simplex virus type 1 (HSV-1), a human DNA virus, incorporates DDX3X into its mature particles and that DDX3X is required for optimal HSV-1 infectivity. The present work focuses on this host-pathogen relationship. The data reveal a dependence between viral propagation and optimal DDX3X protein levels while the virus caused DDX3X aggregation. Surprisingly, DDX3X from incoming viral particles was not required for the early stages of HSV-1 entry but rather the protein controlled the assembly of novel viral particles. This appeared largely independent of the previously reported capacity of DDX3X to stimulate interferon type I production. Instead, DDX3X acted by disturbing viral gene transcription and thus subsequent genome duplication. This suggests DDX3X can impact DNA viruses such as HSV-1 by an interferon-independent pathway.

Author Summary

It is well established that viruses can incorporate in their mature particles proteins from their hosts. For instance, we previously reported the identification of up to 49 cellular proteins in Herpes simplex virus type I extracellular virions, many of which affect viral propagation. This includes DDX3X, a protein modulating RNA metabolism, RNA-based viruses and innate immunity but whose role in the spread of the virus was unclear. The present data explore this host-pathogen relationship and show a dependence of the virus on the right levels of DDX3X. An in-depth analysis revealed that DDX3X acted on viral gene transcription and that this was independent of its ability to stimulate the interferon pathway. These findings hint at a novel mechanism whereby DDX3X interacts with the genome of a DNA-based virus. This thus extends the already long list of activities attributed to this cellular protein and the range of viruses it can act upon.

Introduction

The human DDX3 protein is a member of a large family of DEAD-box ATP-dependent RNA helicases. In humans, it is encoded by the X (DDX3X) and Y (DDX3Y) chromosomes, albeit the latter is restricted to testis [1]. It participates in different stages of cellular gene expression, such as transcription, mRNA maturation, mRNA export and translation [2]. Given these crucial roles in RNA biology, several RNA viruses interact with DDX3X, often with important consequences for viral replication. This includes hepatitis C virus (HCV), novorirus, West Nile virus and Japanese encephalitis virus [3-7]. This is also the case for HIV and hepatitis B virus (HBV), a peculiar DNA virus that relies on an RNA template and reversed transcription to replicate its genome [8, 9]. In parallel, DDX3X also contributes to innate immunity against these viruses. DDX3X thus stimulates interferon type I production through binding IKK□ (I-kappa-B kinase epsilon) and TBK1 (tank-binding kinase 1), leading to IRF3 phosphorylation and activation [10, 11]. These findings position DDX3X as a critical player for the replication and immunity against RNA-based viruses. However, its role is not restricted to these viruses since it also impacts innate immunity against DNA viruses. For instance, the HBV viral polymerase competitively binds IKKε and blocks its ability to interact with DDX3X, thus faltering interferon production to the benefit of the virus [12]. Similarly, the vaccinia virus (VACV) K7 protein binds and sequesters DDX3X and prevents its interaction with the above kinases, once again blocking interferon activation to favor viral propagation [13]. DDX3X is thus an important mediator of host-pathogen interactions that acts via multiple and likely parallel routes on many RNA and at least two distinct DNA viruses. We thus hypothesized that DDX3X partly modulate viral expansion by interferon-independent routes.

Herpes simplex virus type 1 (HSV-1) is a ubiquitous human pathogen that is dormant in 80-90% of the population but causes clinical symptoms in roughly a third of humans. It is primarily associated with cold sores but is also responsible for severe infections in both immunocompetent and immunodeficient individuals [14]. Replication of herpesviruses occurs in the cell nucleus, where they efficiently take over the host gene expression machinery during an active infection. Three classes of HSV-1 genes, namely the immediate early (IE), early (E), and late (L) genes are transcribed in a sequential manner by the cellular RNA polymerase II and a collection of transcriptional and translational cellular factors [15]. This gene expression

cascade is stimulated by ICP0, ICP4 and VP16, three tegument components present in mature viral particles that promptly act as transactivators following viral entry in the cell [16-20]. Thus, as for other viruses, HSV-1 gene expression is clearly dependent on both viral and host proteins.

Past studies from our laboratory revealed that HSV-1 incorporates DDX3X into its mature particles [16]. Most interestingly, depleting either the viral or the cellular pool of DDX3X significantly impairs HSV-1 infectivity [21]. However, as the exact function of DDX3X for the HSV-1 replication cycle is not clear, we opted to examine more closely the nature of this host-pathogen interaction. The present data reaffirm with multiple assays the implication of DDX3X on the propagation of HSV-1. They also reveal that the portion of DDX3X present in the incoming virions is not required for the early stages of HSV-1 entry, pointing to a downstream effect, albeit this function could still technically be undertaken by the cellular pool of DDX3X. However, depletion of DDX3X from the cell impacted viral particle assembly and intracellular and extracellular viral yields. Interestingly, either reducing or overexpressing the cellular pool of DDX3X both negatively impacted HSV-1 protein expression. This dependency of the virus on DDX3X protein levels was corroborated in rescue experiments with siDDX3X depleted cells that concomitantly expressed a siRNA-resistant DDX3X construct. At the mechanistic level, DDX3X was found to modulate the transcription of immediate-early, early and late genes, including the aforementioned viral transactivating proteins ICP0, ICP4 and VP16. This appeared independent of the ability of DDX3X to promote interferon β production. We conclude that DDX3X modulates HSV-1 yields by a novel mechanism implicating viral gene transcription independently of the interferon pathway.

Results

DDX3X is required for optimal viral yields

We previously reported by proteomics that HSV-1 incorporates the host protein DDX3X in mature virions [16] and that both the cellular and virion-incorporated pools of DDX3X influence viral yields [21]. To independently validate the role of the cellular DDX3X in viral replication, we resorted to the BHK21-derived tsET24 cell line, which harbors a temperature-sensitive DDX3X inactive at 39,5ºC but functional at 34ºC [22]. Infection of the tsET24 and parental BHK21 cell lines with wild type HSV-1 showed that viral output was strongly reduced at the non-permissive temperature in the tsET24 cell line but not affected in the parental cell line (fig. 1). This ruled out nonspecific off-targets that are possible with siRNA reagents and confirmed that DDX3X is indeed a modulator of the HSV-1 life cycle.

HSV-1 yields are sensitive to DDX3X levels

Given that viral yields were lowered upon depletion of the host protein DDX3X, we probed whether overexpression of the protein would have the opposite effect on HSV-1. An initial examination by immunofluorescence microscopy indicated that overexpression did not influence the subcellular localization of DDX3X and that the level of exogenous DDX3X was in general similar to its endogenous counterpart (fig. S1). Thus cells were first transfected with wild type DDX3X then infected and extracellular virus production measured by plaque assay. Unexpectedly, an excess of DDX3X also perturbed the release of mature virions (fig. 2A). This suggested that optimal viral replication required a carefully controlled level of DDX3X. To examine this issue at the single-cell level, we monitored viral propagation by fluorescence microscopy (fig. 2B). Interestingly, all cells overexpressing DDX3X appeared uninfected, while viral gene expression and cytopathic effects were readily detectable in all untransfected cells. Thus HSV-1 propagation seemed to depend on a critical level of DDX3X in the cells.

To better define the relationship between DDX3X and the infection, we opted to rescue DDX3X in siRNA depleted cells. To this end, we first determined which of the 4 siRNA targeting DDX3X worked best (fig. S2) and generated a DDX3X mutant resistant to this specific

siRNA. We then treated cells with this unique siRNA to reduce endogenous DDX3X levels and rescued them with the above siRNA resistant DDX3X construct. Cells were subsequently infected with HSV-1 K26GFP and the presence of DDX3X and virus monitored by fluorescence microscopy and quantified. Note that the exogenous DDX3X was expressed at similar levels as its endogenous counterpart and thus doubled the total DDX3X in the absence of siRNA (compare GS-DDX3X and no transfection in Fig 3A and B). The results also revealed that, as expected, silencing DDX3X significantly reduced both DDX3X and GFP expression (fig. 3A, B). In agreement with our previous findings, overexpression of DDX3X nearly abolished HSV-1 production. Rescuing the depleted cells with exogenous DDX3X restored the infection up to 63% compared to the untransfected cells (fig. 3A, B). Thus the virus appeared sensitive to the levels of DDX3X.

The virus functionally interacts with endogenous DDX3X

To understand the role of DDX3X in the viral life cycle, we next examined whether the virus influenced DDX3X levels. To this end, we probed DDX3X expression by Western blotting in three cell lines commonly used to study HSV-1, namely HeLa, 143B and Vero cells. HSV-1 infection had no impact on DDX3X protein levels in HeLa and Vero cells, but reduced them by half in 143B cells (Fig. 4). We also examined whether the virus altered the subcellular localization of DDX3X. As shown in figure 5 (left panels), endogenous DDX3X was primarily found in cytoplasmic granules in uninfected cells. Attempts to identify these granules with a panoply of markers unfortunately failed to unambiguously identify them. In agreement with previous studies, some DDX3X could also be detected in the nucleus (see insets), consistent with its shuffling across the nuclear envelopes [23, 24]. Upon infection, DDX3X levels were once again unaffected in HeLa and Vero cells but reduced in 143B cells as reported above. However, DDX3X was somewhat aggregated in all three cell lines (fig. 5A, compare left and right panels). Furthermore, the GFP tagged viral particles often but not always co-localized with DDX3X in the cytoplasm (fig. 5B), in agreement with the incorporation of this cellular protein in mature virions [21]. A time course revealed that DDX3X started to aggregate between 3 and 6 hours post-infection and this intensified with time (fig. 6). As noted above, DDX3X

occasionally colocalized with the viral marker used to identify infected cells. Altogether, we conclude that the viral particles functionally interacted with DDX3X during the infection.

DDX3X down regulation alters novel viral particle assembly

A number of scenarios may justify the presence of a host protein in mature virions. One is that it might be needed immediately after cell entry to initiate an infection. A second one would be boosting viral replication post-entry. A third one is that DDX3X virion incorporation may be a bystander effect of a previously occurring interaction taking place during viral particle assembly or transport towards the cell periphery. Note that these options are not mutually exclusive. To address the first of these scenarios, we directly probed whether the virion-associated pool of DDX3X is required for viral entry. To this end, we used a viral strain that encodes a luciferase under a constitutive CMV immediate early promoter [25]. We thus infected cells with the above virus, wild type virus (i.e. without the luciferase cassette) or no virus at all (mock infection). To specifically probe the role of DDX3X during entry, we additionally infected the cells with the luciferase coding virus depleted for DDX3X, or as a control, depleted for VP16 [21]. To synchronize the infection, viral adsorption was performed at 4°C for an hour and the cells subsequently transferred to 37°C for an additional hour. Some control cells were alternatively maintained at 4°C throughout the experiment to prevent viral entry. Entry of the virus into the cells was then measured with a commercial luciferase kit (see materials and methods). Not surprisingly, viruses devoid of the luciferase gene, mock-infected cells or the luciferase-coding virus incubated at 4°C all gave background signals (fig. 7). In contrast, incubation of the untreated luciferase-positive virus at 37°C gave a strong signal, which was arbitrarily set to 100%. Infection by VP16 or DDX3X depleted luciferase-positive virions gave very similar strong signals, indicating their presence in the mature virus was not essential for this early phase of the infection. This also suggested that the CMV immediate early promoter driving the luciferase is insensitive to VP16. Altogether, this suggested DDX3X acted downstream of viral entry.

DDX3X modulates viral gene expression

Given the reduced production of viral particles upon DDX3X depletion and that DDX3X is a known modulator of host gene transcription and translation, we wondered if DDX3X could also regulate viral gene expression. To address this, we probed representative candidates of the three viral kinetics classes, namely ICP0 and ICP4 (immediate early proteins), ICP8 and pUL23 (early proteins) and VP16 and pUL31 (late proteins) under normal, reduced (siDDX3X) or enhanced (DDX3X overexpression) conditions. A 9 hour midpoint was chosen since it is an intermediate time when both early and late viral proteins are detectable. Both DDX3 conditions negatively affected the expression of all classes of viral proteins, including once again VP16 (fig. 10A). Quantification of several independent experiments, which were normalized against a γ-tubulin loading control, confirmed these findings (fig. 10B). To evaluate if the reduced protein levels were the consequence of reduced transcription, the samples were further analyzed by quantitative RT-PCR. The data showed that most of the viral gene transcripts were negatively impacted by both reduced and overabundant DDX3X (fig. 11), albeit slightly less than the corresponding protein levels (Table 1). Although, one might be concerned that WB is not as quantitative as RT-PCR technique. Our attempt to measure the linearity of WB results obtained by ChemiDoc system (Boi-Rad) indicated that these results are perfectly linear (R^2=0.99) for tested proteins, DDX3X and γ-Tubulin (fig S4). However, knowing this, we still cannot rule out the fact that RT-qPCR is more quantitative and Table 2.1 remains a modest comparison.

