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DOCTORAL THESIS

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SVEUČILIŠTE U RIJECI
FAKULTET BIOTEHNOLOGIJE I RAZVOJA LIJEKOVA

Adwait Anand Parchure

Istraživanje uloge ADAR1 u modulaciji urođenog imunološkog odgovora tijekom produktivne HSV-1 infekcije

DOCTORSKI RAD

Mentor: prof. dr. sc. Igor Jurak

Rijeka, 2026

Mentor of the doctoral thesis: Professor Igor Jurak, Faculty of Biotechnology and Drug Development, University of Rijeka

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1. Dr. Christian Andrew Reynolds, FABRI (President of the committee)

2. Dr. Dragomira Majhen, IRB (Member)

3. Dr. Vjekoslav Tomaić, IRB (Member)

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Summary

Herpes simplex virus 1 (HSV-1) is an important human pathogen extensively studied in fundamental virology and serves as model for understanding viral biology including entry, replication, latency and reactivation as well as virus-host interaction. During productive infection, HSV-1 exhibits extensive transcriptional activity and generates double stranded RNA (dsRNA) structures that can activate host innate immune response. ADAR1 (Adenosine deaminase acting on RNA 1) play an important part in dsRNA homeostasis and regulation of innate immune signalling through adenosine-to-inosine (A-to-I) RNA editing as well as dsRNA binding. Although the functions of ADAR1 have been widely studied in RNA viruses, its role in DNA viruses, particularly during HSV-1 productive infection remains poorly understood. In this study, we investigated the role of ADAR1 in regulating innate immune responses during productive HSV-1 infection.

This study demonstrates that ADAR1 is required for efficient HSV-1 replication and acts as proviral host factor using knockout and transient depletion models. Loss of ADAR1 resulted in impaired viral replication accompanied by strong activation of cytosolic sensor protein kinase R (PKR) and phosphorylation of its downstream effector eIF2α, leading to translational arrest in cell. This phenotype can be rescued by depletion of PKR, inhibition of eIF2α phosphorylation by ectopic expression of viral antagonist ICP34.5, or pharmacological inhibition of translational arrest using ISRIB. In contrast, other cytosolic sensors had limited effects, identifying PKR mediated translational arrest as a primary antiviral pathway restricting viral replication in ADAR1 deficiency.

The proviral function of ADAR1 is isoform specific. The cytoplasmic p150 isoform of ADAR1 was able to suppress PKR activation and rescue viral replication. This role of ADAR1p150 appears to be independent of editing function but relies on RNA dependent interaction with PKR. Furthermore, the activation of PKR during HSV-1 infection is initiated by immediate-early and /or early viral transcription and translation.

Collectively, this study identifies ADAR1p150 as a proviral host factor during productive HSV-1 infection that suppresses PKR/eIF2α mediated translational arrest, thereby enabling efficient viral replication.

Key words: HSV-1, ADAR1, PKR, innate immunity, translational arrest, virus-host interaction

SAŽETAK

Virus herpes simplex 1 (HSV-1) važan je humani patogen koji se intenzivno proučava u temeljnoj virologiji te služi kao model za razumijevanje virusne biologije, uključujući ulazak virusa u stanicu, replikaciju, latenciju i reaktivaciju, kao i interakcije između virusa i domaćina. Tijekom produktivne infekcije HSV-1 pokazuje izrazito intenzivnu transkripcijsku aktivnost te stvara dvolančane RNA (dsRNA) strukture koje mogu aktivirati urođeni imunološki odgovor domaćina. ADAR1 (adenozin deaminaza koja djeluje na RNA 1) ima važnu ulogu u održavanju homeostaze dsRNA i regulaciji signalizacije urođenog imuniteta putem uređivanja RNA preko konverzije adenozina-u-inozine (A-to-I), kao i vezanjem dsRNA. Iako su funkcije ADAR1 detaljno istražene u RNA virusima, njegova uloga u DNA virusima, osobito tijekom produktivne infekcije HSV-1, još uvijek nije dovoljno razjašnjena. U ovom radu istražena je uloga ADAR1 u regulaciji urođenog imunološkog odgovora tijekom produktivne HSV-1 infekcije.

Ovo istraživanje pokazuje da je ADAR1 nužan za učinkovitu replikaciju HSV-1 te djeluje kao proviralni čimbenik domaćina, što je utvrđeno korištenjem modela knockouta i prolazne deplecije. Gubitak ADAR1 doveo je do smanjene virusne replikacije praćene snažnom aktivacijom citosolnog senzora protein kinaze R (PKR) i fosforilacijom njegovog nizvodnog efektora eIF2α, što rezultira translacijskim zastojem u stanici. Ovaj se fenotip može spasiti deplecijom PKR-a, inhibicijom fosforilacije eIF2α ektopskom ekspresijom virusnog antagonista ICP34.5 ili farmakološkom inhibicijom translacijskog zastoja pomoću ISRIB-a. Nasuprot tome, ostali citosolni senzori imali su ograničen učinak, čime je PKR-posredovani translacijski zastoj identificiran kao primarni antivirusni mehanizam koji ograničava virusnu replikaciju u odsutnosti ADAR1.

Važno je naglasiti da smo pokazali da proviralna funkcija ADAR1 ovisi o njegovoj specifičnoj izoformi. Ekspresijom samo citoplazmatske izoforme ADAR1p150 bilo je moguće suprimirati aktivaciju PKR-a i obnoviti virusnu replikaciju. Naši rezultati također ukazuju se da je ova uloga ADAR1p150 uglavnom neovisna o njegovoj funkciji uređivanja RNA, te se temelji na RNA-ovisnoj interakciji s PKR-om. Nadalje, pokazali smo da se aktivacija PKR-a tijekom HSV-1 infekcije pokreće tijekom neposredne rane i/ili rane faze virusne transkripcije i translacije.

Zajedno, rezultati ovog istraživanja identificiraju ADAR1p150 kao proviralni čimbenik domaćina tijekom produktivne HSV-1 infekcije koji suprimira PKR/eIF2α-posredovani translacijski zastoj, omogućujući time učinkovitu virusnu replikaciju.

Ključne riječi: HSV-1, ADAR1, PKR, urođena imunost, translacijski zastoj, interakcija virus–domaćin

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1. INTRODUCTION

Herpes simplex virus 1 (HSV-1) is a highly prevalent human pathogen. It has complex replication cycle including productive infection, latency and reactivation, making the virus an important model in understanding virus-host interaction and mechanisms of immune evasion. HSV-1 undergoes extensive transcription during productive infection, generating nucleic structures, such as double stranded RNA (dsRNA), that are recognised by host immune sensors. These sensors initiate antiviral response restricting viral replication including interferon signalling and translational arrest.

Like many other viruses, HSV-1 has evolved multiple strategies to evade host immune response. Similarly, host cells employ regulatory mechanisms to prevent aberrant activation of immune sensors and to maintain homeostasis. Among these, adenosine deaminase acting on RNA (ADARs) regulate dsRNA mediated immune response through adenosine-to-inosine (A-to-I) editing and RNA binding. Depending on viral and cellular context, ADARs can exert either proviral or antiviral effects Although role of ADARs have been studies in several viral systems, their role during herpesviruses, particularly in productive HSV-1 infection remains incompletely understood. HSV-1 and the host maintain complex interplay with many interactions profoundly influencing both host as well as virus. Therefore, it is important to understand HSV-1 biology and its interactions with the host cells.

1.1. The biology and replication cycle of herpes simplex virus 1 (HSV-1)

Herpes simplex virus 1 (HSV-1) is a thoroughly studied human double stranded DNA (dsDNA) virus. It is known to cause cold sores, commonly in orolabial region. It is estimated that 40-80% of global population is infected with HSV-1 [1].

HSV-1 follows two distinct phases of infection, viz., productive (lytic) phase and latent phase. Virus predominantly infects oropharyngeal mucosa during lytic or productive infection. Gradually, it transmits to peripheral sensory neurons and establishes lifelong latent infection in trigeminal ganglia. Subclinical reactivation in infected individuals can result in asymptomatic shedding or symptomatic lesions. Although, primary infection often manifests as oral lesions, in some of the cases, infection upon reactivation can lead to ocular herpes or encephalitis which often results in high mortality [2].

1.1.1. Taxonomy and structure of HSV-1

Herpesviruses are frequently found in nature, with most of animals host at least one herpesvirus, often many. More than 200 herpesviruses infecting different species have been identified, of which 9 are known to infect humans. Members of the order *Herpesvirales* family have very distinguished morphological properties including spherical virion, an icosahedral capsid containing double stranded DNA (dsDNA) viral genome, surrounded by tegument and envelope containing glycoprotein spikes. The order is further classified into three families, *Orthoherpesviridae* (herpesviruses infecting mammals, birds and reptiles), *Alloherpesviridae* (herpesviruses of fish and amphibians) and *Malacoherpesviridae* (herpesviruses infecting molluscs). The family *Orthoherpesviridae* is additionally classified into three subfamilies: *Alpha*-, *Beta*-, and *Gammahespesvirinae*, based on host range, replication cycles and genomic structure [2].

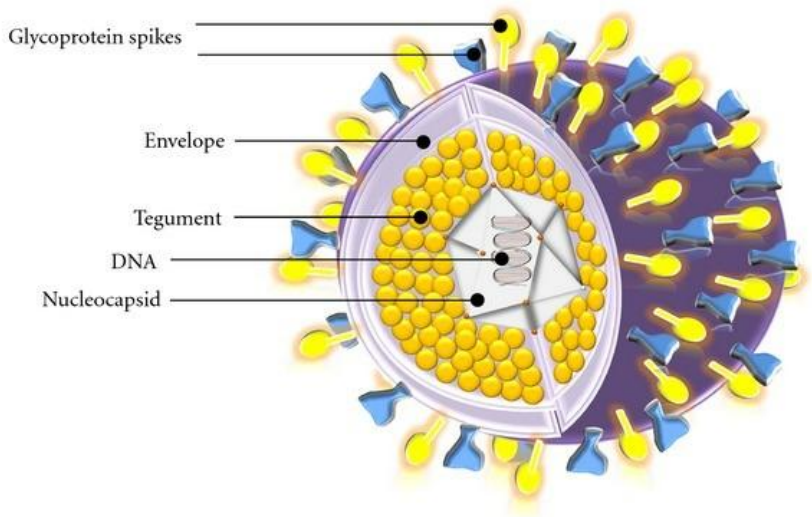


Figure 1. Schematic illustration of HSV-1 virion. Herpes simplex virus 1 virion showing icosahedral capsid containing genomic dsDNA, tegument with viral proteins and envelope with glycoproteins. Adapted from [3].

Of nine herpesviruses infecting humans, herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), and varicella-zoster virus (VZV) belong to *Alphaherpesvirinae*. Human cytomegalovirus (HCMV), human herpesviruses 6 (HHV-6A and HHV-6B) and Human herpesvirus 7 (HHV-7) classified in *Betaherpesvirinae*, whereas Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KHSV) are of *Gammaherspesvirinae* sub-family [2].

The size of mature HSV-1 virion is approximately 120-260nm [4]. The viral core contains linear double stranded DNA, enclosed in icosahedral capsid. The capsid is surrounded by tegument, a protein rich layer containing around 24 viral proteins, which assist initiation of viral replication upon entry [5]. The assembly is enclosed within host derived lipid envelope with embedded at least 12 different viral glycoproteins (gB - gN) mediating viral attachment and entry to the host cell (Fig. 1) [2], [6].

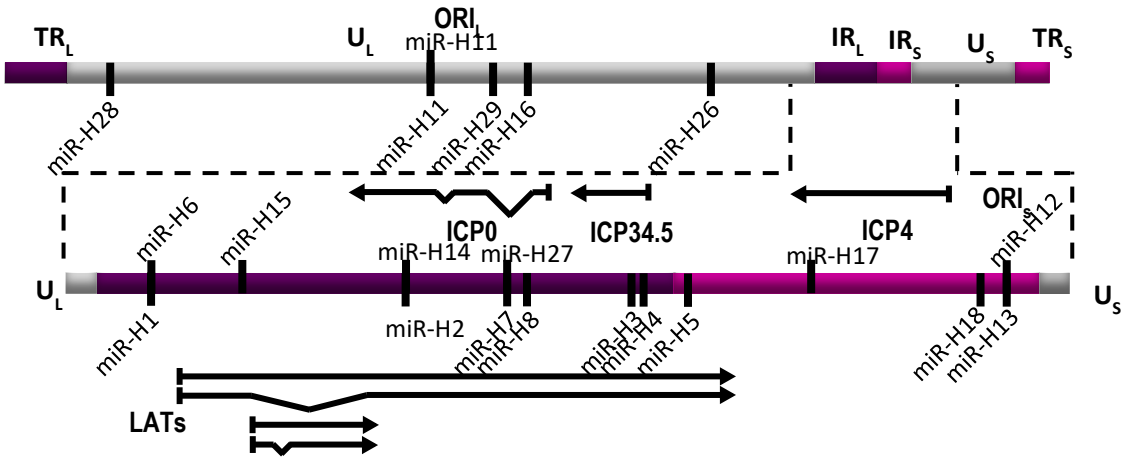


Figure 2. Structure of HSV-1 Genome. HSV-1 genome consists unique long (U_L) and unique short (U_S) regions flanked by terminal repeat (TR_L /TR_S) and internal repeat (IR_L /IR_S) sequences. The genome contains three origins of replication, oriL and two copies of oriS. Immediate early (IE) genes with selected miRNAs are shown, with arrows indicating direction of transcription. The genome encodes around 82 proteins and 18 miRNAs with numerous non-coding transcripts such as latency associated transcript (LAT) involved in involved in regulation of viral replication. Generated by *A. Zubković*.