DDX3X's effect on the virus is independent of interferon β production

Given the role of DDX3X in innate immunity (see introduction), we probed by qRT-PCR the impact of DDX3X modulation on interferon type 1 production using interferon β as a gauge. As reported elsewhere [10], depleting endogenous DDX3X had no effect on the already low level of interferon β mRNA in uninfected cells, while DDX3X overexpression strongly stimulated its expression (fig. 12). Meanwhile, interferon β production was slightly increased by the virus compared to mock treated cells but this was not statistically meaningful, perhaps consistent with the ability of the virus to counteract this innate response. Similarly, depleting the endogenous DDX3X or overproducing in infected cells it did not have any major impact on

interferon β levels (fig. 12). Thus, as previously documented by others, DDX3X positively promoted interferon β levels in uninfected cells but had limited effect in HSV-1 infected cells. Thus the well-known DDX3X ability to modulate the interferon type 1 pathway did not appear to play a critical role, since both DDX3X knockdown or overexpression reduced viral gene transcription. This suggested that DDX3X may act on viral propagation by an interferon independent pathway.

To independently evaluate whether DDX3X significantly impacted HSV-1 propagation via innate immunity, we opted to measure viral genome copies by qPCR under normal, depleted or overexpressed DDX3X conditions. The data illustrated that viral genome duplication was strongly inhibited in both cases (fig. 13), in agreement with the scenario where DDX3X acted on the virus independently of its ability to modulate interferon β production. To finally confirm this hypothesis, we resorted to a well-characterized DDX3X point mutant (K230A) that abolishes its ATPase - and consequently helicase - activities without perturbing its ability to modulate the interferon pathway [8, 10, 13]. Our rationale was that if DDX3X primarily acted via the interferon type I pathway, the mutant should behave like wild type DDX3X and block viral propagation but if the ATPase activity is the main driver, the mutant would be dead and allow the virus to replicate normally. As seen in fig. 14, the data confirmed the impact of wild type DDX3X overexpression on viral release, while the K230A mutant was completely inactive despite being normally expressed (fig. S3). This indicated that DDX3X acted on the virus via its ATPase/helicase activities independently of the interferon pathway.

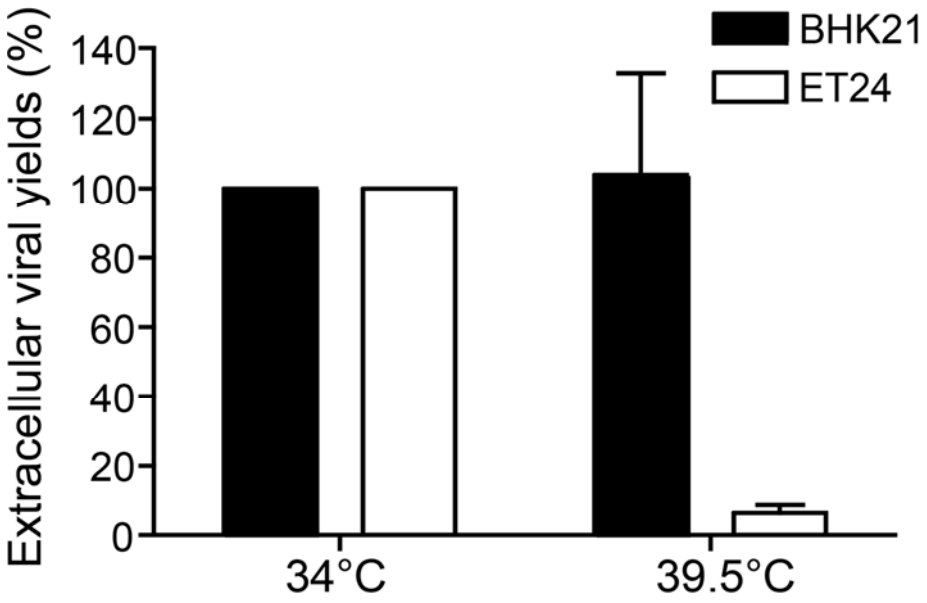

Figure 2.1. DDX3X is necessary for HSV-1 replication.

The DDX3X thermosensitive tsET24 and parental BHK21 cell lines were incubated for 24 hours prior to infection at the permissive (34ºC) or non-permissive (39.5ºC) temperature and infected with HSV-1 K26GFP (MOI of 5) for an additional 24 hours at that same temperature. Afterwards, supernatants were collected and titrated on Vero cells. Titers were normalized to the mean value obtained with samples infected at 34°C (arbitrarily set to 100%). They represent the averages of 2 independent experiments, each titrated in duplicates. The error bars represent the standard deviations to the means.

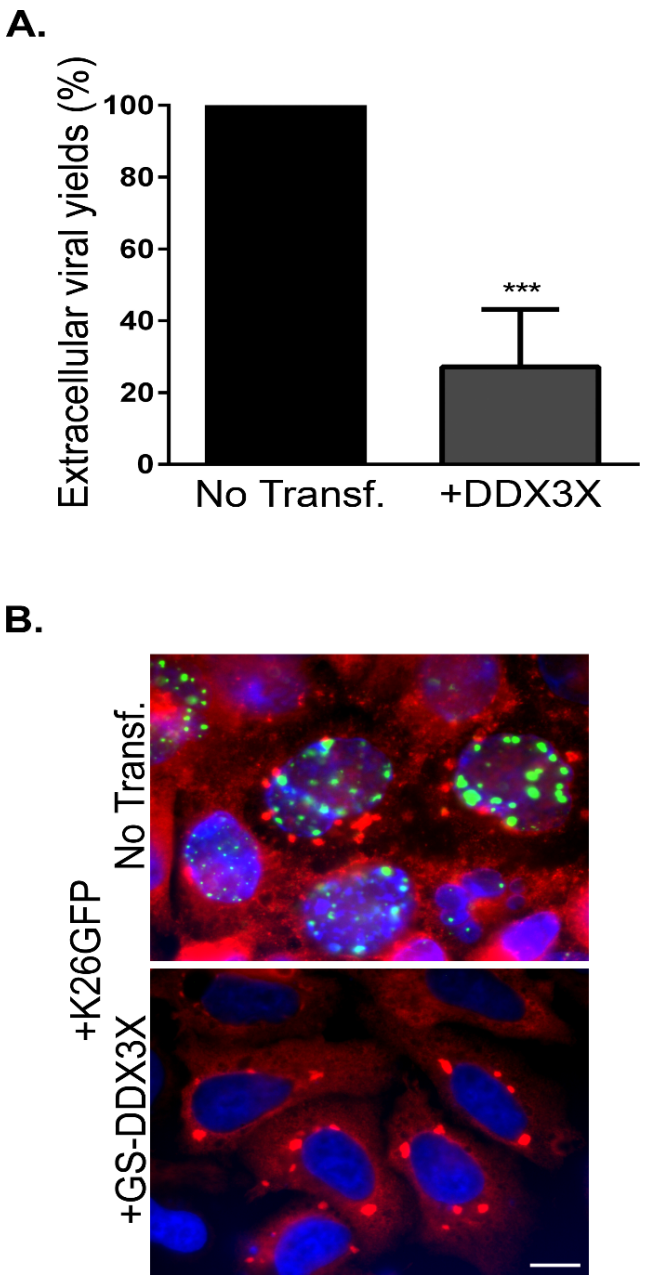

Figure 2.2. DDX3X overexpression hampers viral infection.

A) Effect of DDX3X overexpression on HSV-1 infectivity. Hela cells grown in 6-well plates were either mock treated or transfected with exogenous DDX3X for 24 hours and then infected with wild-type HSV-1 at a MOI of 5 for 18 hours. Supernatants, containing extracellular viruses, were then collected and titrated on Vero cells. Data represents the pool of 5 individual experiments. Bilateral Student's T-tests (with standard deviations

shown) were performed to detect significant hits compared to the transfection agent only control. (***: $p<0.001$). **B) Cells overexpressing DDX3X are refractory to the infection.** HeLa cells grown on coverslips were either mock treated or transfected with exogenous DDX3X as above and infected with HSV-1 K26GP for another 18 hours prior to fixation and examination by fluorescence microscopy. The red signal depicts both endogenous and exogenous levels of DDX3X, while the green signal represents the virus. The data shown is representative of at least 3 individual experiments. Scale bar: 10 μm.

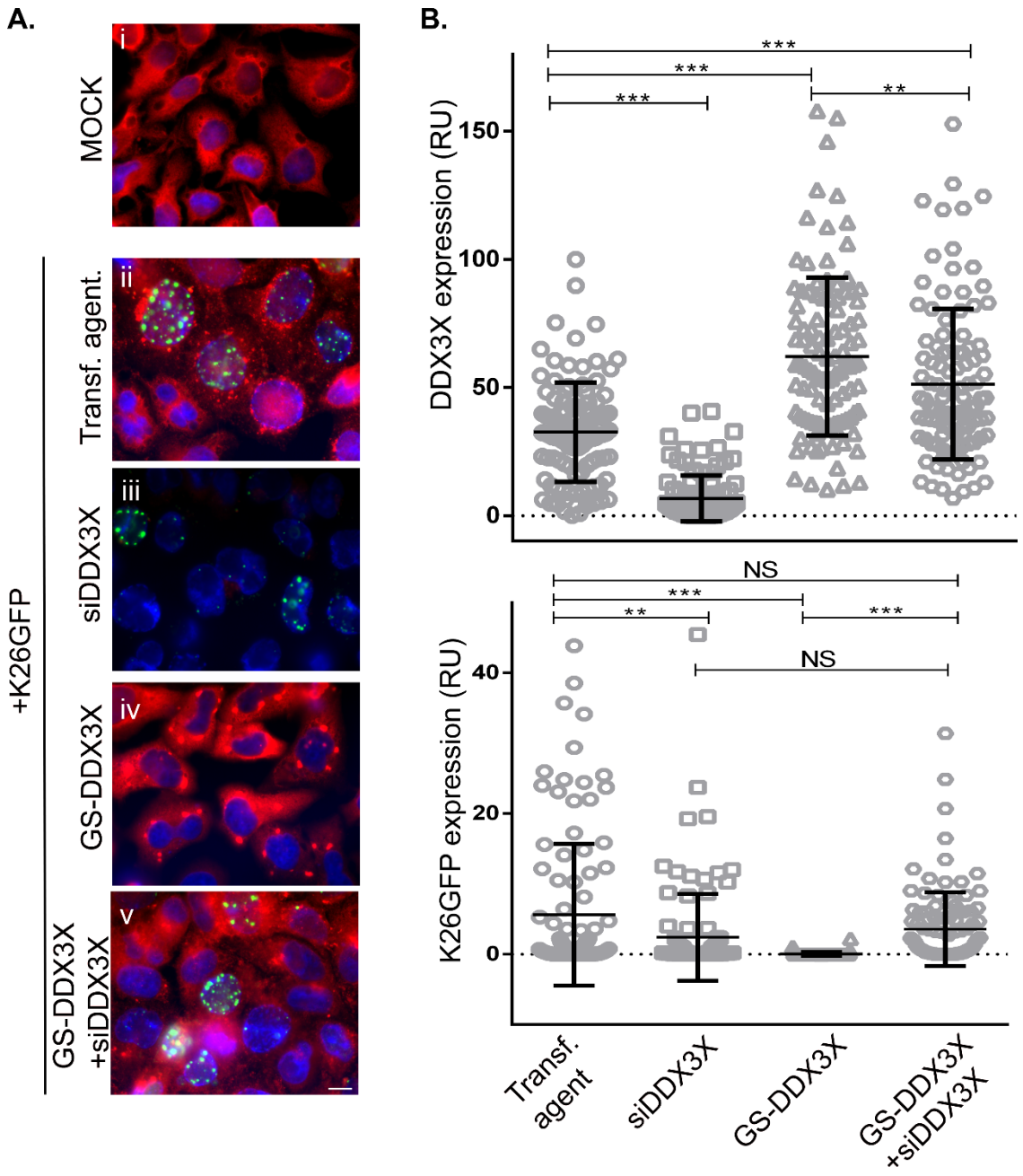

Figure 2.3. Normal levels of DDX3X best support HSV-1 propagation.

A) Hela cells grown on coverslips were sequentially mock transfected or treated with siDDX3Xa in the presence or absence of a plasmid coding for the siRNA resistant DDX3X mutant for 48 hours. All but the mock infected samples were subsequently infected at a MOI of 5 with HSV-1 K26GP (green signal) for 18 hours. The cells were finally fixed and reacted with primary antibodies against DDX3X and appropriate secondary antibody, which detects both

endogenous and exogenous DDX3X (red signal), while nuclei were labeled with Hoechst (blue signal). Samples were analyzed by fluorescence microscopy. **B)** Fluorescence intensities (Relative Units) were quantified for 100 cells using Image J. Scale bar: 10 µm. The data is representative of 3 independent experiments. Bars indicate the means and error bars the standard deviation to the mean (bilateral Student T-tests).