The HSV-1 genome consists of ~152kb long double stranded DNA and has 68-70% GC rich content. It contains two covalently linked regions, unique long (U_L) and unique short (U_S). the unique region encodes proteins for DNA replication, capsid formation and host immune evasion. U_L is surrounded by a long terminal repeat region (TR_L) and long inverted internal repeat region (IR_L), while U_S is surrounded by a short terminal repeat region (TR_S) and short inverted internal repeat region (IR_S). Repeat regions are usually present in two copies and code for proteins to initiate viral replication and latency associated transcripts (LATs) and multiple viral microRNA (miRNA). Both unique (U_L) and repeat regions (IR_S and TR_S) contain replication origins, oriL and two copies of oriS, respectively. Overall HSV-1 genome encodes approximately 82 proteins and number of non-coding transcripts including 18 miRNA (Fig. 2) [2], [6].

1.1.2. HSV-1 replication cycle

The initial site of HSV-1 infection is usually epithelial cells. With the help of viral glycoproteins B and C (gB and gC), virion binds to heparin sulphate proteoglycans (HSPGs) on the cell surface. Further engagement of cellular receptors such as nectin-1, nectin-2, or herpesvirus entry mediator (HVEM) by glycoprotein gD triggers a cascade involving gH/gL and activation of the fusogenic glycoprotein gB. The fusion of virion membrane with cell membrane releases tegument proteins and viral capsid in cytoplasm. The viral capsid is docked to nuclear pores and viral DNA is injected in the nucleus [2], [7].

The viral gene expression occurs in regulated cascade of gene expression. Firstly, immediate-early (IE or α) genes are transcribed, followed by early (E or β) and late (L or γ) genes. Viral tegument protein (VP16) initiates gene expression by creating complex with host proteins Oct-1 (Octamer binding protein 1) and HCF-1 (host cell factor 1). Parallelly, another tegument protein virion host shutoff (vhs or UL41) carries out degradation of host mRNAs and primes the host translational machinery for viral protein production. IE proteins such as ICP4, ICP0 and ICP27 (ICP: infected cell protein) act as transcriptional regulators inducing massive transcription of viral genes as well as modulators of host antiviral immunity. These proteins are essential for efficient transcription of E and L genes. E genes are predominantly involved in viral DNA replication. ICP8, a single stranded DNA binding protein and DNA polymerase UL30, along with UL42 and components of the helicase-primase complex such as UL5 and UL8 are required for DNA synthesis. ICP8 also contributes to transition from early to late phase of infection. L genes encode structural components required for virion assembly such as glycoproteins gC and gD, capsid proteins such as VP5 and tegument proteins such as VP22. The assembled viral particle is transported to the cell membrane and released (Fig. 3) [2], [6], [7]. This tightly regulated cascade results in extensive viral transcription producing large amounts of viral RNA within infected cell [8].

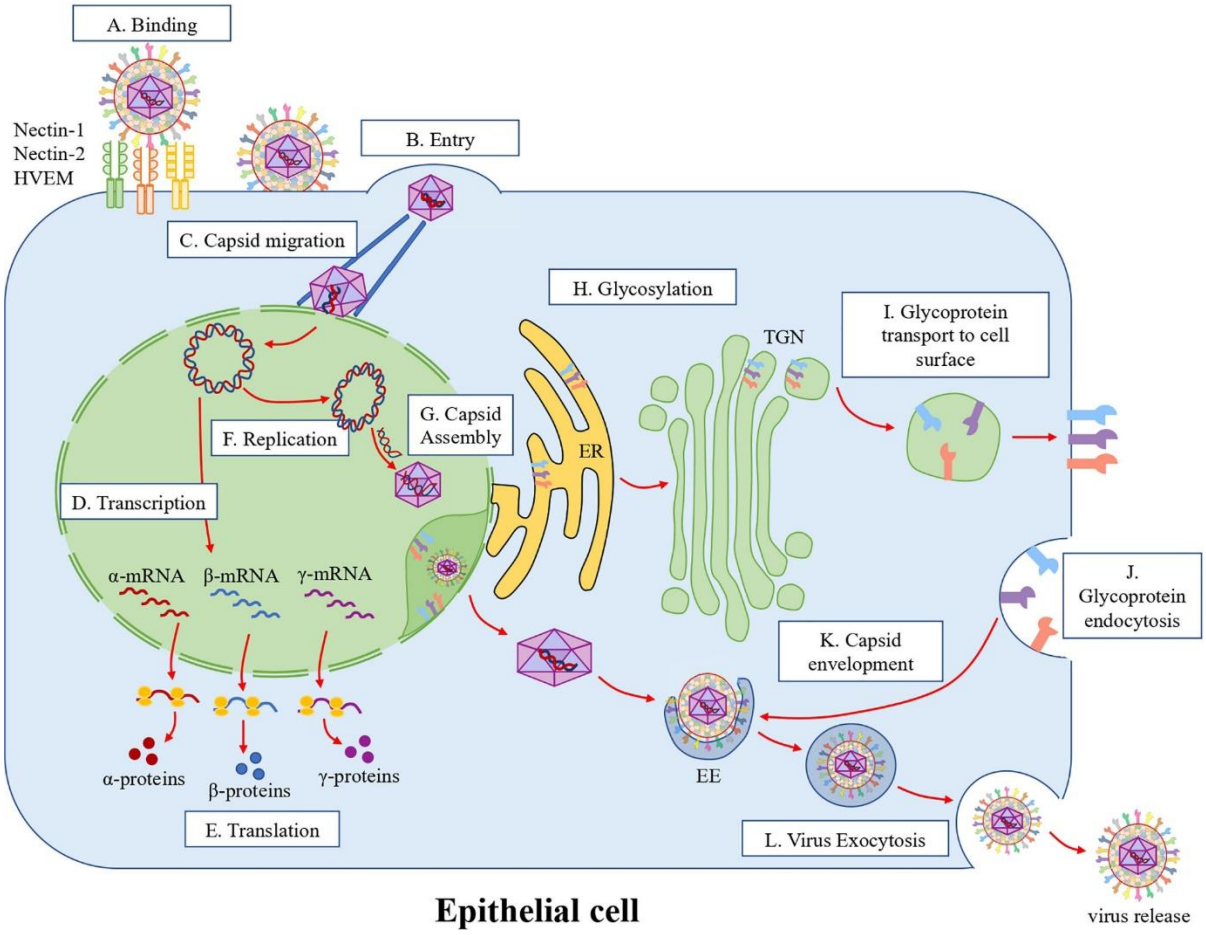


Figure 3. Productive (lytic) replication cycle of HSV-1. The epithelial cell is a usual site of infection. Virion attaches to specific receptor on cell surface (A: binding) and enters the cell by fusion or endocytosis (B: entry). The capsid migrates to nuclear pore releasing viral DNA into nucleus (C: Capsid migration). The cascade of viral gene expression and DNA replication occurs in stages (first immediate early α-mRNA/proteins, then early β- and leading to replication of viral DNA and late γ-gene expression)(D. Transcription, E. translation, F Replication). Assembled capsid is enveloped with surface glycoprotein and released by exocytosis (G-L: Virus exocytosis). Adapted from [7].

Following primary infection in epithelial cells, HSV-1 enters sensory nerves and travels to sensory trigeminal ganglia (TGs) by retrograde transport, where it establishes latent phase of infection. However, exact molecular mechanism of HSV-1 latency is not well understood [2]. During latency, viral DNA takes circular episomal form and a limited number of transcripts are transcribed. One of the highly expressed gene is latency associated transcript (LAT), which originates in repeat region of HSV-1 genome. LAT gives rise to an unstable 8.3kb long primary transcript which upon processing generates 1.5kb and 2kb stable introns and a number of miRNAs. The exact function of these transcripts is not yet fully defined; however, it is known to regulate reactivation, IE gene transcription and inhibit apoptosis [9]. Notably, several miRNAs expressed in

latency, including LAT derived miRNAs, are complementary to IE gene transcripts which regulating initial stages of productive infection, suggesting the role in maintaining latency and supressing reactivation (Fig. 2) [10]. One of the well characterised examples is miRNA-H2, derived from LAT transcripts, is antisense to a key IE gene ICP0. miR-H2 has been shown to inhibit expression of ICP0, a protein which promotes viral transcription activation [11]. Importantly, viral miRNA such as miRNA-H2 are also subject to post transcriptional modification, including adenosine-to-inosine (A-to-I) editing mediated by host enzyme ADAR. Such modifications alter miRNA targeting potential and functional outcomes, highlighting host involvement in regulation of viral replication [12].

A key distinction between productive and lytic infection is extent of viral transcription, protein output and generation of viral progeny. Though transcription in restricted in latency, productive infection is characterised by generation of large amounts of RNA. These transcripts are often overlapping and form complex secondary structures such as double stranded RNA (dsRNA) [8], [13], [14]. Similar to latency, transcript produced during productive infection are also subject to host intervention. Evidence suggest that A-to-I editing occurs in productive infection, albeit at lower frequency than in latency; however, A-to-I editing represents most abundant type of RNA editing in viral transcripts [12]. These diverse and abundant range of viral RNAs represent a key molecular signature for recognition by host antiviral immunity.

1.2. Host response to HSV-1 infection

The host has evolved several distinct mechanisms to recognise viral infection at different stages. Specialised immune cells, such as macrophages and dendritic cells, detect viral infection and contribute to the coordination of innate and adaptive immune response. However, individual host cells also possess intrinsic defence mechanism that enables them to sense viral components in both extracellular and cytosolic compartments. These intrinsic immune response by host cells is broadly categorised under innate immunity and plays a critical role in early detection and restriction of viral infection [6], [15].

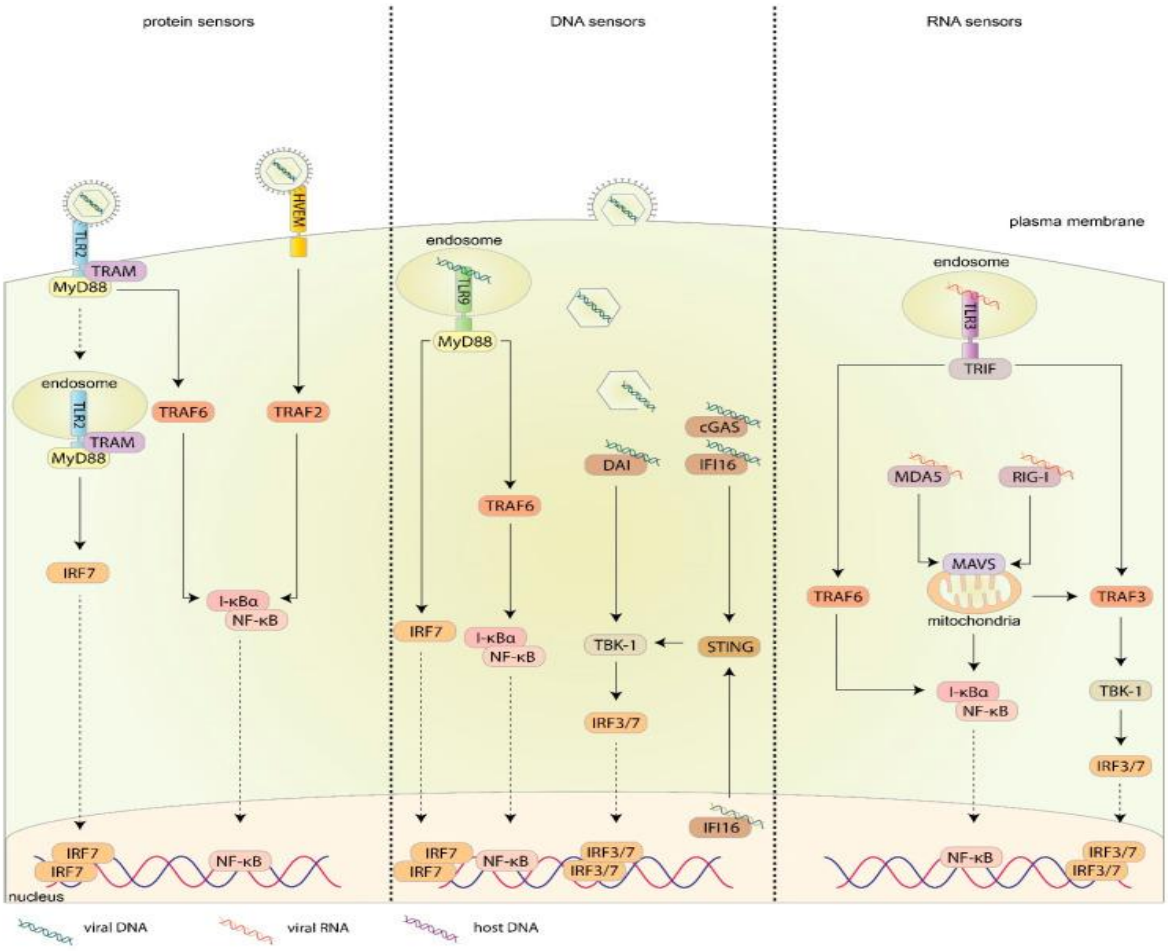


Figure 4. Recognition of HSV-1 by extracellular and cytosolic sensors. Illustration of recognition of different classes of PAMPs, such as viral protein, viral DNA and viral RNA by host sensors. Extracellular and endosomal sensors detect viral glycoproteins and nucleic acids, whereas cytosolic sensors detect viral DNA and RNA generated during infection. Pattern recognition receptors involved in HSV-1 sensing include TLRs, RLRs, cGAS, and FI16. Activation of these sensors induces downstream inflammatory response. Adapted from [16].

1.2.1. Extracellular and endosomal viral detection and response by TLRs

The antiviral response to HSV-1 infection is initiated by cellular sensing systems that recognise viral molecules commonly referred as pathogen-associated molecular patterns (PAMPs). The sensors which recognise PAMPs are known as pattern recognition receptors (PRRs) which initiate downstream signalling upon sensing PAMPs and orchestrate appropriate immune response. Among those, important group of pattern recognition receptors are toll-like receptors (TLRs) in extracellular or non-cytosolic compartments [15], [16].