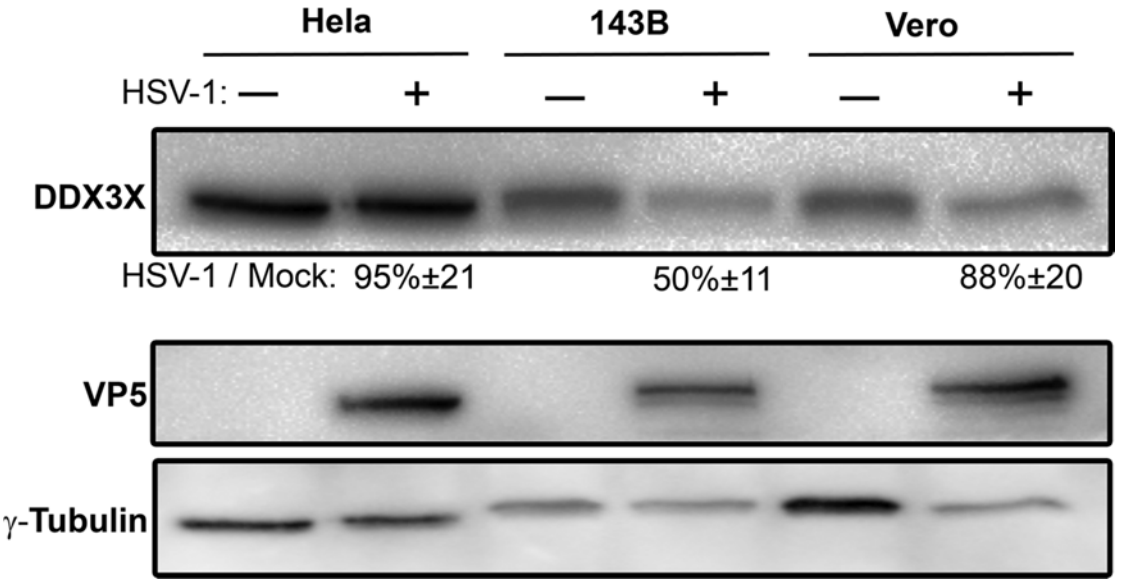

Fig 2.4. Impact of HSV-1 on endogenous DDX3X cellular levels.

Hela, Vero and 143B cells were seeded in 10 cm plates 24 hours prior to infection. Cells were then either mock treated or infected with wild-type HSV-1 at a MOI of 5 for 18 hours. Total cell lysates were then collected and DDX3X cellular levels probed by Western blotting. γ-Tubulin was used as a loading control. Numbers below the blots indicate the average levels of DDX3X, normalized for γ-tubulin, from 5 independent experiments.

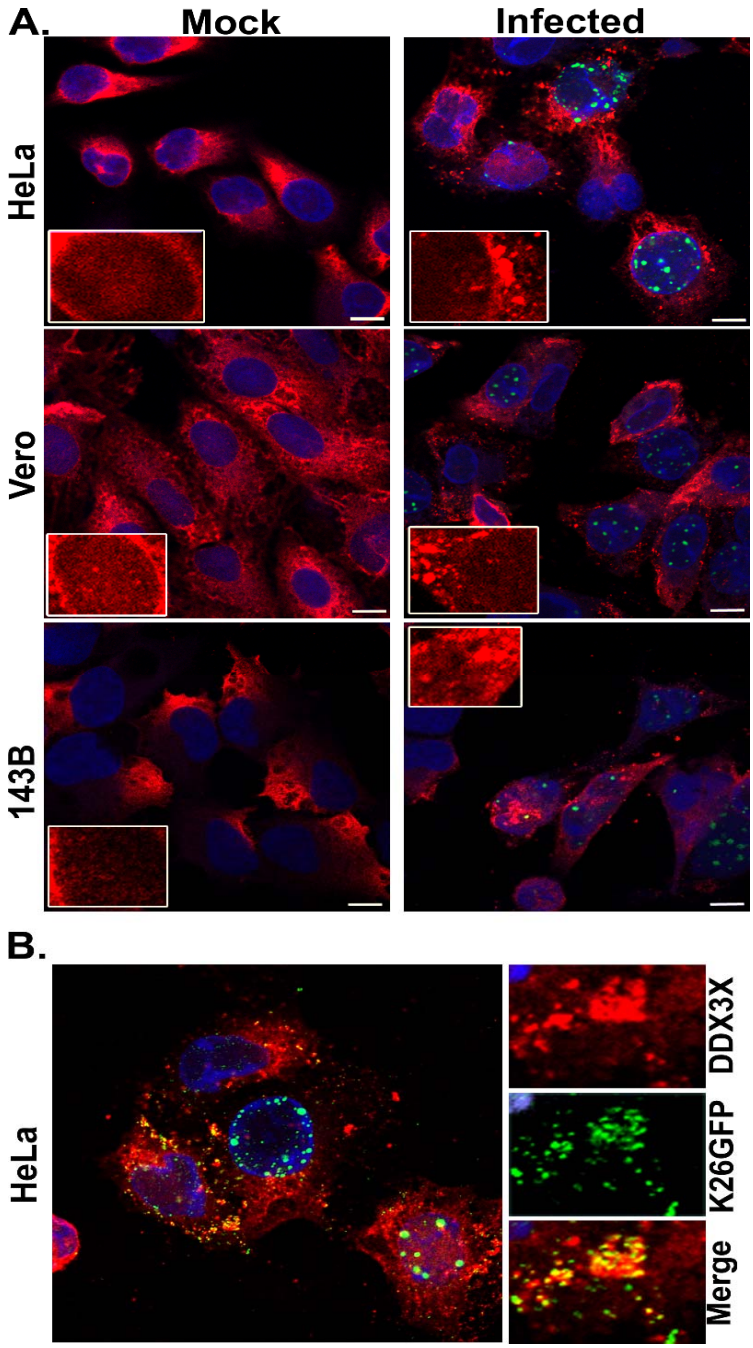

Figure 2.5. Endogenous DDX3X subcellular localization.

A) Hela, Vero and 143B cells were seeded on coverslips 24 hours prior to infection. Cells were then either mock infected or inoculated with HSV-1 K26GFP (green) at a MOI of 5 for 18 hours. Cells were then fixed, permeabilized, and immunostained with an antibody specific for DDX3X

(red) and the nuclei stained with Hoechst (blue). Cells were finally analyzed by confocal laser scanning microscopy. **B)** Close up view in the cytoplasm of infected HeLa cells. Scale bar: 10 μm. Results are representative of 3 independent experiments.

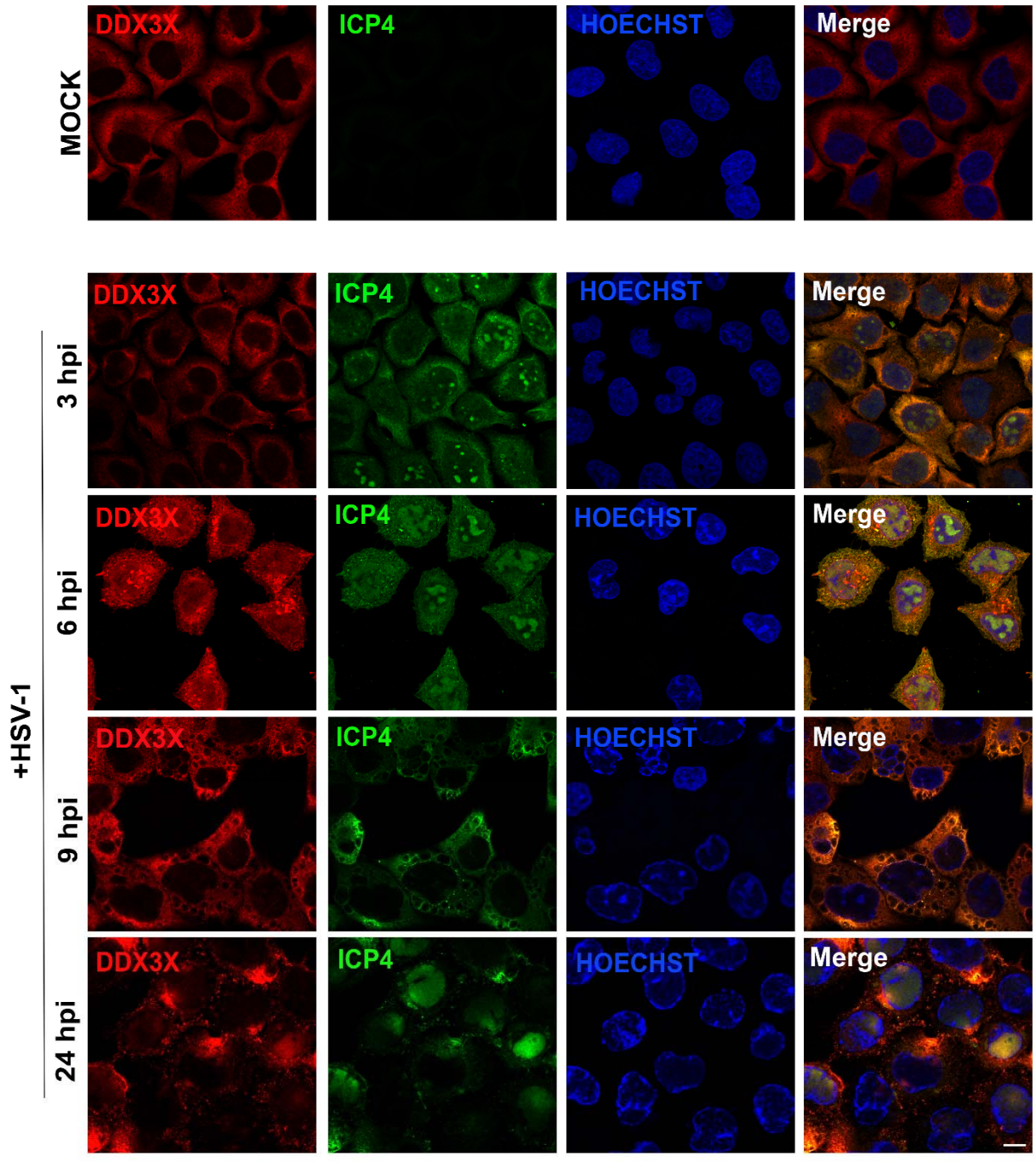

Figure 2.6. Time course of DDX3X aggregation.

Hela cells grown on coverslips were infected with wild-type HSV-1 for 3, 6, 9 and 24 hours. Cells were fixed at the indicated time points and reacted with antibodies against endogenous DDX3X (red) or ICP4 (to delineate infected cells; green), while nuclei were labeled with

Hoechst (blue). Samples were analyzed by confocal laser scanning microscopy. Scale bar: 20 μm. These results are representative of 2 individual experiments.

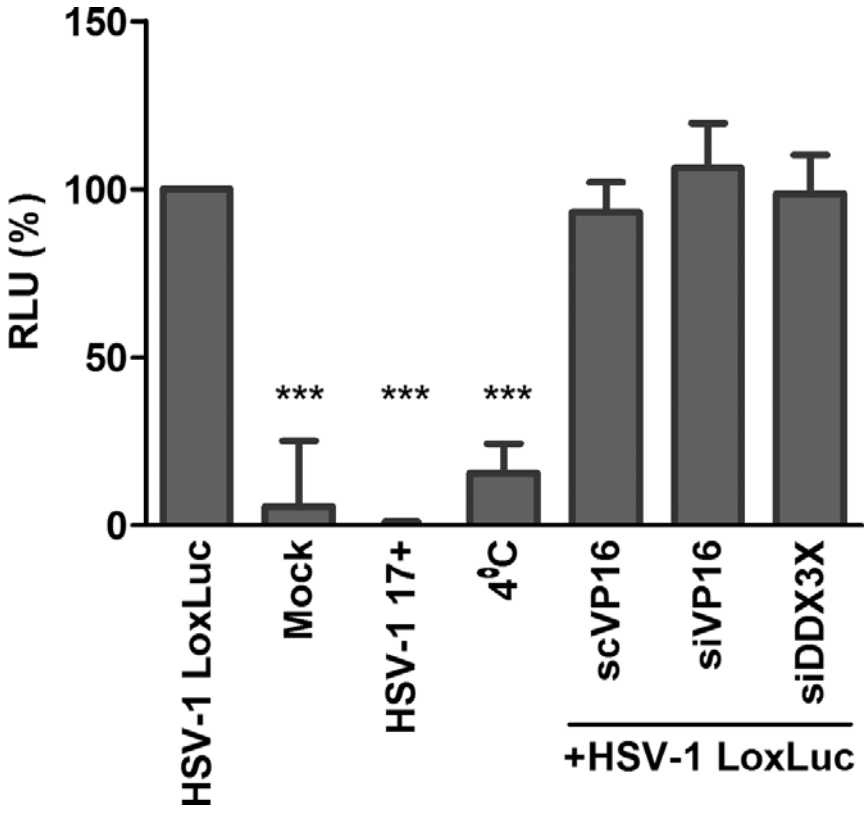

Figure 2.7. Virion-associated DDX3X has no effect on HSV-1 Entry.

143B cells pre-seeded in 24-well plates were mock-infected or infected at a MOI of 30 for 1 hour at 4°C with various HSV-1 viruses as indicated below each bar. These included wild type HSV-1 (luciferase-negative) and untreated, DDX3X or VP16 depleted HSV-1(17^+)Lox-Luc viruses. To enable viral entry, the cells were then incubated at 37°C for another hour and subsequently lysed at room temperature for 30 minutes in the presence of luciferin and energy. As a control, one sample was left at 4°C throughout the experiment to prevent viral entry. Samples were then transferred to 96-well plates and analyzed with a luminometer. Values represent the mean RLU (relative light units) from 5 independent experiments and error bars

depict the standard deviation to the means. Stars indicate the results of bilateral Student's T-tests (***: p<0.001).

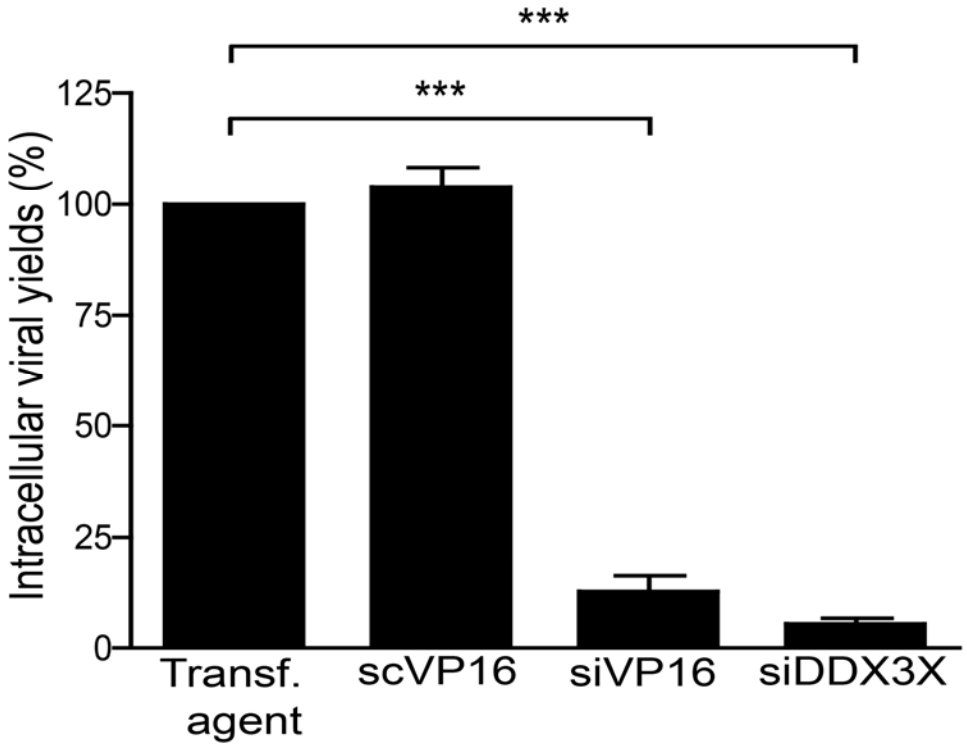

Figure 2.8. Impact of DDX3X knockdown on intracellular virions.

143B cells were transfected with siRNA pools targeting DDX3X or VP16 and infected with K26GFP at a MOI of 5. Cells were collected and lysed and viruses were titrated on Vero cells. The error bars show the standard deviations of the means of two independent experiments. Bilateral Student's T tests were performed to detect significant hits compared to the transfection agent only control (***: p<0.001).

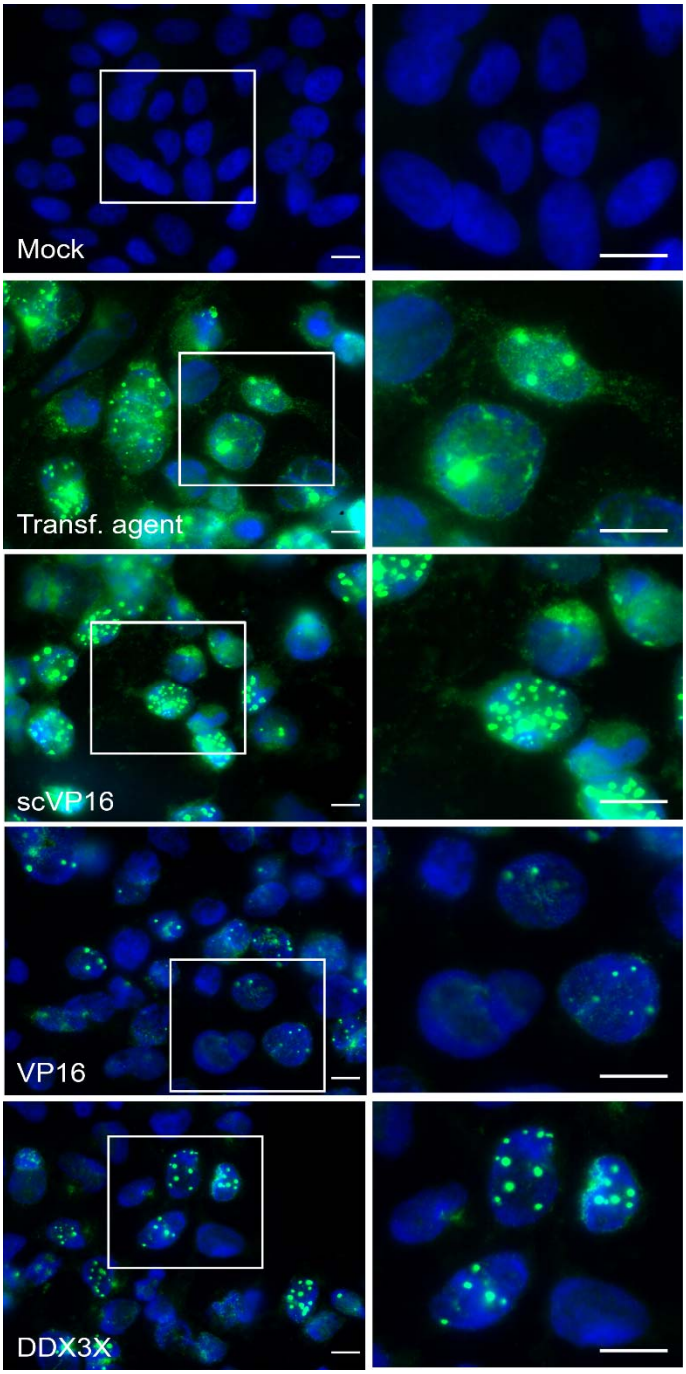

Figure 2.9. Inhibition of DDX3X reduced viral particle assembly.

143B cells were mock infected, mock transfected, treated with non-Targeting siRNA or with pooled siRNAs against DDX3X or VP16 as indicated. All but the mock infected samples were incubated with HSV-1 K26GFP (green signal) at a MOI of 5 for 18 hours. The cells were fixed

and the nuclei stained with Hoechst 33342 (blue). Right panels are enlargements of the indicated sections present in the left panels. Scale bars: 10 μm.

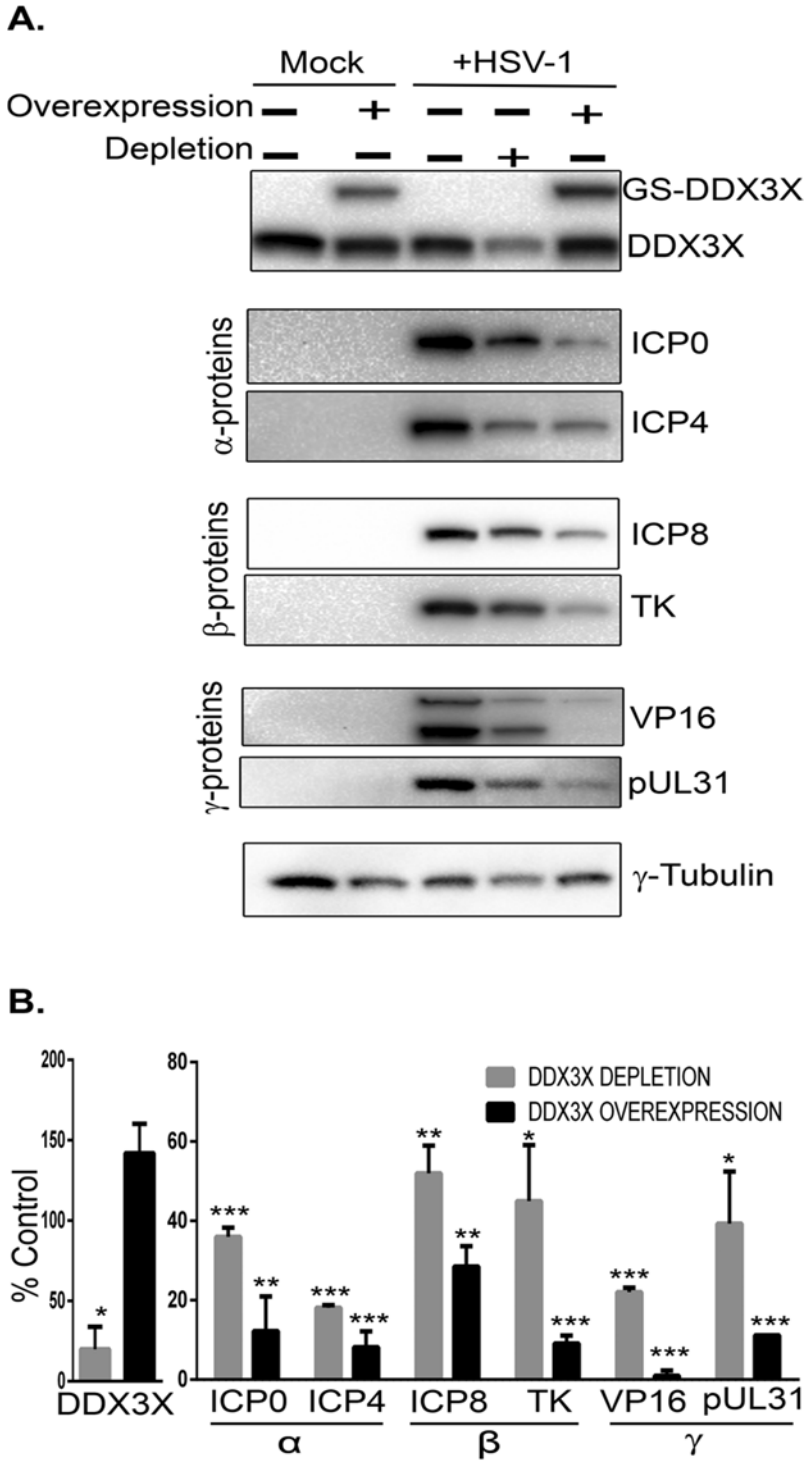

Figure 2.10. Effect of depletion or overexpression of DDX3X on HSV-1 gene expression.

A) Hela cells seeded in 6-well plates were transfected for 48 hours either with pooled siRNA against DDX3X and/or a plasmid coding for siRNA resistant DDX3X. They were subsequently infected at a MOI of 5 with wild-type HSV-1 and harvested at 9 hpi and lysed. Twenty micrograms of the lysates were directly loaded onto SDS-PAGE gels and analyzed by Western blotting. γ--tubulin was used as the loading control (top panels). **B)** Protein expression was evaluated and normalized to γ-tubulin and compared to the values obtained for infected but non-transfected cells (bottom panel). The reported values represent the average of two experiments. The error bars indicate standard deviations of the means. Bilateral Student's T tests were performed (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$). Note that throughout this study, Western blots were quantified on a ChemiDoc MP system with a 4 orders of magnitude dynamic range, not film which has a poor linearity.