TLRs is a class of transmembrane proteins present on plasma membrane as well as within endosomal membrane. They recognise broad spectrum of PAMPs including viral proteins, DNA and RNA [17]. Until now twelve TLRs have been identifies in mammals, however TLR2, TLR3 and TLR9 have been shown to have particular importance in HSV-1 infection [16].

TLR2, present on both plasma membrane and endosomal membrane, detects viral glycoproteins, enabling host cell to detect intact virion. Upon ligand binding, TLR2 signalling cascade is initiated by recruiting myeloid differentiation factor 88 (MyD88) and tumour necrosis factor receptor associated factor 6 (TRAF6). This upstream signal induces degradation of NF-κB inhibitor α (I-κBα) which allows nuclear factor-κB (NF-κB) to translocate to nucleus and induce several pro-inflammatory cytokines. Additionally, endosomal translocation of TLR2 in complex with TRIF-related adaptor molecule (TRAM) and MyD88 can signal through IFN regulatory factor 7 (IRF7) to induce Type I Interferon (IFN I) production (Fig. 4) [16].

Viral DNA present in endosome is recognised by another TLR transmembrane protein TLR9. HSV-1 DNA contains abundant un-methylated CpG motifs detected by TLR9. Similar to TLR2, TLR9 signals through MyD88-TRAF6 to induce NF-κB as well as IRF7 mediated IFN I response. It has been demonstrated that TLR9 and TLR2 work in conjugation to control HSV-1 infection particularly in mucosal tissues (Fig. 4) [16], [18], [19].

TLR3 also an endosomal transmembrane sensor recognises double stranded RNA (dsRNA) accumulated during viral replication. Primarily, TLR3 signalling cascade is MyD88 independent Toll/interleukin-1 receptor-like (TIR) domain-containing adaptor inducing interferon-β (TRIF) and TRAF3 resulting in IRF3/7 mediated IFN I production. Additionally, it can engage through TRAF6 for NF-κB activation similar to TLR2 and TLR9 (Fig. 4) [16]. However, it has not been definitively established exact HSV-1 ligand for TLR3 mediated signalling activation, though it is presumed to be viral dsRNA as they are generated during productive HSV-1 infection [17], [18].

While TLRs play important role in early detection of HSV-1 during viral entry, infected host cells predominantly rely on cytosolic sensing mechanisms in cooperation with TLRs for effective immune response [19].

1.2.2. Cytosolic viral detection and response by RLRs

A major trigger for detection of HSV-1 infection appears to be viral nucleic acid including dsDNA and dsRNA [20]. These nucleic acids are detected by different cytosolic sensors such as retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) [21].

Retinoic acid-inducible gene-I (RIG-I) is viral dsRNA sensor that recognises short dsRNA, typically less than 300bp, with a 5'-triphosphate group. In addition, RIG-I can indirectly sense foreign cytosolic DNA using intermediary system of RNA polymerase III (RNApol III) transcription of DNA into RNA intermediates. Upon binding to ligand, it transmits signal to downstream adaptor protein mitochondrial antiviral-signalling protein (MAVS) leading to activation of NF-κB and IRF3/7 via TANK-binding kinase 1 (TBK1) inducing pro-inflammatory response (Fig. 4) [16], [22], [23].

Melanoma Differentiation-Associated gene 5 (MDA5) is another member of the RLR familythe that recognises long viral dsRNA exceeding 2000bp. Upon activation, MDA5 signals through MAVS, similar to RIG-I, resulting in pro-inflammatory response during HSV-1 infection (Fig. 4) [21], [22], [23]. Laboratory of genetics and physiology 2 (LGP2), another member of RLR family, functions as regulatory protein that promotes MDA5-dsRNA interaction [24].

Apart from RLRs, other cytosolic sensors such as protein kinase R (PKR), which detects dsRNA, and a DNA sensor cyclic GMP-AMP synthase (cGAS) can induce strong antiviral response through complementary but distinct pathways [16].

1.2.3. Cytosolic DNA sensing and associated pathways

Upon successful entry in the host cell, viral capsid is transported to nucleus where it releases viral genomic DNA. During this process, viral DNA from incoming capsids or aberrant DNA present in cytoplasm can be detected by host cytosolic DNA sensors. DNA from degraded capsids during this transport as well as released nuclear DNA can be sensed by Interferon Gamma Inducible Protein 16 (IFI16). Cyclic GMP-AMP synthase (cGAS), another DNA sensor, also binds to cytosolic viral DNA. Both IFI16 and cGAS activate stimulator of interferon genes (STING) pathway, resulting in

activation of IRF3/7 and induction of IFN I response. Though precise ligand for cGAS remains under investigation, it is speculated that cGAS rather detects mitochondrial DNA released during HSV-1 infection (Fig. 4) [16], [23].

Z-DNA binding protein 1 (ZBP1), also known as DNA-dependent activator of interferon-regulatory factors (DAI) signals through TBK1 and STING to IRF3/7, as well as NF-κB, initiating inflammatory response (Fig. 4) [16]. Additionally, ZBP1 can initiate necroptosis by signalling through receptor-interacting serine/threonine-protein kinase 3 (RIPK3). Notably, recent report suggests that ZBP1 mediated necroptosis upon HSV-1 infection relies on nascent viral RNA transcripts rather than viral DNA, highlighting importance of viral RNA in host immune response [25], [26].

1.2.4. PKR mediated antiviral response

Protein kinase R (PKR), a double stranded RNA-dependent serine/threonine kinase is a key component in innate immune response. As dsRNA represents a critical PAMP generated during viral infection, PKR occupies a central role in detecting viral RNA and initiating array of immune response. Although PKR is interferon stimulated gene, many cell types express PKR constitutively enabling rapid response upon dsRNA detection [27], [28].

A range of dsRNA structures, originating from viral transcription as well as endogenous cellular transcripts, can be detected by PKR. Typically, RNA of a minimum length of 79bp forming double stranded structure of ~33bp is required for PKR activation [29], although some studies reporting as short as 30bp [30]. However, small interfering RNA (siRNA) duplexes as short as 19-21bp can also activate PKR. Though PKR is primarily considered as dsRNA binding protein, it can also detect single stranded RNA with very specific structures [31].

Notably, PKR can be regulated by multiple cellular proteins through protein-protein interaction, with one of the most well characterised examples being Protein Activator of PKR (PACT). Under stress conditions in host cell, often due to viral infection, PACT interaction with PKR is enhanced resulting in PKR activation [32]. However, in certain context PACT acts as inhibitor of PKR activation rather than inducer, highlighting its role as regulatory protein of PKR [33].

In response to dsRNA or other stimuli, PKR undergoes dimerization followed by autophosphorylation. Though PKR phosphorylation can occur at multiple residues, phosphorylation at threonine 446 (Thr446) is critical for substrate recognition to initiate downstream signalling [34], [35]. Activated PKR phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α) at serine 51 (Ser51) residue. This modification consequently inhibits formation of protein complex required for delivery of methionyl-tRNA (Met-tRNAi) to ribosome, effectively terminating translation. However, under these conditions selective transcripts such as activating transcription factor 4 (ATF-4) are enhanced making them excellent marker of cellular stress and translational arrest (Fig. 5) [35].

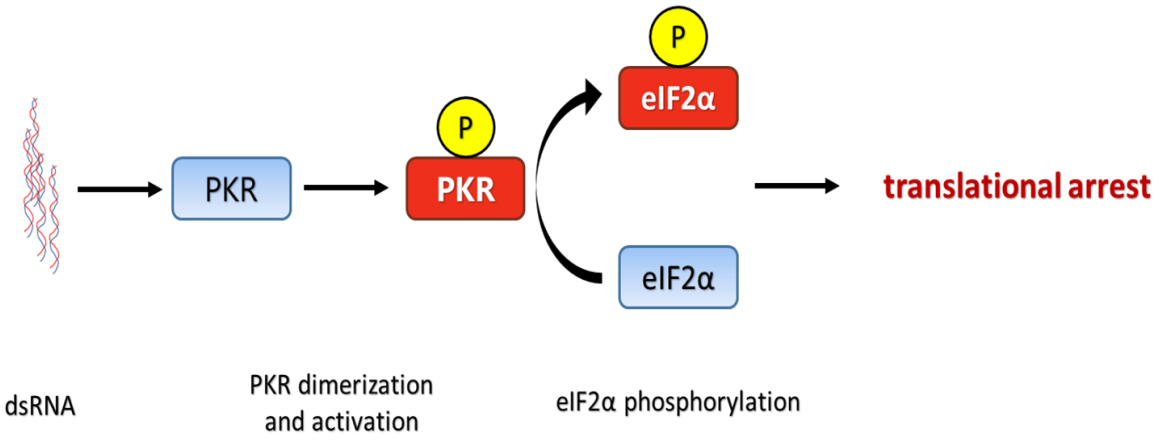


Figure 5. PKR mediated dsRNA sensing. Double stranded RNA binds and induces PKR dimerization and autophosphorylation. Activated PKR phosphorylates eIF2α which leads to translational arrest, interferon response and stress granule formation. Adapted from [27].

PKR mediated eIF2α phosphorylation also promotes formation of cytoplasmic stress granules (SG), a structure containing stalled transcripts, initiation factors and regulatory proteins. The structure maintains transcripts in translationally inactive state further reinforcing translational arrest [36]. Recent studies have also suggested novel mechanism of PKR activation via dsRNA induced condensates termed as dsRNA-induced foci (dRIFs). dRIFs form distinct assembly with PKR with several other dsRNA binding proteins such as PACT and ADAR1, enhancing sensitivity of PKR activation [37].

PKR also interacts with pathway components of multiple other sensors. One of the notable examples is its association with TLR3, an endosomal dsRNA sensor in TLR

family which activates TRIF dependent signalling. PKR is recruited to the downstream signalling complex of TLR3 together with TAK1 and TRAF6 contributing to activation of NF-κB [38]. In the context of MDA5, catalytic activity of PKR has been shown to be required for MDA5 mediated IFN I production [39]. Furthermore, several studies have demonstrated interaction between PKR and IκB kinase complex (IKK complex), leading to activation of NF-κB signalling which is central to many TLR and RLR pathways. Consequently, PKR not only mediates translational arrest but also contributes to stress response and production of inflammatory cytokines, enhancing the immune response [35].

The activity of PKR is closely interconnected with other cytosolic RNA sensors. Circular RNAs (circRNAs) derived from both host cell and viruses tend to form imperfect duplexes. Interestingly, these structure act as inhibitors of PKR activation. These circRNAs are degraded by RNase L, thereby restoring PKR activity [40]. Oligoadenylate synthase (OAS) proteins, cytosolic sensors of dsRNAs, act as upstream activator of RNase L and promote degradation of circRNAs [41], thereby indirectly facilitating PKR activity [34].

HSV-1 is particularly susceptible for PKR mediated immune response, as multiple studies have demonstrated that infection leads to accumulation of substantial amounts of dsRNAs [8], [14]. These dsRNAs can directly activate PKR as a part of host antiviral response. In addition, PKR can also be activated indirectly through other pathways downstream of IFN I induction, given PKR itself is interferon inducible gene. However, constitutive expression of PKR likely contributes significantly to early antiviral response to HSV-1 infection [34], [42].

To counteract such host defence mechanisms, HSV-1 has evolved multiple strategies to inhibit immune sensors [42]. Of note, as PKR can also be activated by endogenous cellular RNA structures, host cell also possesses regulatory mechanisms to prevent aberrant activation such as adenosine deaminase acting on RNA (ADAR) [43].

1.3. Evasion of host immunity by HSV-1

Despite the presence of robust and often redundant host antiviral defence mechanisms, viruses are able to replicate by employing range of evasion strategies to escape the innate immune response. HSV-1, in particular, efficiently counteracts host antiviral pathways, including key sensors such as PKR and interferon signalling complexes, through action of multiple viral proteins. Such strategies enable virus to establish productive infection and successfully complete its replication cycle within host cell.