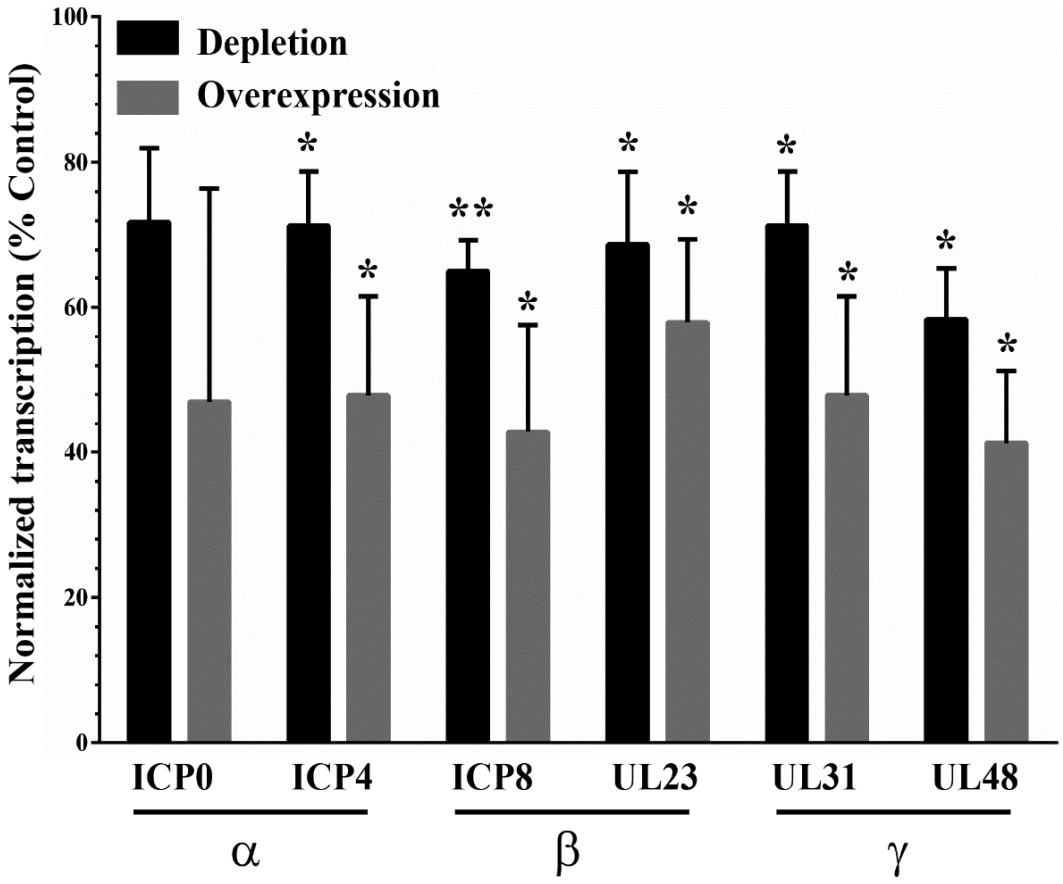

Figure 2.11. Impact of DDX3X on viral gene transcription.

HeLa cells were seeded in 6-wells and transfected for 48 hours either with siDDX3Xa or a siRNA resistant DDX3X plasmid. Following 8 hours of infection with wild type virus at MOI of 5, total RNA was collected and reverse transcribed into cDNA. Expression levels of immediate-early (α), early (β) or late (γ) viral genes were analyzed by RT-qPCR. Values represent the averages of 2 experiments. All the values were normalized on GAPDH. Bilateral Student's T tests were performed to detect significant hits compared to the transfection agent only control arbitrarily set at 100% (*: $p<0.05$ and **: $p<0.01$).

Table 2.1. Effect of DDX3X depletion or overexpression on HSV-1 gene expression.

		Depletion				Overexpression	
Gene	**protein**	**mRNA level**	**protein level**	**Gene**	**protein**	**mRNA level**	**protein level**
RL2	ICP0 *	72 % ± 10	36 % ± 2	RL2	ICP0	47 % ± 29	12 % ± 9
RS1	ICP4 ***	71 % ± 7	18 % ± 0.6	RS1	ICP4 *	48 % ± 14	8 % ± 4
UL29	ICP8	65 % ± 4	52 % ± 6	UL29	ICP8	43 % ± 15	28 % ± 5
UL23	TK	69 % ± 10	44 % ± 14	UL23	TK *	58 % ± 12	9 % ± 2
UL31	pUL31	71 % ± 7	39 % ± 13	UL31	pUL31	48 % ± 14	11 % ± 0.04
UL48	VP16 *	58 % ± 7	22 % ± 1	UL48	VP16 *	41 % ± 10	1 % ± 1

Impact of DDX3X on viral gene transcription. Stars indicate the significant difference between mRNA and protein levels in 2 independent experiments based on bilateral Student's T test (+/- standard deviations to the means). P-values. (*: $p<0.05$ and ***: $p<0.001$).

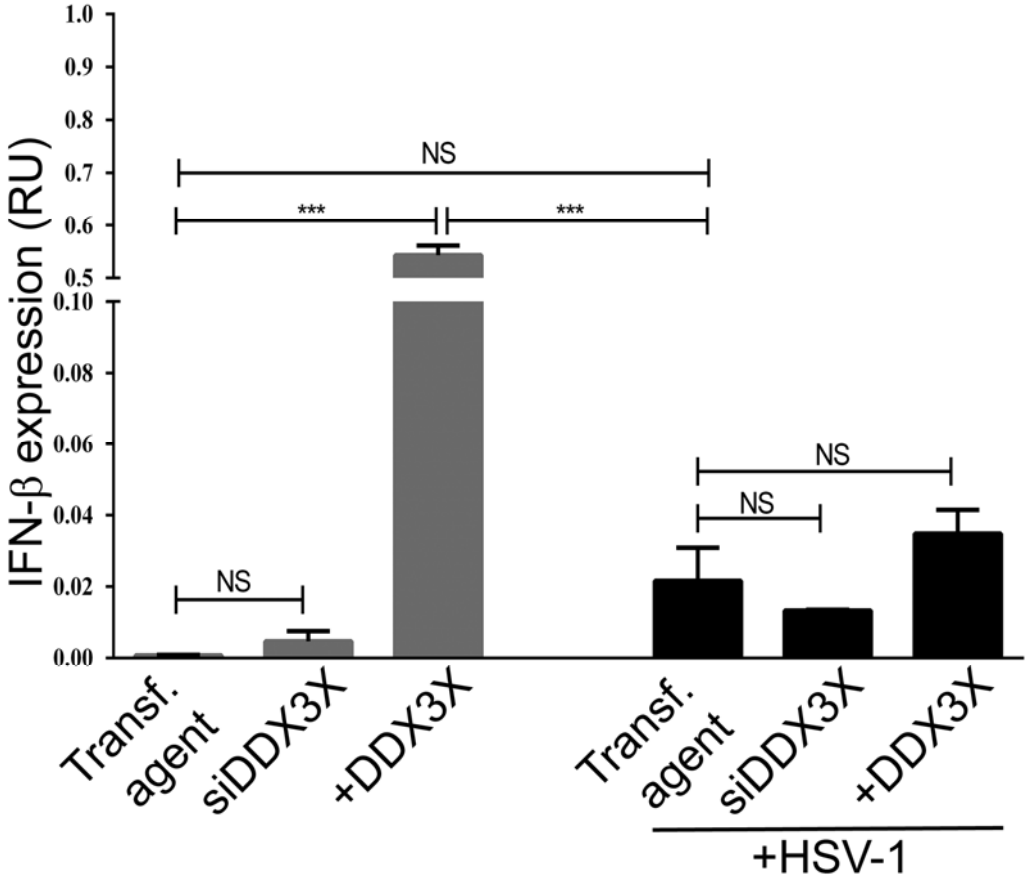

Figure 2.12. IFN transcription levels.

HeLa cells grown in 6-wells were transfected for 48 hours either with siDDX3Xa and/or a siRNA resistant DDX3X plasmid. Cells were either mock treated or subsequently infected with wild type virus for 8 hours at MOI of 5. Total RNA was collected and IFN-β mRNA measured by RT-qPCR. Data represent the average of 2 independent experiments. Bilateral Student's T tests were performed to detect significant hits compared to the transfection agent only control (RU: Relative units; NS: non-significant; ***: $p<0.001$).

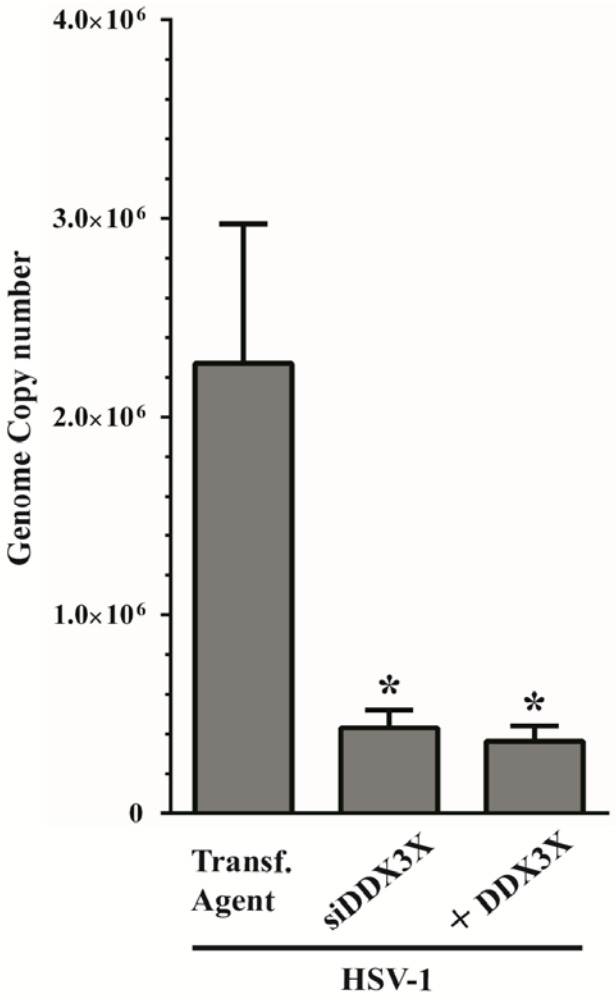

Figure 2.13. Viral genome copies upon siDDX3X or overexpression.

HeLa cells seeded in 6-wells were transfected for 48 hours either with siDDX3Xa and/or a siRNA resistant DDX3X plasmid. Cells were then infected with wild type virus for 8 hours at MOI of 5. Total DNA was then collected and viral genome copy numbers were measured by qPCR. Data represent the average of 2 experiments. Bilateral Student's T tests were performed to detect significant hits compared to the transfection agent only control (*: $p<0.05$).

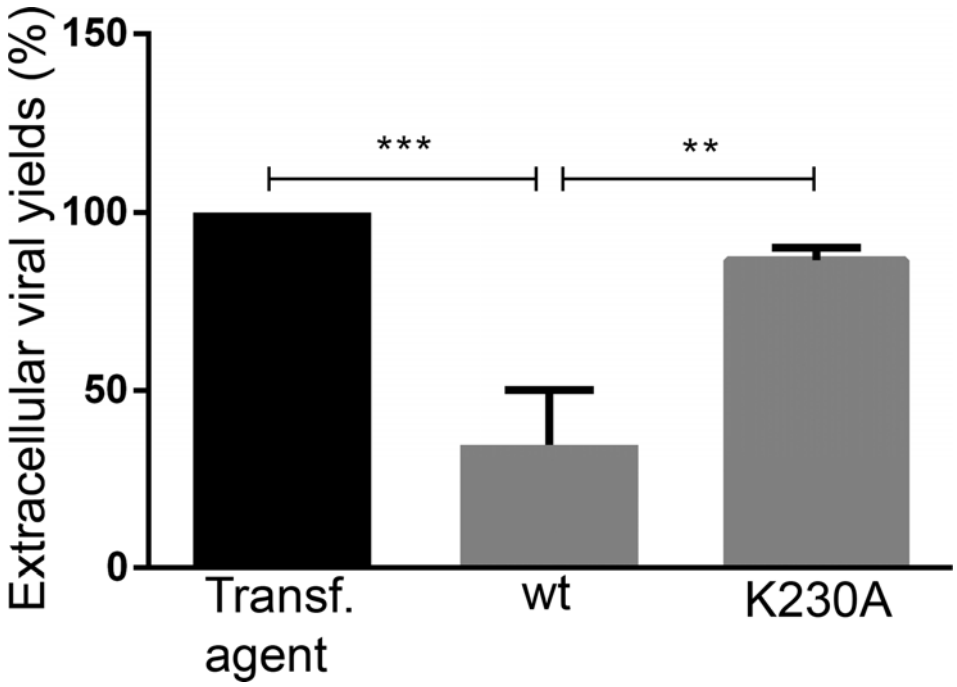

Figure 2.14. Overexpression of ATPase/helicase inactive but interferon competent DDX3X mutant has no impact on viral propagation.

Hela cells grown in 6-well plates were either mock treated or transfected for 24 hours with exogenous wild type DDX3X or the ATPase/helicase deficient but interferon competent K230A mutant. They were then infected with wild-type HSV-1 for 18 hours with a MOI of 5. Extracellular viruses in the supernatant, were then collected and titrated on Vero cells. Data represents the pool of 5 individual experiments. Statistical significance was assessed based on P-values. (**: $p<0.01$ and ***: $p<0.001$).

Discussion

The DDX3X RNA helicase modulates HSV-1 propagation

The present data, along with our past findings [21], indicate that depletion of DDX3X either in cells or in HSV-1 mature viral particles led to a significant reduction of infectious HSV-1 particles. Two independent data sets confirmed that this was specific and not the results of off-target effects associated with RNA interference. First, viral yields were significantly rescued by a siRNA-resistant DDX3X construct (fig. 3). Secondly, orthogonal validation of the results with the tsET24 cell line, where DDX3X is non-functional at the restrictive temperature, further proved that the phenotypes were directly imputable to DDX3X (fig. 1). We thus conclude that DDX3X is required for optimal HSV-1 propagation.

Despite the positive implication of DDX3X in viral yields, we unexpectedly found that overexpression of DDX3X also reduced virus production (fig. 2-3). This was corroborated by the fact that we only detected HSV-1 K26GFP expression in 0.9% of the cells overexpressing DDX3X (fig. 2). This inhibition notably required the ATPase and helicase activities of DDX3X (fig. 14). Though initially counter-intuitive, this apparent contradiction is not unusual and has indeed been reported for hepatitis B and C viruses [9, 26]. We thus presume that reduced or overabundant DDX3X levels exert their effect by distinct mechanisms and postulate that DDX3X indeed positively modulates viral proliferation when rate limiting or in normal amounts. In contrast, overabundant functional DDX3X likely titers out one or a few of its molecular partners and drowns the endogenous DDX3X machinery. This is in line with our inability to generate cell lines stably overexpressing DDX3X (data not shown). In this context, it is worth noting that HSV-1 did not alter the DDX3X protein levels in HeLa and Vero cells (fig. 4) and thus ensured the presence of appropriate levels of DDX3X. Why the virus reduced DDX3X protein levels to a significant extent in 143B remains a mystery though. Nonetheless, our working hypothesis is supported by our rescue experiments whereby transfection of siDDX3X-resistant exogenous DDX3X in siDDX3X treated cells partially rescued both normal levels of DDX3X and viral output (fig. 3). Thus DDX3X appears as an important host interacting partner for HSV-1.

DDX3X acts on HSV-1 gene expression

Distinct non-exclusive scenarios could justify the presence of DDX3X in mature HSV-1 particles. First, the virion-associated DDX3X may be required to initiate an efficient infection. However, this was not the case as viral entry was unperturbed by the depletion of this cellular protein within the viral particles prior to the entry assay (fig. 7). It remains possible though that the cellular pool of DDX3X may accomplish this task. Second, DDX3X incorporation in mature virions could also be the consequence of a prior interaction between the host protein and structural components of the virus and thus merely reflect past role(s) or simply a sticky protein. Although we cannot formally rule out this second option, it is clear that DDX3X plays an active role in the assembly of novel viral particles by regulating the transcription of viral genes (fig. 11). Given the stronger impact of DDX3X on viral protein levels than transcription (Table 1), it is conceivable that DDX3X also acts on other steps of viral gene expression, namely mRNA transport, stability or translation or even viral protein stability. On the other hand, the more pronounced effect of DDX3X on protein levels may simply be the consequence of the relative abundance of viral transcripts, albeit this is hard to directly correlate [27]. We conclude that DDX3X unambiguously modulates HSV-1 gene transcription with potential impacts of other steps of gene expression. The presence of DDX3X in other mature extracellular herpesviruses, including HSV-1, PRV and HCMV [28] suggests this could be true for other viruses. One major outstanding question is why these viruses bother incorporating DDX3X in their viral particles.

Mechanism of DDX3X action

Whether DDX3X directly acts on various viral genes or indirectly via a few select molecules is unclear. Thus far, DDX3X was shown to bind to the interferon β and the tumor suppressor p21 promoters, thus suggesting a direct role as a transcription co-factor [10, 29]. However precise binding motifs have yet to be identified.

An intriguing scenario is the positive role that DDX3X plays in innate immunity upon TBK1 and IKKε binding and IRF3 activation [10, 13, 30]. As expected, modulating DDX3X levels did alter interferon production in uninfected cells but had minimal impacts in infected cells (fig. 12), likely owing to the well documented ability of the virus to circumvent this

pathway [31]. Since either increasing or decreasing DDX3X levels negatively altered viral output (fig. 1-3, 8-10), transcripts (fig. 11 and Table 1) and genome copies (fig. 13), this suggested that DDX3X acted on the virus independently of the interferon pathway. Supporting this model is that the K230A DDX3X mutant, devoid of its ATPase and helicase activities but still able to stimulate interferon production [10, 13], was completely dead in our hands and failed to limit HSV-1 propagation (fig. 14). So, we conclude that DDX3X most likely modulate HSV-1 gene transcription in an interferon-independent manner.

DDX3X is a key player for both RNA and DNA viruses

DDX3X is a multifunctional cellular protein that interacts with viruses at several levels. Much of the current literature focuses on RNA viruses given the RNA helicase activity of DDX3X. Not surprisingly, it was found to promote genome duplication of many of those viruses [32]. However, DDX3X binds TBK1/IKKε, which both stimulates IRF3 activation and interferon type I production thereby stimulating an anti-viral state [10, 11]. DDX3X thus has dual functions that can either facilitate or hamper viruses. Interestingly, this later property is not limited to RNA viruses, but operates with vaccinia virus and hepatitis B virus, two DNA viruses [12, 13]. DDX3X thus influences viral outcome both via innate immunity and RNA viral gene duplication. The present study further suggests that DDX3X also acts on DNA viruses by a third mechanism, namely by modulating viral gene expression.

Materials and Methods

Cells, Viruses and Plasmids. Hela (ATCC CCL-2), Vero (ATCC CCL-81), 143B tk^- (ATCC CRL-8303), tsET24 and BHK21 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 2 mM L- glutamine in 5% CO_2 and either 10% FBS (fetal bovine serum) or 5% bovine growth serum (BGS). 143B cells were also supplemented with 15 µg/ml 5-bromo-2 deoxyuridine (BrdU, Sigma) except prior to transfection and infection. The tsET24 thermosensitive and parental BHK21 cell lines (a kind gift from Dr Takeshi Sekiguchi, Kyushu University; [22]) were passaged at the permissive temperature of 34°C.

HSV-1 K26GFP (strain KOS; provided by Prashant Desai, Johns Hopkins University) [33]) is a fluorescent virus tagging the minor VP26 capsid component. Wild-type HSV-1 strain F was obtained from the American Type Culture Collection (ATCC VR-735). The HSV-1(17^+)Lox-Luc viral strain is derived from strain 17^+ and encodes a luciferase gene under the constitutive immediate early CMV promoter positioned between the UL55 and UL56 HSV-1 genes [25]. All viruses were propagated on BHK cells and titrated on Vero cells as previously described [34].

The pGS-TAP-tagged DDX3X (DDX3X fused to protein G and streptavidin binding peptide), pSG-N-4xHA-TEV-N-term DDX3X (HA tagged DDX3X) and pSG-N-4xHA-TEV-N-term K230A (defective mutant) eukaryotic expression plasmids were a kind gift from Dr G. Superti-Furga (Research Center for Molecular Medecine; [10]). A siRNA-resistant plasmid was derived from pGS-TAP-tagged DDX3X using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) using forward primer 5'-GGAAAGAGGAAAGATTGGATTAGACTTTTG**T**AA**G**TA**T**CTGGT**C**C**T**G**G**ATGAAGC TG ATCGGATGTTGGATATGGG-3' and reverse primer 5'-CCCCATATCCAACATCCGATCAGCTTCATC**C**A**GG**ACCA**GA**TA**C**TT**A**CAAAAGTCT AATCCAATCTTTCCTCTTTCC-3', as per manufacturer's instructions, where bold sequences indicate the silent point mutations.

Antibodies. Primary antibodies were as follows: the anti-human DDX3 rabbit R648 polyclonal serum was a kind gift from Dr A. Patel (University of Glasgow Centre for Virus Research; [35]). Viral antibodies were generously provided by several laboratories and used at

the indicated dilutions for Western blotting: VP16 (Dr Helena Browne), TK (Dr James R Smiley) and pUL31 (Dr Joel D. Baines). All other antibodies were purchased from commercial vendors, including anti-VP5 (Cedarlane), γ-tubulin (Sigma-Aldrich), ICP4, ICP8 and ICP0 (all three from Abcam). All secondary antibodies were purchased from Molecular probes.

Fluorescence microscopy. Hela, Vero or 143B cells were seeded overnight on coverslips in 24 well plates at a concentration of 1.5×10^4 cells/well. Cells were mock treated or infected with HSV-1 K26GFP at a multiplicity of infection (MOI) of 5. After a one hour adsorption at 37°C, cells were washed twice in phosphate-buffered saline (PBS), fresh medium was added and the cells incubated for another 18 hours prior to fixation and permeabilization with 3% paraformaldehyde and 0.1% Triton X-100. The cells were then incubated with a rabbit anti-DDX3X antibody for one hour and further incubated for 45 minutes with a goat anti-rabbit antibody coupled to Alexa Fluor 568 (Molecular Probes). The samples were finally stained with Hoechst 33342 (Sigma-Aldrich) and examined on an Axiophot epifluorescence or LSM700 confocal microscope (Zeiss). Fluorescent intensities were calculated by image J by subtracting the background signal then divided this net signal by the cell.

Western blotting. Hela, Vero or 143B were mock treated or infected with wild type HSV-1 at a MOI of 5 and collected 18 hours later by scraping them in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 1% Igepal and a cocktail of protease inhibitors). After a centrifugation of 5 min at 4ºC and 500xg, cell pellets were resuspended in lysis buffer and incubated on ice for 1 hour. Then they were passed through 27G 1/2 needles 3 times and treated with DNase for 30 minutes at 37ºC (500 U/ml, Roche). Cell debris were removed by a 2500xg spin of 10 minutes and cell lysates collected. Samples (typically 20 μg) were loaded on 8% acrylamide SDS-PAGE gels in protein sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol and 2% β-mercaptoethanol). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, which were then incubated for 1 hour in blocking buffer (5% nonfat dry milk, 13.7 mM NaCl, 0.27 mM KCl, 0.2 mM KH_2PO_4, 1 mM Na_2HPO_4, and 0.1% Tween 20). The membranes were ultimately reacted with antibodies as indicated in each figure legend. When indicated, protein expression levels were quantified with a ChemiDoc MP System (Bio-Rad), which has a 4 orders of magnitude dynamic range, and Image lab version 5.0 software (Biorad).

Thermosensitive DDX3X. Thermosensitive tsET24 and parental BHK21 cells were plated in 6-well plates and incubated at the permissive temperature of 34°C or the non-permissive temperature of 39.5°C for 24 hours. The cells were then mock treated or infected with HSV-1 K26GFP at a MOI of 5 at 34°C or 39.5°C. Cells and supernatants were collected at 24 hours post-infection (hpi) and HSV-1 production titrated on Vero cells by plaque assays.

DDX3X Overexpression. Hela cells were seeded on coverslips in 24 well plates at a concentration of 2.0×10^4 cells/well. The next day, cells were transfected for 24 hours with 0.5 µg/well of pGS-TAP-tagged DDX3X using the lipoD293 (SignaGen). Cells were subsequently infected for a further 18 hours with wild type HSV-1 K26GPF at a MOI of 5. The samples were finally fixed and permeabilized as described above and observed on an Axiophot epifluorescence microscope (Zeiss). To measure the impact of DDX3X overexpression on viral yields, Hela cells were seeded at a concentration of 5.5×10^5 cells/well in 6 well plates and transfected with pSG-N-4xHA-TEV-N-term DDX3X or pSG-N-4xHA-TEV-N-term K230A for 24 hours with 4 µg/well of DDX3X constructs. Cells were then infected for a further 18 hours with wild type HSV-1 at a MOI of 5. Supernatants, containing extracellular viruses were collected and titrated on Vero cells.

RNA interference. siRNA against human DDX3X were either used as a SMARTpool of 4 siRNA (Dharmacon) or individually tested as indicated in the respective figure legends. These siRNA were: siDDX3Xa: GCAAATACTTGGTGTTAGA, siDDX3Xb: ACATTGAGCTTACTCGTTA, siDDX3Xc: CTATATTCCTCCTCATTTA and siDDX3Xd: GGTATTAGCACCAACGAGA). 25 nM of siRNA was transfected into 143B or Hela cells using either Pepmute (Signagen) or lipofectamine 2000 (Thermofisher) for 48 hours.

Generation of depleted virions. To produce depleted virions, $1x10^6$ cells 143B cells were seeded in 10 cm dishes for 24 hours. These cells were then either transfected or mock treated for 48 hours with 25 nM of siRNA against DDX3X, VP16 or scVP16 (scrambled) [21] using lipofectamine 2000 (Invitrogen). Cells were then infected for another 24 hours with HSV-1(17^+)Lox-Luc or wild type virus at a MOI of 5. In order to separate the intra- from extracellular virions, the tissue culture medium was first removed and cells scraped, centrifuged at 250g for 5 minutes at 4°C and resuspended in 100 mM MNT (30 mM morpholinoethanesulfonic acid, 100 mM NaCl and 20 mM Tris, pH 7.4). Meanwhile, the extracellular medium was concentrated

at 40,000g for 40 minutes at 4ºC and viral pellets resuspended in the above buffer. All viruses were then titrated on Vero cells.