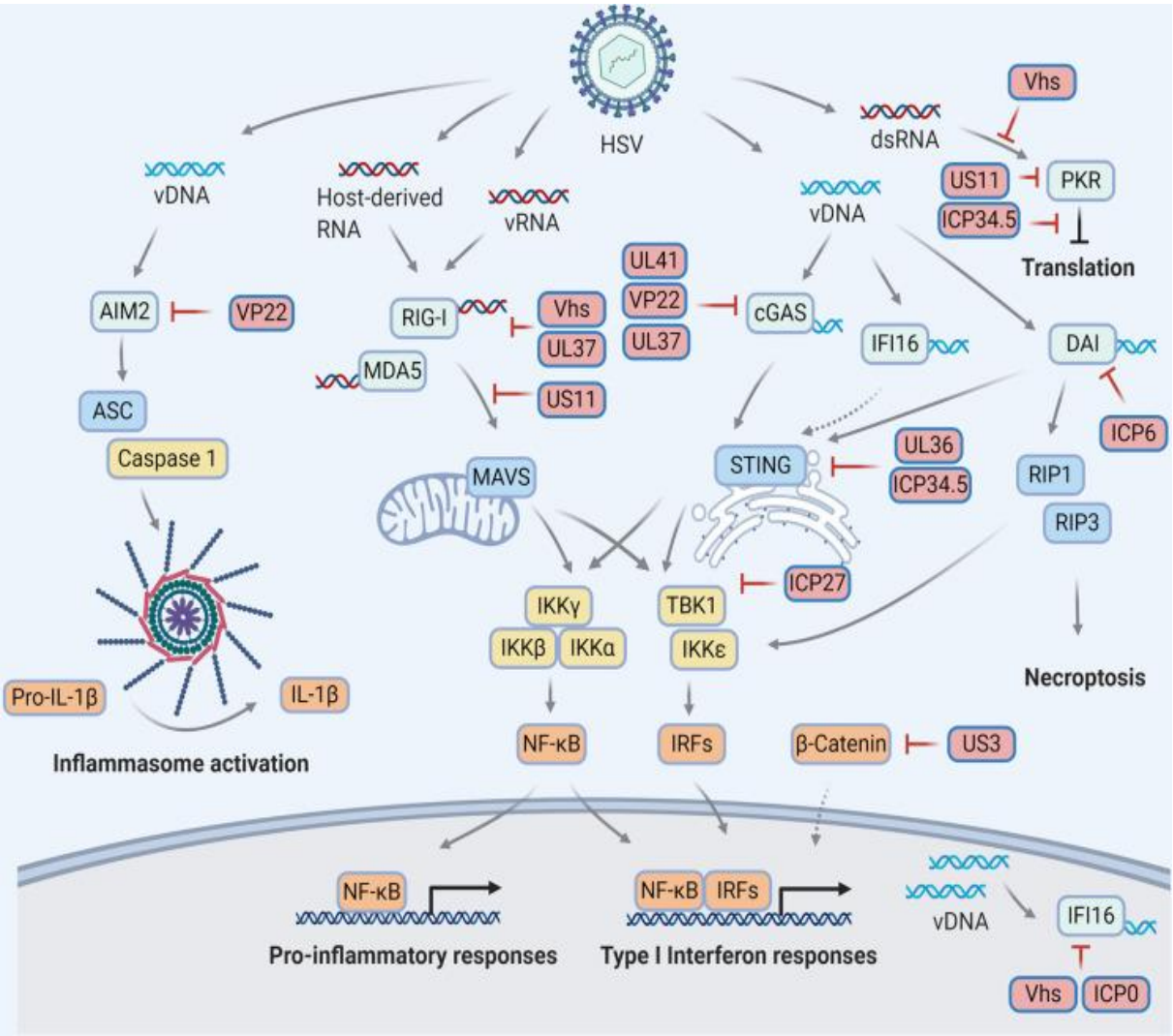


Figure 6. Evasion of host immunity. HSV-1 infection triggers activation of multiple cytosolic pattern recognition receptors (PRRs) and sensors initiating host antiviral immune responses including cytokine production, inflammasome activation, translational inhibition, and necroptosis. HSV-1 encodes multiple proteins including, ICP0, ICP27, ICP34.5, UL36. UL41/vhs, US3 and US11, which redundantly inhibit these immune pathways activated by sensors and the downstream effectors, collectively facilitating viral replication. Adapted from [44].

1.3.1. Inhibition of TLR and RLR pathways

TLR3, a dsRNA sensor, is inhibited by HSV-1 protein US3 by downregulating the expression of TLR3 [45]. US3 also targets TRAF6, downstream effector of multiple TLR pathways such as TLR3, TLR9 and TLR1 [46]. MyD88 a key adaptor molecule of multiple TLR signalling pathways is reduced by immediate early protein ICP0 (Fig. 6) [47]. Together, these observations suggest that HSV-1 effectively suppress TLR mediated immune response.

HSV-1 directly targets RLR receptor molecules. The helicase domain of RIG-I is modified by a tegument protein deamidase, UL37, blocking its ability to trigger immune response [48]. Additionally, dsRNA binding viral tegument protein US11 directly interacts with both RIG-I and MDA5 inhibiting their association with MAVS, a downstream effector, disrupting RLR signalling (Fig. 6) [49]. Though HSV-1 genome lacks features typically required for binding RLR like RNA viruses, the dsRNA generated during productive infection warrants counteraction of RNA sensing pathways, underlining the importance of dsRNAs as well as host sensors in HSV-1 replication and host regulation [44].

Interestingly, while HSV-1 actively represses RLRs during productive infection, it strategically exploits same pathway during latency. Small non-coding RNA (sncRNA) arising from LAT activate RIG-I, to induce IFN I and NF-κB, promoting viral establishment and latency in neuronal cells. This striking contrast highlights distinct biological requirements of the different phases of viral life cycle and ability of virus to reprogram the host response to its advantage [50].

1.3.2. Inhibition cytosolic nucleic acid sensors

Apart from countering TLR and RLR pathways, HSV-1 also inhibits other cytosolic nucleic acid sensors that detect viral DNA and RNA.

The cytosolic DNA sensor IFI16 is specifically targeted for proteasomic degradation by HSV-1 IE protein ICP0, particularly at later stages in infection [51]. Similarly, cGAS is antagonised by multiple HSV-1 proteins. UL41 downregulates cGAS mRNA levels,

while VP22 directly inhibits its enzymatic activity. Similar to RIG-I inhibition, UL37 also targets cGAS by deamidation (Fig. 6) [44].

ZBP1, a cytosolic DNA sensor that induces necroptosis by forming complex with RIPK3. A viral ribonucleotide reductase ICP6, disturbs the formation of ZBP1-RIPK3-MLKL complex, known as necrosome, preventing host cell from inducing necroptosis [52]. Similarly, viral proteins also act on common downstream signalling adaptor proteins to achieve broader immune suppression; a prominent example is multifunctional IE protein ICP27, which targets TBK1-STING signalosome to inhibit IFN I production (Fig. 6) [53]. Collectively, these findings reveal the multi-layered and aggressive evasion strategies employed by HSV-1 to dismantle host antiviral defences.

1.3.3. Inhibition of PKR mediated host response

PKR plays a crucial role in innate immune response by detecting broad range of dsRNA structures derived from both virus and the host. Its ability to induce translational shutdown in cell poses a significant threat to not only a viral replication but also the host cell itself under certain circumstances. The impact of PKR is further amplified as it is an interferon stimulated gene also indirectly contributing to interferon production generating positive feedback loop. Accordingly, numerous DNA and RNA viruses encode antagonists targeting PKR directly as well as downstream signalling effectors further solidifying potency of its antiviral function [28], [34].

US11, a late protein of HSV-1 present in tegument, plays major role in the direct inhibition of PKR. As both US11 and PKR are dsRNA binding proteins, their interaction largely RNA-dependant. US11 inhibits PKR either by sequestering dsRNA or by directly binding to PKR and preventing its autophosphorylation and subsequent phosphorylation of eIF2α [54], [55]. Additionally, the interaction between PKR with US11 also inhibits PKR activation by PACT [56].

HSV-1 deploys additional proteins to counteract consequences of PKR activation. Phosphorylation of eIF2α by activated PKR prevents translation in cell. lCP34.5 interacts with protein phosphatase 1 (PP1) redirecting it to dephosphorylate eIF2α alleviating effects of PKR activation [57]. Another example of nullifying effect of PKR

is virion host shutoff protein (vhs), a tegument protein with RNase activity, which promotes selective degradation of host mRNAs [58]. However, vhs have been shown to reduce dsRNA accumulation within infected cell, effectively regulating PKR and other RNA sensors which limits host immune response [59]. Together, these mechanisms highlight HSV-1 not only inhibits PKR activation but also mitigates its downstream effect to sustain immune response occurred due to high dsRNA accumulation during its replication (Fig. 6).

Additionally, HSV-1 proteins US11 and ICP34.5 are also known to inhibit other nucleic acid sensing pathways. US11 interacts with RNA sensors RIG-I and MDA5 inhibiting their interaction with MAVS, whereas ICP34.5 targets TBK1 and STING, effectors downstream to multiple nucleic acid sensors (Fig. 6) [16]. These observations reiterate necessity of countering RNA sensor activities for efficient replication and survival.

In addition to viral derived dsRNA, host cells are also susceptible to unintended activation of immune response, driven by cytosolic sensors such as PKR or RLRs, due to presence of endogenous RNA structures arising from sources including Alu repeats or non-coding transcripts. To prevent aberrant activation of immunity, one such mechanism involves adenosine deaminase acting on RNA (ADAR) mediated adenosine-to-inosine (A-to-I) deamination, which modifies and masks endogenous RNA reducing its immunogenic potential [60]. Notably, A-to-I modifications have been observed in HSV-1 transcripts [12], suggesting a broader role of such RNA alterations and ADAR in modulating virus-host interaction.

1.4. ADAR mediated regulation of dsRNA and innate immunity

The pattern recognition receptors (PRRs) such as PKR and their activation needs to be tightly regulated to prevent aberrant triggering, as certain endogenous RNA structures, including those derived from repetitive elements and mitochondrial transcripts resemble to viral RNA [61]. Uncontrolled activation of these pathways can induce severe autoimmune responses. This raises the fundamental question about ability of cell to distinguish between self and foreign RNA. A critical process involved in this distinguishing is RNA editing. Broadly, two major forms of RNA editing by deamination occurs. The conversion of cytosines to uracil is carried out by the

apolipoprotein B mRNA editing enzyme catalytic subunit (APOBEC) and the deamination of adenosine to inosine (A-to-I editing) catalysed by adenosine deaminase acting on RNA (ADAR) [62].

1.4.1. The ADAR protein family

Adenosine deaminase acting on RNA (ADAR) enzymes were first discovered in *Xenopus laevis* by observing apparent unwinding activity on dsRNA. Originally ADARs were named as unwindase, where subsequent studies discovering that RNA was not unwinded but rather modified through by A-to-I conversion, which destabilized dsRNA structure to become more single stranded. Based on the activity and substrate, enzyme names were standardised as ADAR [63], [64]. Further studies discovered three ADAR genes that are present in mammals. ADAR1 encoded by gene ADAR, ADAR2 encoded by gene ADARB1 and ADAR3 encoded by ADARB2 [65].

ADAR1 is ubiquitously expressed across cell types in two major isoforms. The ~110kDa isoform (p110) is constitutively expressed, whereas the ~150kDa isoform (p150) is inducible by interferon. These two forms of ADAR1 are generated from alternative exon structure controlled by different promoters. The Induction of p150 occurs via Janus kinase-signal transducers (JAK)-signal transducer and activator of transcription (STAT) pathway. The interferon inducible p150 promoter, PiA, is activated by STAT1-STAT2-IRF9 transcription activator complex [66]. The PiA promoter contains a kinase-conserved-like element initially identified in PKR promoter that enhances transcriptional activities, suggesting shared regulatory mechanisms between ADAR1p150 and PKR expression [67].

ADAR1 contains three dsRNA binding domains (dsRBDs) and a catalytic deaminase domain. The p150 isoform possesses two Z-DNA binding domains (Zα and Zβ), while p110 is a truncated version of p150 having single Z-DNA binding (Zβ) domain. The N-terminus Z-DNA binding domain Zα contains a nuclear export signal (NES) resulting in predominantly cytoplasmic location of p150 isoform, however nuclear localization signal (NLS) is present in dsRNA binding domain III present in both isoforms, which contributes to nuclear localization of p110 (Fig. 7) [62]. ADAR1 is expressed in higher

levels than other ADAR family members therefore making it responsible for most of the editing activity in cells [68].

ADAR2 contains two double stranded RNA binding (dsRBD) domains and a catalytic deaminase domain. It is highly expressed in brain tissues but also present in other cell types. Whereas, expression of ADAR3 is restricted to the brain. Although ADAR3 is structurally similar to ADAR2, with a key difference is that it lacks in deaminase activity, therefore considered to function as a negative regulator of RNA editing. Both ADAR2 and ADAR3 contain nuclear localization signal (NLS) and predominantly localized in the nucleus (Fig. 7) [69], [70] .

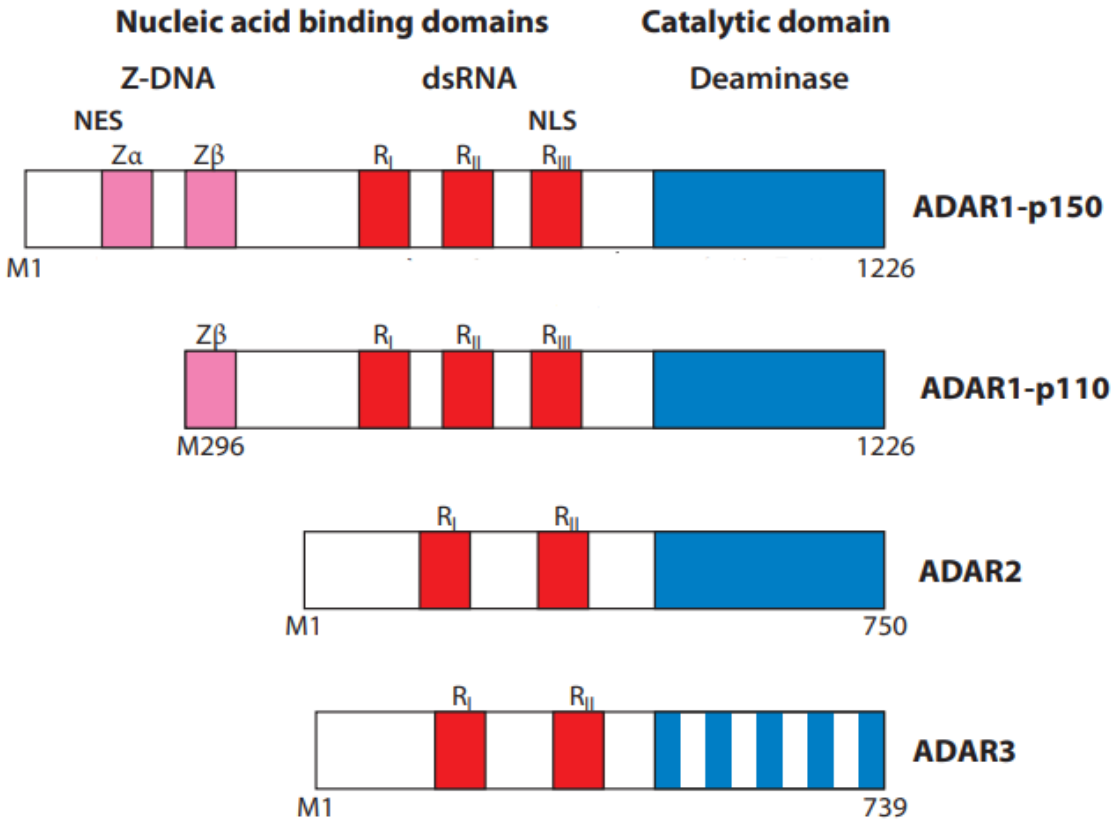


Figure 7. Schematic representation of ADAR proteins. Three members of ADAR protein family are present in humans. ADAR1 exists in two major isoforms p110 and p150, which differ in their N-terminal region and cellular localization. ADAR proteins contain dsRNA binding domains (dsRBD: RI, RIII and RIII) and catalytic domain for A-to-I RNA editing. Z-DNA/RNA binding domain (Zα/β) is unique to ADAR1. ADAR2 and ADAR3 lack Z-DNA binding domain, and catalytic deaminase domain of ADAR3 is inactive. NLS denote nuclear localization signal and NES denotes nuclear export signal. M1 and M296 indicate alternative translation initiation sites of p150 and p110 isoforms of ADAR1, respectively. Adapted from [71].