DDX3X Rescue. Hela cells were seeded on coverslips at a concentration of 2.0×10^4 cells/well. Cells were transfected for 24 hours with 25 nM of siRNA against DDX3X (siDDX3Xa) or with transfection agent only as control. Cells were then transfected a second time with a siRNA-resistant DDX3X construct using lipoD293 for a further 24 hours. They were finally infected with HSV-1 K26GFP at a MOI of 5 for 18 hours. The cells were then fixed and permeabilized as described above and examined with an Axiophot epifluorescence microscope. Quantification of fluorescence signals was done with Image J (version 1.48) and values normalized per cell areas.

Entry assay. 143B cells were seeded in 24-well plates 24 hours before infection at a concentration of 1 $x10^5$ cells/well. Cells were inoculated with wild type HSV-1, untreated HSV-1(17^+)Lox-Luc, depleted viruses (see above) or as control no virus at all under conditions that enable the virus to bind the cells but not penetrate them (MOI of 30, 4ºC, 1 hour). Samples were then shifted to 37ºC for another 1 hour and lysed for 30 minutes at room temperature with 100 µl/well of lysis buffer from the firefly luciferase assay kit (Biotium). Samples were then transferred to 96-well plates and analyzed by LUMIstar Galaxy luminometer (BMG Labtech). Luminescence was quantified using LUMIstar Galaxy software version 4.30-0.

RT-qPCR. Following DDX3X depletion or overexpression (see above), Hela cells were infected with wild type HSV-1 for 8 hours. Total RNA was extracted using SV Total RNA Isolation System (Promega). The RNA was then reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The cDNA was then analysed by quantitative PCR using a LightCycler 480 (Roche). Viral (ICP0, ICP4, UL23, ICP8, VP16 and UL31) or cellular genes (DDX3X and IFN-β) were quantified using the standard curve method and normalized to an endogenous control (GAPDH). All PCR reactions were performed using SybrGreen (Molecular Probes) and primers as indicated in Table 2.

Table 2.2. PCR primers

Gene	protein	Forward primer *(5' → 3')*	Reverse primer *(5' → 3')*
RL2	ICP0	CTGTCGCCTTACGTGAACAA	CATCCAGAGGCTGTTCCACT
RS1	ICP4	CGACACGGATCCACGACCC	GATCCCCCTCCCGCGCTTCGTCCG
UL23	TK	GTAATGACAAGCGCCCAGAT	ATGCTGCCCATAAGGTATCG
UL29	ICP8	ACATTACGTTCACGGCCTTC	GGCCATCGACACGATAGACT
UL48	VP16	GGACGAGCTCCACTTAGACG	AGGGCATCGGTAAACATCTG
UL31	pUL31	GTGAAGACCACTCCCGTCTC	ATCGTGTTGATCTGCTGCAC
UL55	gB	TTTGTGTACATGTCCCCGTTTTAC	AGAAGCCGTCGACCTGCTT
DDX3X	DDX3X	TGCTGGCCTAGACCTGAACT	TTGATCCACTTCCACGATCA
GAPDH	GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
IFN-β	IFN-β	AAACTCATGAGCAGTCTGCA	AGGAGATCTTCAGTTTCGGAGG

Viral gene copies. DDX3X was either depleted or overexpressed in Hela cells as detailed above. Cell were then infected with wild type HSV-1 for 8 hours. Total DNA was purified from each condition using GenElute Mammalian Genomic DNA Miniprep Kits (Sigma) as per manufacturer's instructions. For the qPCR analysis, gB specific primers (Table 2) were used with the above SybrGreen assay using GAPDH as the internal control.

Statistical analysis. Virus titers, protein abundance, fluorescence and flow cytometry data were normalized to the values obtained for the controls as mentioned in each figure legend and analyzed with bilateral Student's T tests using GraphPad Prism version 5 (GraphPad Software).

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Supplementary Data

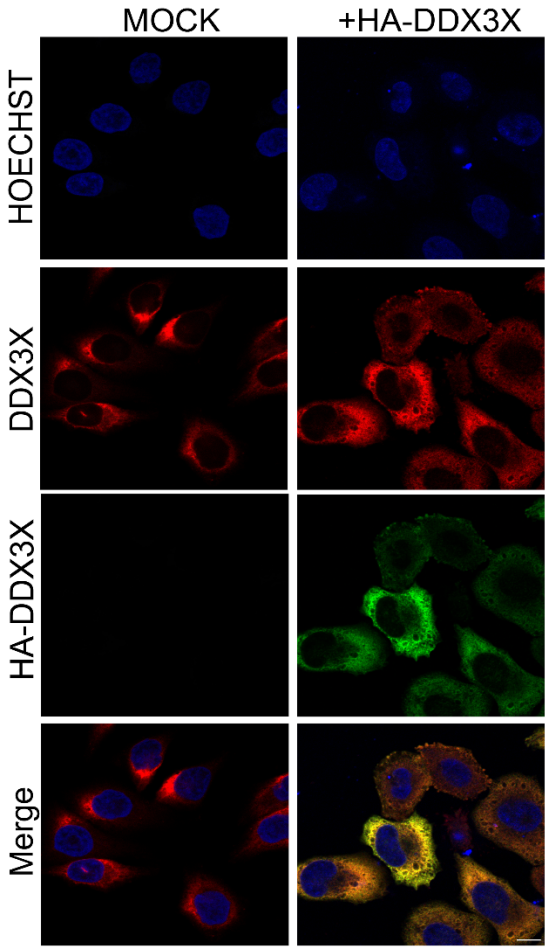

Figure S1. Overexpressed DDX3X co-localizes with endogenous DDX3X.

Hela cells grown on coverslips were mock treated or transfected with pSG-N-4xHA-TEV-N-term DDX3X for 24 hours. Cells were fixed and reacted with antibodies against total DDX3X (red) or HA specific antibodies to detect exogenous DDX3X (green). Nuclei were labeled with Hoechst (blue). Samples were analyzed by confocal laser scanning microscopy. Scale bar: 10 μm. These results are representative of 3 individual experiments.

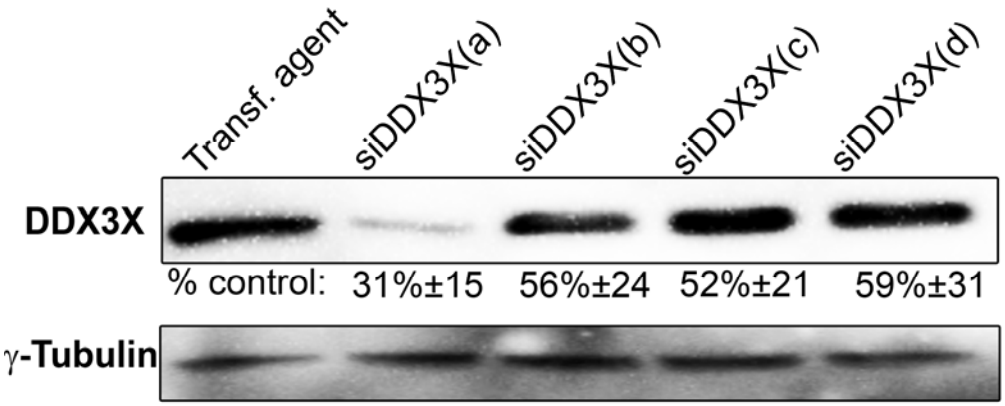

Figure S2. RNA interference efficiency.

HeLa cells seeded in 24 wells were individually transfected for 48 hours with each of the different siRNAs against DDX3X (a, b, c and d) that are present in the SMARTpool. Cell lysates were collected and Western blotting was done as described before. Values represent the amounts with respect to the untransfected control. The data is representative of 2 individual experiments.

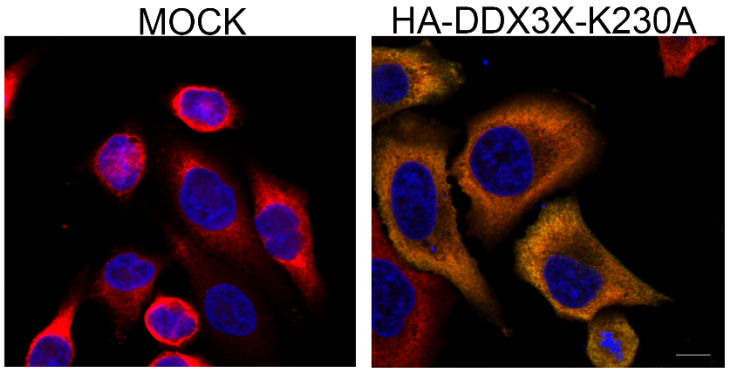

Figure S3. The K230A DDX3X mutant is expressed at similar levels than wild-type.

Hela cells grown on coverslips were mock treated or transfected with pSG-N-4xHA-TEV-N-term K230A for 24 hours. Cells were fixed and labeled with antibodies against total DDX3X (red) or HA specific antibodies to detect mutant DDX3X (green). Nuclei were labeled with Hoechst (blue). Samples were analyzed using confocal laser scanning microscopy. Scale bar: 10 µm. Images are representative of 3 individual experiments.

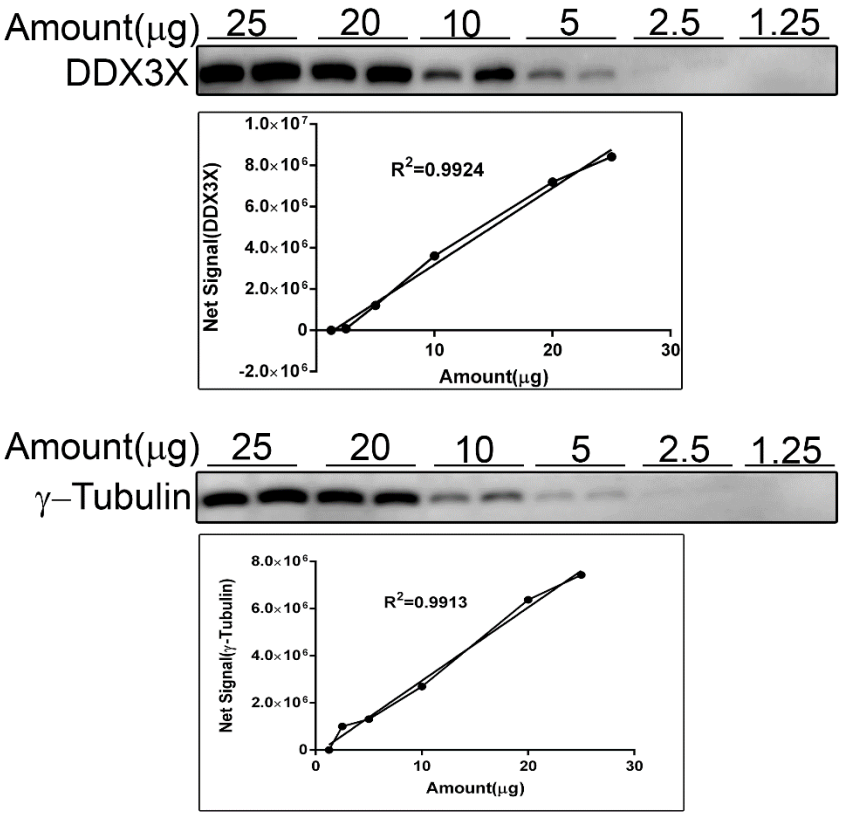

Figure S4. The linearity of WB results using ChemiDoc MP System (Bio-Rad).

Hela cells were seeded in 10 cm petri dish for 24 hours, cell lysate were then collected using 3 cycles of freeze and thaw. Different amounts of cell lysate were load on a SDS-PAGE in duplicates to exclude the potential loading errors. Western blotting was done using DDX3X or γ-Tubulin antibodies. Linearity of the results was assayed by measuring the signal intensity of each point (mean of the duplicates). R squares were measured using GraphPad Prism 6.

Chapter 3. Discussion

3.1. DDX3X is a determinant host factor in various viral life cycles

The host-pathogen interactome has become the topic of interest for many studies in the recent years and is constantly evolving. Pathogens as parasites have adapted countless strategies to recruit their host's cellular machinery and evade their immune defense (210). These strategies create high complexity to eliminate these foreign agents. Therefore, by better understanding proteins like DDX3X that interact with wide spectrum of viruses from several families, we will be well-equipped to target many pathogens all at once.

Shortly after the discovery of the DEAD-box protein DDX3X (159), it was revealed that this protein is associated with viral replication using HCV (174, 185). In fact, the majority of DDX3X cellular functions have been discovered in the context of infection (168, 171, 173, 185, 206, 228, 236-239). Although DDX3X, as a RNA-helicase has been mostly associated with RNA viruses, its contribution is not only limited to these pathogens and it has been demonstrated to play essential roles in the replication of DNA viruses such as HBV (205-207), VACV (173, 211) and HCMV (212).

HSV-1, like many other enveloped viruses highjacks several host factors. Our comprehensive characterization of HSV-1 mature virions, identified DDX3X among 49 other cellular proteins (39). Further screening established the requirement of DDX3X for HSV-1 infectivity, as the depletion of DDX3X from either viral or cellular pools drastically reduced viral yields (137). These results, along with the fact that many other pathogens manipulate DDX3X, made this host factor a very interesting target for us. Most importantly, the use of a temperature sensitive species of DDX3X and rescue with a siRNA resistant DDX3X clone proved our siRNA screen results to be perfectly valid and not just an off-target effect (Fig 2.1 and 2.3).