1.4.2. A-to-I RNA editing

The A-to-I editing activity is widely present in eukaryotic cells. Both intermolecular and intramolecular dsRNA of more than 20bp can serve as substrates for ADAR, including double stranded regions such as stems and loops. In longer dsRNA molecules, more than 100bp in length, up to ~50% of adenosines are converted in inosine residues [72]. Some reports suggest that homodimerization of ADAR is required for efficient editing activity, likely mediated by protein-protein interaction between two monomers [73].

Upon binding to its substrate, ADAR catalyses hydrolytic deamination of adenosine residue at sixth carbon (C6) position occurs releasing ammonia and converting adenosine residue into inosine residue (Fig. 8) [74]. The resulting inosine base pairs

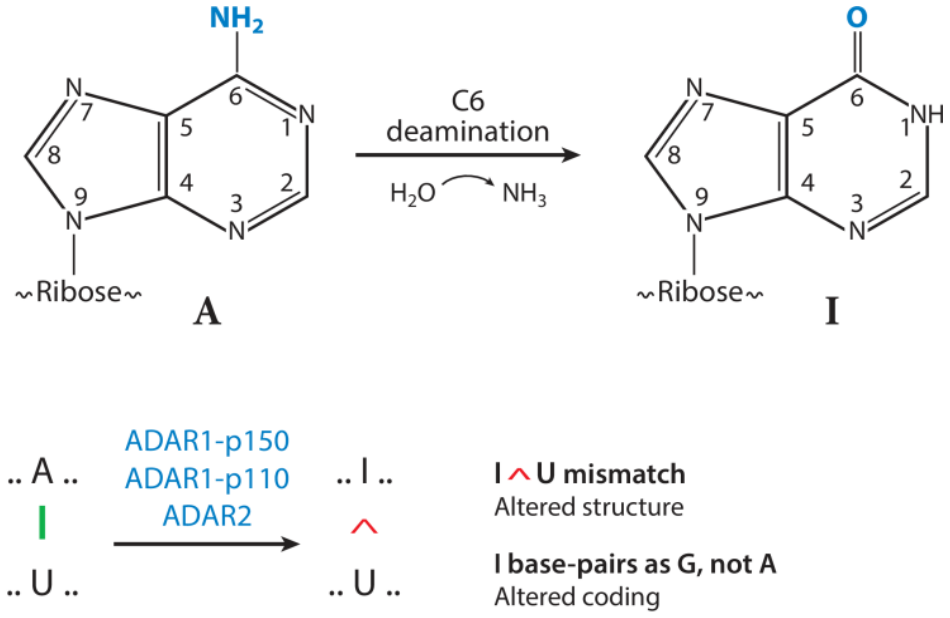


Figure 8. A-to-I editing. ADAR1 and ADAR2 convert adenosine to inosine by deamination at C6 position. This conversion creates a mismatch in the structure. Adapted from [71].

similarly as guanosine, and it is recognised as such by translational machinery, thereby altering RNA structure and functional potential [70].

In the case of mRNA, editing often results in protein diversification through co-existence of proteins from both edited as well as unedited transcripts. A functionally

significant example is observed in glutamate receptor (GluR) ion channel subunit. ADAR2 mediated A-to-I converts codon CAG coding for glutamine (Q) to CIG (read as CGG) coding arginine (R) (known as the Q/R site) making channel impermissible to calcium ions expanding functional diversity of receptors [75]. Importantly, ADAR2 deficiency is lethal in mice, and only introduction of a genomic Q/R point mutation is sufficient to rescue the phenotype [76]. This highlight the influence of site-specific RNA editing that can modulate cellular physiology (Fig. 9).

Beyond editing in coding regions, ADAR enzymes are involved in multiple functions that influence RNA metabolism. The editing regulates pre-mRNA splicing by modifying conserved adenosines in splice site sequence. In context of RNA viruses, ADAR mediated modifications influence genome stability and RNA products during replication. Additionally, effects of editing on miRNA biogenesis and targeting have been widely reported (Fig. 9) [77].

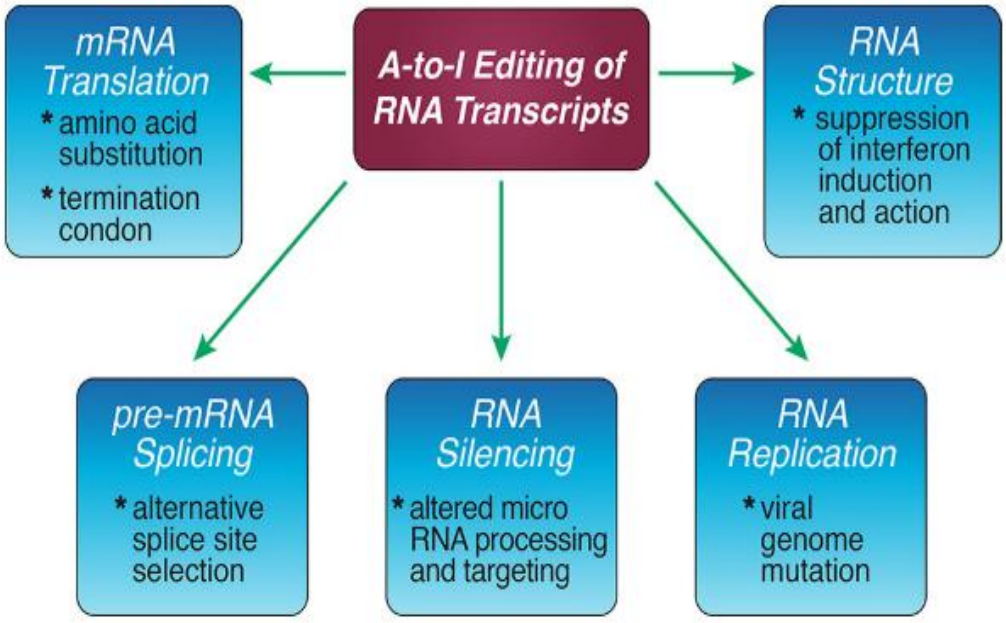


Figure 9. Consequences of A-to-I editing. Editing of RNA transcripts with double stranded structure affects array of biological functions including stability and translation. Adapted from [77].

However, one of the most consequential function of ADAR enzymes is editing of repetitive elements, particularly Alu sequences, which form intramolecular dsRNA

structures. Approximately 15000 editing sites identified in humans across ~2000 different genes are located within inversely oriented repetitive Alu elements. It has been estimated that more than 85% of pre-mRNA within intronic and UTR regions may be edited by ADARs [78].

Loss of A-to-I editing has severe implications on organism's growth and development as well as maintaining cellular homeostasis. For instance, insufficient Q/R site editing in GluR subunit is associated with multiple neurological disorders such as amyotrophic lateral sclerosis (ALS) and linked to glioblastoma proliferation, due to excessive calcium ion (Ca^{2+}) influx. Similarly, inactive ADAR1 results into embryonic lethality in mice due to aberrant inactivation of immune system [70]. Perhaps the most compelling example highlighting importance of ADAR1 in immune regulation is Aicardi-Goutières syndrome (AGS). AGS is an autoinflammatory disorder caused by loss of ADAR1 function, leading to accumulation of cytoplasmic dsRNA generated by genomic repetitive elements. These endogenous dsRNA are aberrantly recognised by cytosolic RNA sensors, resulting in sustained upregulation of interferon signalling and consequential neurological damage in affected individuals [79]. Together, these observations highlight importance of the regulation of immune sensing pathways by ADARs and maintaining cellular homeostasis.

1.4.3. Interaction of ADARs with innate immune sensors

The RLR signalling pathway represents a key mechanism detecting cytosolic dsRNA. ADAR1 plays a critical role in regulating this pathway, particularly by supressing MDA5 and its downstream adaptor MAVS. In absence of ADAR1, mice exhibit embryonic lethality at ~ 13.5 days due to interferonopathy. However, concurrent deletion MDA5 or MAVS together with ADAR1 restored embryonic viability suggesting critical role of ADAR1 supressing aberrant activation of IFN response. Notably, study also suggests that the binding activity of ADAR1 to its substrate dsRNA is sufficient to reduce immune response with editing enhancing the suppressive effect (Fig. 10) [80]. Although similar effects were not observed for RIG-I in vivo, ADAR1 RNA binding activity has been shown to inhibit RIG-I dependant RNA sensing and subsequent IFN production in primary mouse embryonic fibroblast (MEF) and HEK293 cell models (Fig. 10) [81]. Collectively, these findings indicate that not only ADAR1 mediated RNA

editing but also its RNA binding activity is essential for maintaining cellular homeostasis.

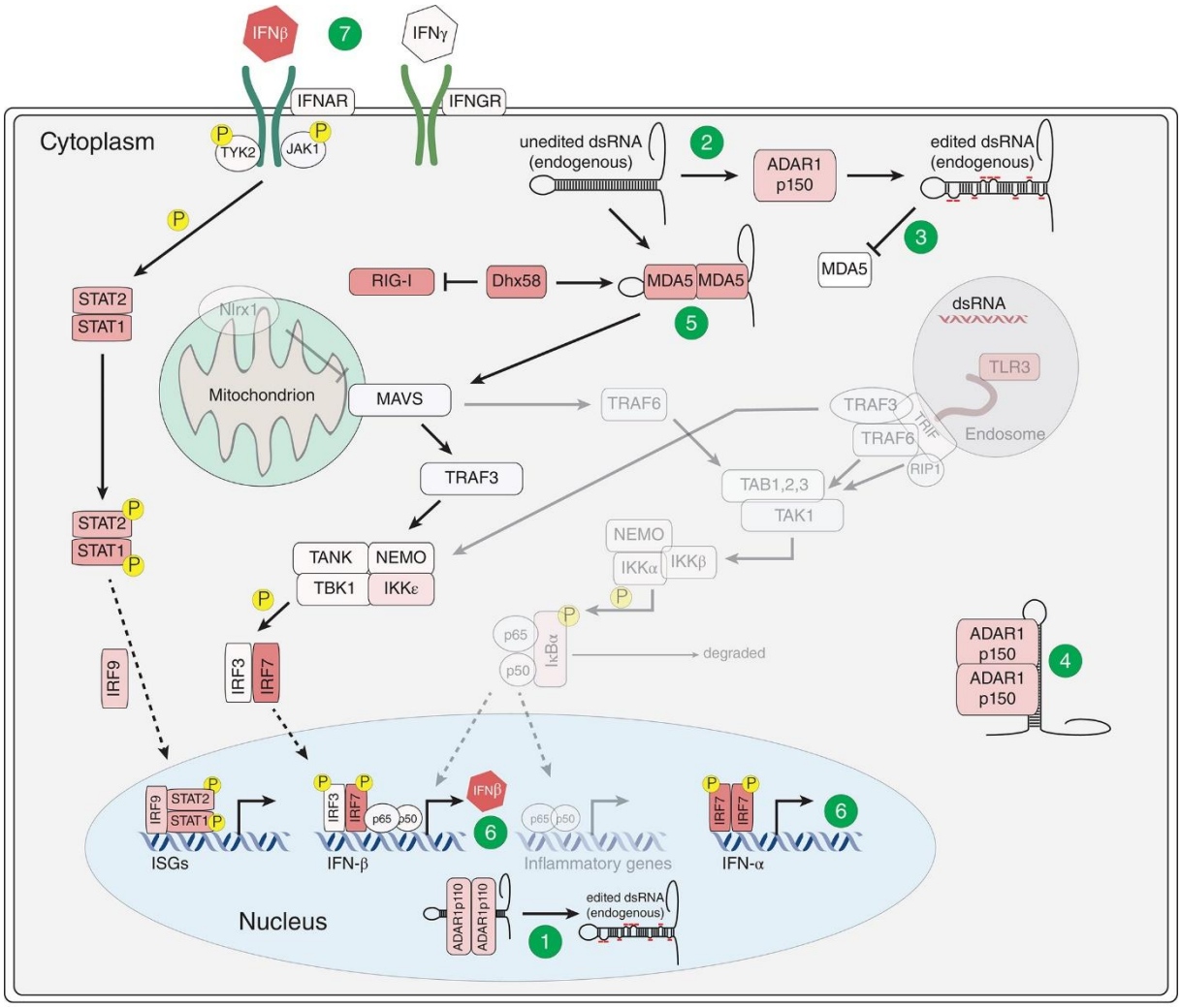


Figure 10. Role of ADAR1 in MDA5-RIG-I mediated dsRNA response. ADAR1 edits dsRNA in nucleus or cytosol reducing its recognition by innate immune sensors. In absence of editing or binding from ADAR1 dsRNA is detected by sensors leading to induction of interferon response. Adapted from [82].

Another sensing pathway induced upon detecting cytosolic dsRNA is the IFN inducible oligonucleotide synthase (OAS)- RNase L pathway. In human lung adenocarcinoma A549 cell line, cell death due absence of ADAR1 can be rescued by deleting RNase L, even in presence of MDA5 and MAVS signalling. ADAR1 was shown to be primary regulator of RNase L activity by preventing OAS activation, particularly for the studied cell model [83].

ZBP1 together with ADAR1, are only two proteins in mammals that possess Z-DNA binding domain. ADAR1 plays a critical role in regulating ZBP1 mediated cell death responses. Loss of ADAR1 sensitizes cells to ZBP1 dependent apoptosis and necroptosis, likely due endogenous dsRNA including Alu duplexes, which can be sensed by ZBP1. Furthermore, mutations in Z-DNA binding domain of ADAR1 are associated with Aicardi–Goutières syndrome (AGS) [84].

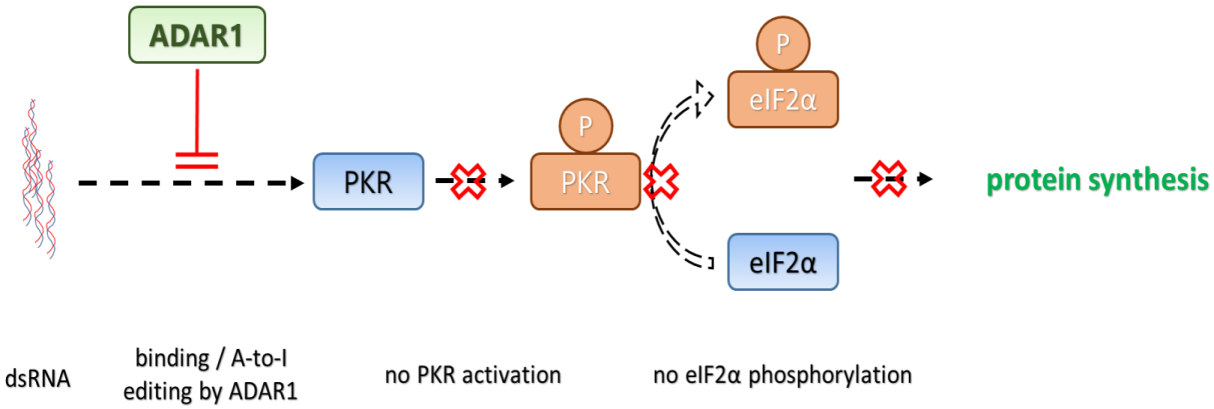


Figure 11. Inhibition of PKR mediated dsRNA sensing. In presence of ADAR1, activation of PKR is suppressed, preventing phosphorylation of eIF2α maintaining translational activities in cell. Adapted from [77].

Protein Kinase R (PKR) shares mechanistic and structural similarities to ADAR1 being an IFN inducible gene and possessing of double stranded RNA binding domains (dsRBD) [85]. Though deletion of MDA5 or MAVS rescues embryonic lethality in mice, infants die shortly after birth suggesting indicating involvement of additional pathways. In further studies PKR has emerged as critical factor. Loss of ADAR1 or p150 specifically affected protein levels of interferon stimulated genes (ISG) but not mRNA levels, suggesting defect at translational level. This phenotype is associated with increased activation of PKR and phosphorylation of downstream effector eIF2α. Upon simultaneous deletion PKR and ADAR1 levels of mRNA as well as proteins were restored indicating PKR mediated translational shutdown was the primary driver. Furthermore, inhibition of transcription in ADAR1 deficient cells resulted in reduced PKR activation suggesting that newly transcribed endogenous RNA was responsible for triggering PKR activation. Results from the same study showed that more than 90% of A-to-I editing occurred in Alu repeats, suggesting that ADAR1 mediated dsRNA

editing or binding prevents PKR from recognising endogenous RNA species and triggering translational block (Fig. 11) [60]. These observations underline the role ADAR1 in maintaining cellular homeostasis and preventing aberrant activation of innate immune response.

Overall, these studies highlight the central role of ADARs in regulating immune activation through modulation of dsRNA recognition. This function is particularly relevant in context of viral infections, where presence of viral dsRNA structures necessitates tightly balanced in immune response.

1.5. ADAR in virus-host interactions

While viruses actively counteract host immune defences, ADAR proteins are essential for maintaining immune balance by preventing excessive activation of these defence mechanisms and limiting host damage. The regulatory activity of ADARs mediated through deamination and binding of dsRNA structures, spans from endogenous to viral substrates and ranges from site specific to extensive hyperediting. These activities result in diverse functional outcomes that vary depending on the virus, the stage of the replication and the host cell type, leading to either proviral or antiviral effects (Fig. 12) [71].

1.5.1 Role of ADARs in RNA virus infections

Due to intrinsic stage in the life cycle generating dsRNA structures, the role of ADARs have been extensively studied in RNA viruses, even prior to identification of ADARs as enzymes catalysing A-to-I editing [71].

Measles virus (MeV), a negative single stranded RNA (-ssRNA) virus, is one of the earliest characterized examples of ADAR-mediated RNA editing, where A-to-I hyperediting of viral RNAs was observed [86]. Although the editing could disrupt viral protein production exhibiting antiviral effects, editing dsRNA species generated by MeV to counter sensors such as PKR, ADAR1 supressed activation PKR leading to proviral effects (Fig. 12) [87].

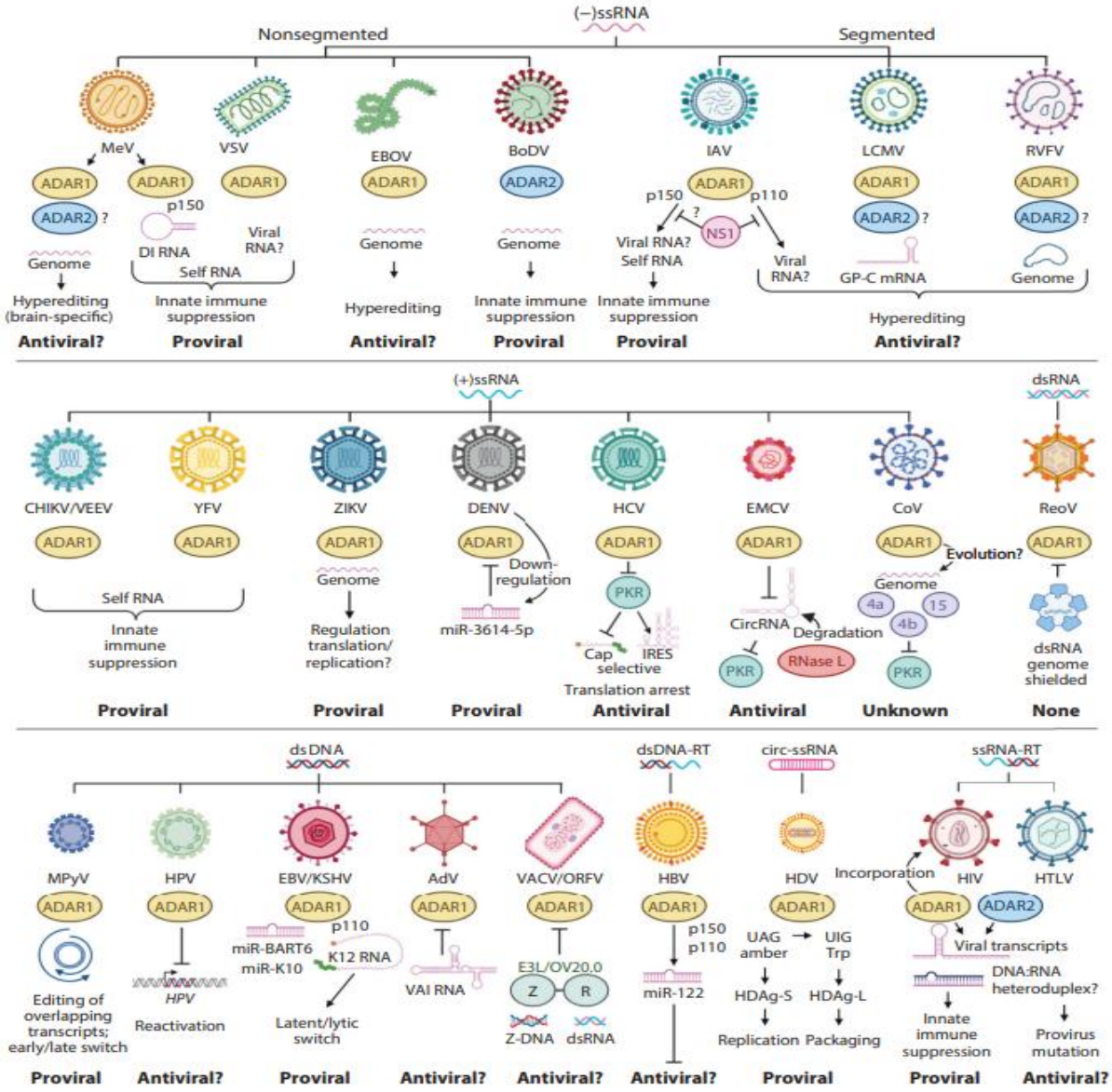


Figure 12. Diverse roles of ADAR1 in virus-host interaction. ADAR1 is the primary regulator of host response to viral infection, acting through binding and A-to-I editing both viral and host dsRNA structures, while ADAR2 may contribute in specific viral infections. Depending on the virus and cellular context, ADAR1 can exert either proviral or antiviral effects. The figure illustrates representative interactions of ADAR1 with RNA viruses, including influenza A virus (IAV), hepatitis C virus (HCV), measles virus (MeV), vesicular stomatitis virus (VSV), as well as DNA viruses such human cytomegalovirus (HCMV), Epstein–Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV). Adapted from [71].

However, effects of ADAR1 are not only viral dependent but also isoform dependent. Influenza A virus (IAV) is a well characterised example of this isoform dependent functional diversion. The cytoplasmic isoform, ADAR1p150, promotes the viral replication by supressing dsRNA mediated innate immune response including MDA5 and RIG-I signalling. Interestingly, the binding activity of ADAR1p150 alone is

sufficient to supress RIG-I activation [88]. In contrast, nuclear isoform, ADAR1p110 has been associated with antiviral effects, likely through editing viral RNA (Fig. 12).

Hepatitis C virus (HCV), a positive single stranded RNA (+ssRNA) virus, further highlights complex role of ADAR1. Upon HCV infection IFN response is induced leading to PKR mediated translational arrest. However, HCV utilizes internal ribosome entry site (IRES) driven translation to bypass of the translational block, therefore, promoting viral replication. However, by editing and binding dsRNA structures ADAR1 supresses PKR activation, thereby having negative effects on viral replication exhibiting antiviral effect (Fig. 12) [89].

Beyond the examples discussed above, a wide range of RNA viruses have been studied in the context of ADAR activity. However, outcomes in many cases is often inconsistent and inconclusive, highlighting the complexity of ADAR mediated immune regulation and leaving significant gaps in our current understanding (Fig. 12). Given that many DNA viruses also generate large amounts of dsRNA during the replication cycle, ADAR is more likely to play an important role in their infections .

1.5.2. Role of ADARs in DNA virus infections

In contrast to RNA viruses, where characterization of the role of ADAR mediated editing has been established, its functions remain comparatively understudied in DNA virus infections. However, even among DNA viruses, the role of ADARs is highly context dependent. In Hepatitis B virus (HBV), ADAR1 has been shown to inhibit replication, while in Murine polyomavirus (MPyV), ADAR1 rescues cells from virus induced cytotoxicity (Fig. 12) [71]. Human herpesviruses, representing one of the most extensively studied DNA viruses with ability to generate substantial amount dsRNA structures, provide relevant system to investigate role of ADAR proteins [90].

The role of ADAR activity in herpesviruses was reported in Epstein-Barr virus (EBV) through editing of miRNAs. A-to-I editing of primary miRNA transcripts, especially, pre-miR-BART6, impairs further processing during miRNA biogenesis and alters downstream targets, such as miRNA processing protein Dicer. This, in turn, influences global miRNA regulation and contributes to maintenance of viral latency. The specific ADAR responsible for this editing is yet to be known, but given the low expression of

ADAR2 in cells with latent EBV, it is speculated to be ADAR1p110 [91]. In addition to miRNAs, long noncoding RNA (lncRNA) expressed during EBV reactivation have been shown to interact with ADAR1, suggesting potential modulation in innate immune response, however role of editing activity is yet to be described [92].

Early evidences of potential editing phenomenon was first noted in Kaposi's Sarcoma-Associated herpesvirus (KSHV), where A-to-G variations (due to A-to-I posttranscriptional editing) in miRN-K12-10 were reported [93]. Later studies revealed ADAR1 mediated editing of K12 locus and associated miRNAs impacted viral latency, however precise biological functions remain under investigation [90].