3.2. It is all about DDX3X levels

Since DDX3X knockdown has a negative impact on HSV-1 infectivity (137), we reasoned that overexpression of this protein would conversely favor viral replication. However, to our surprise, the overexpression of DDX3X almost killed (~80% inhibition) the HSV-1 infection (Fig 2.2A). This was also validated at the single-cell level by immunofluorescence (Fig 2.2A), where no sign of infection was found upon DDX3X overexpression. These observations prompted us to rescue DDX3X cellular levels by using a siRNA resistant DDX3X plasmid. Co-transfecting cells with this DNA construct and siDDX3X restored the infection up to 70%, where single transfection with either plasmid or siRNA hampered viral replication, as expected (Fig 2.3). This experiment validated our hypothesis that endogenous levels of DDX3X is highly important for efficient HSV-1 propagation. Indeed, this is not the first time that specific levels of DDX3X seem to be critical for viruses. For example; overexpression of DDX3X has been shown to inhibit HBV in a dose-dependent manner (202) and it has been suggested that norovirus alters DDX3X levels upon infection (196). Furthermore, HSV-1 does not affect the cellular expression of DDX3X in HeLa or Vero cells, but surprisingly down-regulates it by 50% in 143B cells (Fig 2.4). 143B cells are osteosarcoma cells and some studies have shown that these cells are very sensitive to interferon expression, which blocks their growth (211, 212). In contrast, Vero cells do not express any interferons type 1, because they lack interferon genes (213-215) and for HeLa cells the induction of IFN type I, seems to have no particular effect on cell proliferation. As mentioned earlier, DDX3X is an interferon type I stimulator molecule (181), therefore one tempting explanation is that the virus alters the expression of DDX3X in 143B cells so that the cells stay viable for its propagation. Further evidence for this claim comes from experiments where we tried to overexpress DDX3X in 143B cells but we faced a drastic cell loss shortly after transfection (data not shown). Finally, preliminary western blot quantification of HeLa cells depleted for or overexpressing DDX3X in the context of HSV-1 infection for different times (3, 6, 9 and 18 hpi) suggested that the virus tries to rescue DDX3X protein levels back to its normal preinfection cellular levels (data not shown). However, this will need further validation to draw a conclusion.

3.3. DDX3X and HSV-1 Interplay

3.3.1. DDX3X redistribution upon infection

Viral pathogens tend to redistribute proteins upon infection to either take advantage of the protein or inhibit its negative effects on the viral. Our results initially indicated that HSV-1 is affected by DDX3X but the question remained, whether the virus changes the cellular location of DDX3X. Therefore, we investigated the distribution of DDX3X in the HSV-1 infected cells versus mock cells in 3 different cell lines (Fig 2.5A). This showed us that virus displaces DDX3X in a cell-type independent manner.

Tracking of a protein during infection can reveal the moment where protein localization is affected by a virus. Interestingly, close monitoring of DDX3X during the HSV-1 infection suggests it may change location on numerous occasions. For instance, DDX3X appears to be predominantly cytoplasmic early on (Fig 3.1; panel 3 hpi blow up), similar to the normal cellular localization of the protein. Interestingly, somewhere between 3 and 6 hpi, DDX3X migrates from the cytoplasm to the nucleus (Fig 3.1; panel 6 hpi blow up). However, the protein is again mostly cytoplasmic later on (Fig 3.1; panel 9 hpi blow up). It is of note that at this time point (Fig 3.2), DDX3X shows a peculiar distribution which resembles a lot to the localization of actively translating ribosomes in HeLa cells (216). Although, this is a tempting assumption, we will need to validate the colocalization of DDX3X with a ribosomal marker at this time point. Very late during the infection (Fig 3.1; panel 24 hpi blow up), DDX3X is aggregated in the cytoplasm, aggregates that colocalize with the virus (Fig 2.5B), possibly reflecting the incorporation of DDX3X into mature HSV-1 virions.

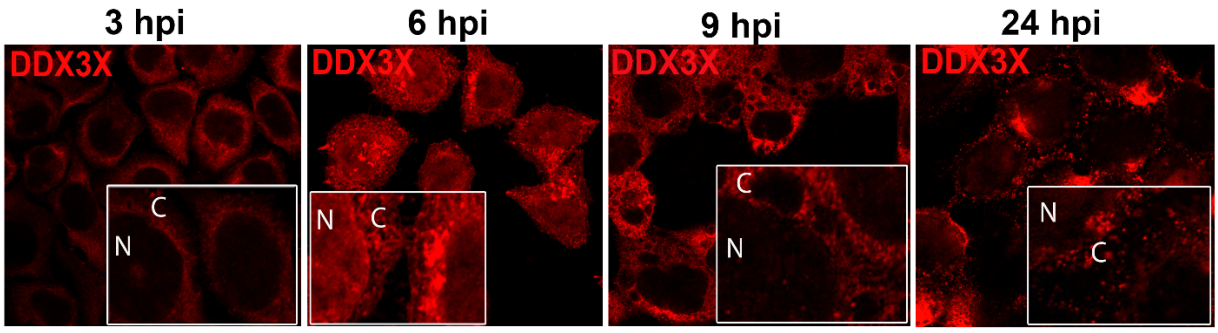

Figure 3.1. Closer look at DDX3X distribution during HSV-1 infection

DDX3X localization at different time points during HSV-1 was isolated from figure 2.6 and some blow ups where added to emphasize on the location of DDX3X at each time point. N: nucleus, C: cytoplasm.

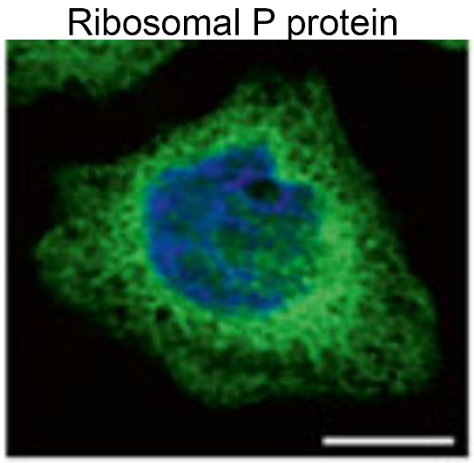

Figure 3.2. Actively translating ribosomes.

Hela cells were treated with puromycin (PMY) to freeze the translation elongation (ribopuromycylation method (RPM)). This method is able to capture the actively translating ribosomes. Ribosome are labeled with antibodies against large ribosome subunit acidic P proteins and observed under the confocal microscope. The figure is adapted from: *David, A. et al; Methods Mol Biol. 2015; 1228:133-42.* (217).

3.3.2. HSV-1 gene expression is affected by DDX3X

In the search of the mechanism behind the impaired infectivity of HSV-1 that results from the imbalanced levels of DDX3X, we discovered the importance of DDX3X in viral gene transcription (Fig 2.11). This is in line with the temporary relocation of DDX3X to the nucleus that was discussed in the previous section. As DDX3X has been shown to regulate transcription of some genes such as E-cadherin and p21 by directly binding to their promoters (170, 182, 183), it is possible that that its effect on viral transcription is also through the direct interaction with viral promoters. However, this effect can also be through interactions with cellular transcription factors (218) or viral transactivator proteins like VP16, ICP0 and ICP4.

By comparing the WB results (Fig. 2.10) and RT-qPCR data (Fig. 2.11), we noticed that the alteration of DDX3X expression might inhibit the gene translation more profoundly than transcription of some viral genes (Table 2.1). This raises the possibility that DDX3X, in case of these viral genes, contributes to viral mRNA translation independent of transcription as well as the other aspects of RNA metabolism such as splicing, RNA export and RNA or protein stability. DDX3X, like all the other RNA helicase can directly unwind/rearrange RNA strands or cause the disassociation of RNA from RNPs, therefore contributing to the stability of RNA structures (140). To this end, it seems logical to assume DDX3X can be involved in the posttranscriptional events of HSV-1 mRNA metabolism. Interestingly, DDX3X has been linked to the inhibition of cap-dependent translation (219). Thereby, given the stronger impact of DDX3X overexpression on viral protein translation (Fig 2.10 and table 2.1), it is possible that too much DDX3X suppress viral translation through this route. On the other hand, DDX3X helps the assembly of ribosomal subunits. Therefore, DDX3X knockdown could also negatively affect viral gene translation at this level. It is noteworthy that the ribosomal-like localization of DDX3X at 9 hpi (Fig 3.1 and 3.2) is in line with the time point where we see major translation of viral proteins. It is thus possible that DDX3X acts on HSV-1 gene expression at multiple levels.

Given that both the depletion and overexpression of DDX3X have similar effects on viral gene expression (Fig 2.10-2.11), we should not forget that DDX3X is an ambivalent protein, i.e. stimulating some pathways and inhibiting others. Therefore we cannot rule out the likelihood of two or even more mode of action in the modulation of HSV-1. For example, the

lack of sufficient amounts of DDX3X could impacts the assembly of ribosomal subunits (220), while excess of DDX3X might block the cap-dependent translation (221) or another important factor for the viral gene expression. Only further work will clarify these aspects.

3.3.3. Could DDX3X contribute to viral entry?

To this day, DDX3X has not been associated with any entry events. Since HSV-1 mature particles incorporate DDX3X, it seemed logical that virus would recruit this protein late during the infection to promote subsequent entry of the newly made viral particles. Surprisingly, depleting DDX3X from mature virions had no effect on viral entry (Fig 2.7). However, we cannot neglect the possible role of cellular pool of DDX3X in the viral entry and it will be interesting to probe a null DDX3X cell line with the depleted virus. Alternatively, it is possible that the presence of DDX3X in viral particles is just the remnant of an interaction of DDX3X with viral proteins during other stages of the infection or even that DDX3X is simply a sticky protein.

3.3.4. DDX3X is not acting through the interferon pathway

Since DDX3X is an active modulator of interferon response (150, 155, 156, 167, 179, 181), its up-regulation strongly induces the expression of IFN-β. However, it has been shown that many viruses, such as HBV and poxvirus, can evade this negative impact of DDX3X (202, 208). Not surprisingly, we found that HSV-1 can also efficiently shut off this response (Fig 2.12), which is in line with reported ability of HSV-1 in suppressing immune responses. Therefore, the normal propagation of the viral particles observed in the presence of the ATPase and helicase deficient DDX3X mutant (K230A), which can still stimulate IFN-β production (Fig 2.14) and form stress granules (176), strongly suggests that DDX3X does not modulate HSV-1 transcription through the IFN-β response but rather directly on viral transcription.

Chapter 4. Conclusion and Perspectives

It is fascinating how HSV-1 is able to suppress the DDX3X induced immune response and instead use the protein to its advantage. The goal of this project was to understand how DDX3X can impact HSV-1 replication. What we have found is that viral gene expression strongly depends on the proper amounts of DDX3X. One would imagine that since knockdown of DDX3X blocks the HSV-1 replication, its up-regulation would favor the infection. However our experiments showed that these interactions are not black and white and the right amount of DDX3X is absolutely essential for HSV-1 replication.

Another interesting observation in this study is the dynamic redistribution of DDX3X in the course of infection. At the beginning of the infection, DDX3X is substantially observed in the cytoplasm, but then relocates towards the nucleus, where it might help viral gene transcription. Later during the infection, DDX3X is again mostly observed in the cytoplasm. At this time, the distribution of DDX3X resembles that of ribosomes, supporting the potential contribution of this protein in viral protein translation.

Although, the present project took us one step forward in understanding the molecular basis of interaction of HSV-1 with DDX3X, many questions remain unanswered. Firstly, our previous works revealed that HSV-1 incorporates DDX3X and this viral pool of DDX3X is as important for the viral replication as the cellular pool of the protein (39, 137). However, our assays indicated that this viral pool of DDX3X has no impact on the early stages of the replication, i.e. viral entry. Therefore it will be very interesting to figure out why the virus bothers to incorporate this host protein into its mature particles and whether the cellular pool of DDX3X is important for viral entry. Unraveling these physical interactions will guide us toward the precise mechanisms that DDX3X control viral replication. Moreover, it will be interesting to examine the viral assembly in depth while depleting or overexpressing DDX3X using transmitting electron microscopy (TEM). Another aspect of DDX3X-HSV-1 interaction that needs more clarification is to distinguish between the effect of DDX3X on transcription, post-transcription, translation or post-translation events of the virus. For example, the effect of DDX3X depletion or overexpression on HSV-1 mRNA decay can be studied. Finally, we have to develop a better understanding of how HSV-1 is capable of escaping DDX3 stimulated

immune response, this will be possible through the recognition of viral partners of DDX3X and whether they block the DDX3X domains that are essential for the stimulation of interferon response or simply the comparison. Finding these interacting partners will also help us to decipher the exact role of DDX3X in the viral mRNA translation.

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