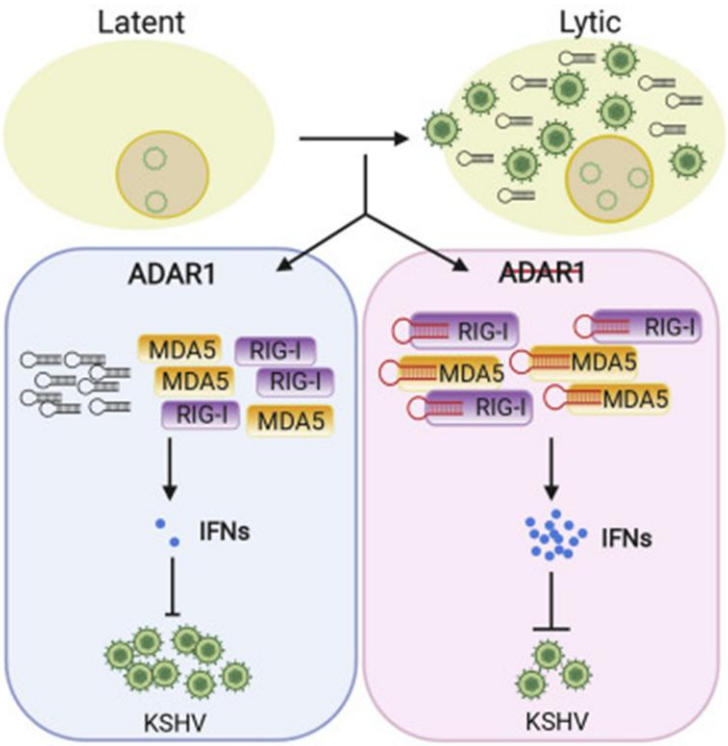


Figure 13. Role of ADAR1 during KSHV reactivation. ADAR1 limits activation of RLR pathway resulting in controlled IFN production and efficient KSHV reactivation. In absence of ADAR1 limited reactivation occurs dur to enhanced dsRNA sensing by MDA5 and RIG-I. Adapted from [94].

Beyond its editing dependent effects on viral transcripts, ADAR1 is reported to regulate innate immune functions, attributed to its editing independent role. During the reactivation of KHSV from latent to lytic stage, loss of ADAR1 function increased IFN I production limiting viral reactivation. However, depletion of RIG-I or its downstream effector MAVS together with ADAR1 rescues KSHV reactivation by reducing TBK1-

IRF3 mediated IFN production. Depleting MDA5 in ADAR1 deficient cells could rescue reactivation, though in lesser extent compared to RIG-I (Fig. 13) [94]. Although the exact RNA ligand triggering RIG-I activation is unknown, this study provides important insights into editing independent modulation of innate immunity and proviral role ADAR1 plays during KSHV infection.

Apart from *gammaherpesviruses*, the role of ADAR1 has also been studied in human cytomegalovirus (HCMV), a *betaherpesvirus*. HCMV selectively upregulates nuclear isoform ADAR1p110. This leads to increases A-to-I editing in host miRNA such as miR-376a, altering target specificity and modulating NK cell mediated immune responses [95]. However, this is one of the very few studies reported and broader role of ADAR1 in HCMV infections remains unclear.

In context of herpes simplex virus 1 (HSV-1), earlier studies have reported that editing events occur during latent infection. During latency, HSV-1 expresses several miRNAs particularly derived from latency associated transcript (LAT). After analysing sequencing data from human trigeminal ganglia, miR-H2-3p in particular has been shown to undergo A-to-I editing with higher frequency. This editing occurs in its seed region altering the targeting potential and enabling regulation of key viral IE genes such as ICP0 and ICP4 essential for initiating viral replication. However, specific ADAR protein mediating this editing activity has not been conclusively determined [12].

Collectively, numerous studies have demonstrated diverse and context dependent roles that ADARs play in virus infections. Particularly within herpesviruses, ADAR activity can influence viral gene expression and modulate host innate immune response. Though previous studies in herpesviruses have provided insights into ADAR function, many aspects remain inconclusive and poorly understood. In context of HSV-1, although RNA editing events have been reported, the specific roles of ADARs have not been clearly defined and contribution of ADARs in HSV-1 productive infection remains largely unexplored.

1.6 Knowledge gaps and rationale for the study

Despite extensive research demonstrating the diverse roles of ADARs in regulating virus-host interaction, their precise functions during HSV-1 infection are not well understood. While ADAR mediated miRNA editing in HSV-1 latency have provided initial insights into their importance, the extent, dynamics and biological consequences of ADARs in productive infection, characterised by extensive transcription, remains unexplored.

At the primary site of HSV-1 infection, epithelial cells, ADAR1 is the predominantly expressed member of ADAR family with well-established roles in regulating cytosolic sensing of dsRNA structures of both host and viral origin. In contrast, ADAR2 expression is more restricted and primarily associated with neuronal tissues. In multiple viral systems, including KHSV as closely related herpesvirus to HSV-1, ADAR1 has been shown to modulate innate immune response and promote viral replication through suppression of dsRNA sensing pathways.

HSV-1 infection is characterised by extensive transcription and accumulation of substantial amounts of dsRNA structures, which potentially activate host antiviral pathways such as PKR and RLR signalling. Among these, PKR represents a key pathway due to its ability to induce global translational arrest in response to dsRNA. Notably, HSV-1 encodes several proteins to downregulate these pathways, highlighting the importance of dsRNA sensing during infection. Although ADAR1 is reported to regulate innate immune responses to cytosolic dsRNA sensing pathways, its specific contribution to HSV-1 productive infection has not yet been defined.

Based on emerging evidences supporting the roles of ADARs in HSV-1 biology, this study aims to investigate role of ADAR1 in modulating innate immune response in productive HSV-1 infection. Specifically, this work focuses on defining contribution of ADAR1 in productive HSV-1 replication including isoform specific functions, and elucidating underlaying molecular mechanisms regulating dsRNA mediated innate immune response.

2. RESEARCH HYPOTHESIS AND AIMS

2.1 Hypothesis

HSV-1 productive infection involves extensive viral gene expression which activates host immune response largely through recognition of viral RNA, which require tight regulation to inhibit unnecessary activation. ADAR1 is a crucial regulator of RNA homeostasis and innate immune signalling. In multiple viral systems, ADAR1 has been shown to modulate host immune response through both RNA editing-dependant and editing-independent manner.

We have discovered ADAR-mediated posttranscriptional modification of HSV-1 miRNAs in latently infected ganglia, indicating an important role of ADAR proteins in HSV-1 latency. However, the function of ADARs in productive HSV-1 infection remains poorly defined. Furthermore, our preliminary studies suggest ADAR-mediated editing of viral transcripts during productive HSV-1 infection, which lead us to hypothesize that,

1. ADAR1 plays a significant role in regulating HSV-1 replication during productive infection
2. Loss of ADAR1 leads to activation of host antiviral pathways that restrict viral replication
3. ADAR1 regulates key antiviral pathways such as viral RNA sensing pathways which affects viral gene transcription and translation
4. The role of ADAR1 in productive HSV-1 infection is mediated through dsRNA binding capacity and/or protein interactions

2.2 Aims and objectives of the study

This study was designed with following aims to test the framed hypotheses.

I. Analysis of the Phenotype

1. **To determine the requirement for ADAR1 in productive HSV-1 infection**
   This objective establishes the requirement of ADAR1 in productive HSV-1 infection and defines the stages the viral life cycle affected by its deficiency.

2. **To determine the innate immune pathways responsible for affected HSV-1 replication in ADAR1 deficient cells**

   This objective determines the host antiviral mechanisms contributing to restriction in viral replication in absence of ADAR1.

II. Functional Characterization

3. **To investigate contribution of PKR signalling and translational control to the antiviral phenotype**

   This objective assesses PKR activation, downstream signalling events and the extent of translational arrest accounts to inhibition of viral replication.

4. **To define isoform specificity and functional requirement of ADAR1**

   This objective evaluates roles of ADAR1 p110 and p150 isoforms in productive HSV-1 infection and their contribution to its proviral function.

III. Investigations into Molecular Mechanism

5. **To elucidate the molecular mechanism of the regulation of PKR activation by ADAR1**

   This objective examines the potential interaction between ADAR1 and PKR and characterizes molecular nature of this interaction.

6. **To investigate the viral and cellular triggers of activation of PKR during productive HSV-1 infection**

   This objective aimed to define upstream signalling responsible for activation of PKR with emphasis on role of viral transcription.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Cell lines

Following cells were used in this research:

- Human Embryonic Kidney cells (HEK-293, ATCC CRL-1573) Wild type and CRISPR generated ADAR1 knockout kindly provided by Jonathan Maelfeit (CRIG, Ghent University)[96]
- Epithelial cells of the African green monkey kidney (Vero, ATCC CCL-81)
- Primary human foreskin fibroblast cells (HFF), kindly provided by Professor Stipan Jonjić, (MEDRI, University of Rijeka)
- epithelial cervical adenocarcinoma cells (HeLa, ATCC CRM-CCL-2)
- lung fibroblast cells (MRC-5 ,ATCC CCL-171)
- epithelial lung carcinoma cells (A549, ATCC CRM-CCL-185)

3.1.2. Cell culture media

All culture media that were used for this work are based on the commercially available DMEM (PAN-Biotech), and to complete media, the following supplements listed in Table 1 were added.

Table 1 : Complete media used in the cell culture

Cells maintained in complete media	Base medium/type	• Supplements	Concentration
- HEK-293 (WT and ADAR1 KO) - Vero - HFF - HeLa - MRC-5 - A549	Dulbecco's Modified Eagle Medium (DMEM)	- Fetal bovine serum (PAN-Biotech) - L-glutamine (Capricorn) - Penicillin/Streptomycin (Capricorn) - Sodium Pyruvate (Capricorn)	10% 2 mM 100 µg/µl 1 mM

3.1.3. Viruses

- Wild-type HSV-1 laboratory strain KOS (kindly provided by Professor Donald M. Cohen, Harvard Medical School, Boston, USA) was prepared in Vero cells and stored at -80°C. [97]
- HSV-2 a thymidine kinase (TK)-negative mutant of HSV-2 strain 186syn+,186ΔKpn (HSV-2) (kindly provided by David M. Knipe, Harvard Medical School) [97]

3.1.4. Buffers, reagents and kits

3.1.4.1. Buffers gel electrophoresis of nucleic acids

Table 2: Buffers used for the nucleic acid handling

Tris-acetate (TAE) buffer, 50X	Tris (Carl Roth) Glacial acetic acid EDTA (pH 8.0) (Carl Roth)	2 M 1 M 50 mM
TE buffer	Tris (pH 8.0) (Carl Roth) EDTA (pH 8.0) (Carl Roth) RNase A (Macherey-Nagel)	10 mM 1 mM 10 µg/mL
DNA loading buffer	Bromophenol-blue (MilliporeSigma) Xylene cyanol (MilliporeSigma) Glycerol (Carl Roth)	2.5 g/L 2.5 g/L 1 mL
agarose gel	Agarose (Carl Roth) 1x TAE buffer	0.8-3%

Note: All buffers were made by dissolving the ingredients in double distilled water and were then sterilized by filtration if needed.

3.1.4.2. Buffers for protein isolation, SDS-PAGE and Western Blot

Table 3. Buffers used for the protein handling

RIPA (Radioimmunoprecipitation assay) lysis buffer	NaCl (Carl Roth) NP-40 (Thermo Scientific) Na deoxycholate (Carl Roth) SDS (Carl Roth) Tris (pH 8.0) (Carl Roth) Complete Protease inhibitor (Roche)	150 mM 1% (v/v) 0.5% (w/v) 0.1% (w/v) 50 mM
Sample Buffer	4x Laemelli Buffer (Biorad)	1x
Separating Gel Buffer	Tris (pH 8.8) (Carl Roth)	1.5 M

Stacking Gel Buffer	Tris (pH 6.8) (Carl Roth)	1 M
SDS-PAGE Electrophoresis Running Buffer (10x)	Tris (Carl Roth) Glycine (Carl Roth) SDS (Carl Roth)	25 mM 192 mM 0.1% (w/v)
Transfer Buffer (10x)	Tris (Carl Roth) Glycine (Carl Roth)	25 mM 192 mM
Transfer buffer (1x)	1x transfer buffer Methanol (Carl Roth)	 20%
Tris-Buffered Saline (TBS) 10x (pH 7.6)	Tris (Carl Roth) NaCl (Carl Roth)	10 mM 150 mM
TBS-T	1x TBS Tween-20 (Carl Roth)	 0.05% (v/v)
Ponceau S	Ponceau S (Carl Roth) Glacial acetic acid (Carl Roth)	0.1% (w/v) 1% (v/v)
Milk blocking buffer	Milk powder (Carl Roth) 1x TBS	5% (w/v)

Note: All buffers were made by dissolving the ingredients in double distilled water and were then sterilized by filtration if needed.

3.1.4.3. Buffers for immunofluorescence

Table 4. Complete buffers used for immunofluorescence

Formaldehyde solution	Paraformaldehyde (Carl Roth) NaOH (Carl Roth)1x PBS 1x PBS	4% (w/v) 1N
Permeabilization buffer	Triton-X-100 (MilliporeSigma) 1x PBS	 0.5% (v/v)
Bovine serum albumin (BSA) blocking buffer	BSA (Capricorn) Triton-X-100 1x PBS	3% (w/v) 0.2% (v/v)

3.1.4.4. Plaque assay solutions

Table 5. Complete buffers used for plaque assay

2x methylcellulose	Methylcellulose (MilliporeSigma) FBS (PAN-Biotech)	2.4% (w/v) 3% (v/v)

	5x DMEM (Gibco) Penicillin/Streptomycin (Capricorn) Sodium Pyruvate (Capricorn)	 2 mM 0.5 mM
5x DMEM (pH 7.0)	DMEM powder (Gibco) Sodium hydrogen carbonate (Carl Roth)	 1.85 % (w/v)
Fixation buffer	Methanol (Carl Roth) Glacial acetic acid 1x PBS	5% (v/v) 10% (v/v)
Staining buffer	Giemsa (Carl Roth) 1x PBS	5%

3.1.4.5. Reagents, Kits and other materials (Miscellaneous list)

The following kits and reagents were used according to the manufacturer's instructions.

A. Analysis of nucleic acids

- GelStar Nucleic Acid Gel Stain 10,000x (Lonza) diluted in DMSO 1:100
- Gel Loading Dye (6x) (New England Biolabs)
- Quick-Load Purple 2-Log DNA Ladder (0.1-10.0 kb) (New England Biolabs)
- NuceloSpin Gel and PCR Clean-Up Kit (Macherey-Nagel)
- NucleoSpin Plasmid, Mini kit for plasmid DNA (Macherey-Nagel)
- NucleoBond XTRA Midi kit for transfection-grade plasmid DNA (Macherey-Nagel)
- Chloroform (Carl Roth)
- Isopropanol (Macron)
- Ethanol (Carlo Erba)
- TRIreagent (Invitrogen)
- RNase ZAP (Ambion)
- miRNeasy Micro Kit (Qiagen)
- High Capacity cDNA Reverse Transcription Kit (Applied Biosystems)
- low rox SybrMix (PCR Biosystems)
- Nytran Supercharge membrane (Whatman)
- dsRNA positive control (Jena Bioscience)
- ssRNA negative control (Jena Bioscience)

- dot blot blocking solution (DIG High Prime labelling and detection starter Kit II, Roche)

B. Analysis of proteins

- Nitrocellulose membrane (Machary Nagel)
- Enhanced Chemiluminescence (ECL) Detection System (Cytiva)
- SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher)
- Dynabeads Protein G immunoprecipitation kit (Life Technologies)
- RNase A (Promega)
- Shortcut RNase III (New England Biolabs)

C. Cell culture and transfections reagents

- Lipofectamine 2000 (Invitrogen)
- Lipofectamine RNAiMAX (Invitrogen)
- OptiMEM (Gibco)
- 1x Phosphate Buffer Saline (PBS) (Pan Biotech)
- Matrigel (BD)
- 10x Phosphate Buffer Saline (PBS) (Carl Roth)
- Antibiotics Ampicillin, Kanamycin (Carl Roth)
- DMEM high glucose methionine free media (Gibco)

D. Modulation and pharmacological reagents in cell culture

- rIFN-β (R&D Systems)
- Acyclovir (ACV) (MilliporeSigma)
- Cycloheximide (MilliporeSigma)
- Actinomycin D (MilliporeSigma)
- Staurosporin (Roche)
- zVAD (Invivogen)
- ISRIB (Merck)
- O-Propargyl-Puromycin (OPP) (Jena Biosciences)

E. Functional assays in cell culture

- PE Annexin V Apoptosis detection kit (BD)
- Caspase-Glo 3/7 assay (Promega)
- Click-iT Metabolic Labeling Reagents for Proteins (Molecular Probes)
- Click-iT Protein Reaction Buffer Kit (Molecular Probes)
- HRP-Linked Streptavidin (Cell signaling)
- ProLong Gold antifade reagent (Life Technologies)
- Azide conjugated to Alexa Fluor 594 (Molecular Probes)
-

F. Imaging and microscopy

- Microscope slides (Carl Roth)
- Coverslips (Carl Roth)
- Aqua-Poly/Mount mounting medium (Polysciences)
- 4',6-diamidino-2-phenylindole (DAPI) stain (10 000x) (MilliporeSigma)

3.1.5. Bacterial strains and medium

Commercially acquired *Escherichia coli* DH5α cells with required plasmid were cultured in Luria Broth (LB) medium (Carl Roth) and LB agar (Carl Roth) plates.

Ampicillin (100 µg/mL) and Kanamycin (100 µg/mL) were used when needed for the selection of transformed cells.

3.1.6. Plasmids

Following plasmids were used:

- pEGFP-N1 – Vector for fusing EGFP to the C-terminus of a partner protein (Clontech)

- pmGFP-ADAR1-p110 (Addgene #117928)

- pmGFP-ADAR1-p150 (Addgene #117927)

- pmGFP-ADAR2 (Addgene #117929)

- pc-FLAG-ICP34.5 contains the ICP34.5 gene (ORF ICP34.5 HSV-1 strain 17) constructed in laboratory of Donald M. Coen [98]

3.1.7. Antibodies used for Western blot, immunofluorescence and immunoprecipitation

Table 6. Antibodies used for Western blot

Antibody	Company	Catalog No.	kDa	Dilution WB
ADAR1	Cell Signalling	14175S	110, 150	1:1000
ADAR p150	Cell Signalling	32136S	150	1:1000
EIF2α	Cell Signalling	9722S	38	1:1000
Phospho EIF2α (Ser51)	Cell Signalling	3398S	38	1:1000
NFκB p65	Santa Cruz Biotech	sc-372	65	1:1000
Phospho -RELA/NFκB p65 (27.Ser 536)	Santa Cruz Biotech	sc-136548	65	1:1000
MAVS	Cell Signalling	3993T	75,52	1:1000
MDA5	Cell Signalling	5321T	130	1:500
OAS1	Santa Cruz Biotech	sc-515518	40,44	1:500
PKR	Cell Signalling	12297S	74	1:1000
Phospho PKR (T446)	Abcam	ab32036	68	1:1000
ATF-4	Cell Signalling	11815S	49	1:500
gC	Abcam	ab6509	98	1:2000
ICP4	Abcam	ab6514	175	1:2000
ICP27	Santa Cruz Biotech	sc-69807	63	1:2000
TK	Pan et al [99]	Pan et al [99]	42	1:2000
ICP8	Abcam	ab20194	128	1:2000
ICP0	Abcam	ab6513	120	1:2000
VP16	Abcam	ab110226	58	1:2000
Actin	Merck	MAB1501	42	1:10000
GFP	Santa Cruz Biotech	sc-9996	26	1:2000
J2 Anti-dsRNA	Jena Biosciences	RNT-SCI-10010200	-	1:200 (for dot blot)
FLAG-M2	Cell Signalling	1479S	-	1:2000
Anti-Rabbit IgG HRP Linked Secondary	Cell Signalling	7074S	-	1:2000
Anti-Mouse IgG HRP Linked Secondary	Cell Signalling	7076S	-	1:2000

Table 7. Antibodies used for immunofluorescence

Antibody	Company	Catalog No.	Dilution IF
J2 Anti-dsRNA	Jena Biosciences	RNT-SCI-10010200	1:200
AlexaFluor 555 Goat Anti-Mouse IgG	ThermoFischer	A21425	1:500

Table 8. Antibodies used for immunoprecipitation

Antibody	Company	Catalog No.	Conc. in IP
PKR	Cell Signalling	12297S	1:50
GFP	Santa Cruz Biotech	sc-9996	1:50
Normal Rabbit IgG	Cell Signalling	2729S	1:50
Normal Mouse IgG	Santa Cruz Biotech	sc-2025	1:50

3.1.8. siRNA

Table 9. siRNAs for transient knock-down

siRNA	Company	Catalog No.
RIG-I	Santa Cruz	sc-61480
LGP-2	Santa Cruz	sc-93967
MAVS	Santa Cruz	sc-75755
OAS-1	Santa Cruz	sc-61241
ZBP-1	Santa Cruz	sc-61823
cGAS	Santa Cruz	sc-95512
MDA5	Santa Cruz	sc-61010
PKR	Santa Cruz	sc-36263
ADAR-1	Invitrogen	4390824 s1007
CTRL	Invitrogen	4390843
ADAR-1 p150	-	Kind gift from J. Maelfeit

3.1.9. PCR Primers

Table 10. Specific primers used in PCR

Gene	Organism	FORWARD	REVERSE	Source (REF)
ICP4	HSV-1	TCGAGAGTCCGTAGGTGAC	TTGTTCTCCGACGCCATC	[99]
ICP0	HSV-1	AGCGAGTACCCGCCGGCCTG	CAGGTCTCGGTCGCAGGGAAAC	[99]
ICP27	HSV-1	GTGTGCAGCCGTGTTCCAA	AGCGACCGGGCCCGAATC	[99]
ICP8	HSV-1	AAGCTGGTTGCGTTGGAG	TTTCTGCTGAAGCAGTTCCA	[100]
TK	HSV-1	ACCCGCTTAACAGCGTCAACA	CCAAAGAGGTGCGGGAGTTT	[100]
VP16	HSV-1	TTTGACCCGCGAGATCCTAT	GCTCCGTTGACGAACATGAA	[100]
gC	HSV-1	GCCCATTTCGTACGACTACA	GGTGCTCTAGAACGGGAATC	[99]
18S	Human	GTAACCCGTTGSSCCCCATT	CCATCCAATCGGTAGTAGCG	[101], [102]
ADAR1p110	Human	GGCAGCCTCCGGGTG	CTGTCTGTGCTCATAGCCTTGA	[103]
ADAR1p150	Human	CGGGCAATGCCTCGC	AATGGATGGGTGTAGTATCCGC	[103]
ADAR2	Human	CGCAGGTTTTAGCTGACGC	GCATCTTTAACATCTGTGCCTGT	[104]
ATF-4	Human	TTCTCCAGCGACAAGGCTAAGG	CTCCAACATCCAATCTGTCCCG	Origene#HP205494

3.2. Methods

3.2.1. Cell culture

All cells were cultured in indicated growth medium (Table 1), incubated in cell culture incubator maintaining 5% CO2. Cell were observed daily and passaged after reaching 70-90% confluency depending on cell line.

For passaging, old media was removed and cells were directly incubated in 2X trypsin. Upon observing detachment, cells were washed out with culture media, collected and centrifuged at 200g for 5min. Supernatant was removed and cells were resuspended in fresh media, counted and seeded at desired density.

For storage, cells were passaged, centrifuged and resuspended directly into cell freezing media (90% FBS + 10% DMSO) and transferred to cryotubes. Overnight cryo-tubes were kept in cryo-boxes at -80°C and next day transferred to liquid nitrogen for long term storage.

For reviving, cryotubes were removed from liquid nitrogen and contents were thawed at 37°C in water bath. Immediately thawed content was added to prewarmed culture media, centrifuged and cells pellet were resuspended in prewarmed culture media and seeded directly in cell culture plasticware for maintenance.

For counting, 10µL sample was loaded in Neubauer chamber. All four quadrants were counted, average of count was corrected by multiplying correction factor 10000 and volume in mL from which sample was collected. Cell count is denoted as per mL.

For experiments, all cells were seeded day before infection or transfection. HEK293 cells were seeded on Matrigel coated plates. Briefly, Matrigel was freshly diluted 1:200 in DMEM without FBS and added to culture plates to cover entire surface. Plates containing diluted Matrigel were incubated at 37°C for 1h. Matrigel media was removed and cells were directly seeded on coating.

3.2.2. Infection

Cells were seeded a day before infection. Where indicated, cells were pre-treated or transfected with inhibitors, siRNAs, plasmids or other reagents before infection. Reagents used for cell treatment in the experiments were added 30 minutes to 1 hour before the infection and maintained during the time course of the infection. Cells were infected with HSV-1 (or other indicated viruses) at indicated MOI (multiplicity of infection) with infectious media prepared with adding calculated virus stock in culture media. For mock-infection, equal amount of culture media was added without virus. After 1hr infectious media was replaced with fresh culture media, and the timepoint was defined as 1hpi. (1-hour post infection). Samples were collected at indicated hpi for downstream experimentation.

3.2.3. Virus stock preparation

Virus stock were prepared as described [105]. Briefly, Vero cells were seeded to reach the 100% confluency and infected with previous stock at MOI 0.01. Two days after reaching the complete infection stage, cells were removed with scraping the surface and centrifuged to remove most of the supernatant with 1-2mL remaining. The pellet was quick frozen and thawed 3 times by switching between dry ice-ethanol paste and water bath at 37°C to release the virus. Lysed solution was centrifuged again to remove cell debris and supernatant was collected, aliquoted and stored at -80 until use. Stock titre was determined using plaque assay.

3.2.4. Plaque assay

Plaque assay was performed as described previously [102]. Briefly, vero cells were seeded to reach a monolayer with 100% confluency in required TC plate. Monolayers were infected with 10-fold serially diluted sample in culture media. After 1 hr infectious media were replaced by overlaying methylcellulose media and were incubated in cell culture incubator till the plaques were observed under microscope, or until 72 hours. Cells were then fixed in fixing solution for 2h and stained with 5% giemsa in 1xPBS for 2h. Number of plaques were counted, and titre is represented as plaque forming unite (PFU) per mL.

3.2.5. Protein extraction and Western blot

Cells were lysed with RIPA buffer with protease inhibitor and centrifuged at 11000g for 5min at 4°C to remove cell debris. Supernatant was mixed with 4x laemmlli buffer with betamercaptoethanol and heated at 95°C for 7 min. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. Membrane was blocked with 5% blocking milk for 30min and incubated in desired antibody (Table 6) in blocking milk overnight at 4 with gentle rolling. Next day, membranes were washed 3x for 10min each with 1x TBS-T. Washed membranes were incubated in secondary HRP linked antibody in blocking milk for 1 hr at RT followed by 3x washes with 1x TBS-T. Blots were visualised with ECL or Supersignal with Chemidoc.

3.2.6. RNA extraction and RT-qPCR

Cells were lysed in TRIreagent and stored at -80°C until processed. Total RNA was extracted as per manufacturer's instruction and dissolved in Nuclease free water and concentration was determined. cDNA was prepared from equal amount of RNA from all samples with reverse transcriptase kit as per manufacturer's instruction. Genes were detected by quantitative targeted amplification with qPCR kit as per manufacturer's instruction. All genes were normalised to cellular 18S and additionally all viral transcripts were normalised to expression of respective transcripts at 1hpi.

3.2.7. Transfection

In siRNA screen or siRNA mediated knock-down experiments, Cells were transfected in complete culture media with 20pmol of indicated siRNA (Table 9) with RNAimax as per manufacturers protocol. After 6h media with transfection was replaced with fresh culture media. After overnight incubation, cells were utilized for further experiments. In complementation by ectopic expression with plasmids, cells were transfected in complete culture media with Lipofectamine 2000 as per manufacturer's instructions. Cells were incubated overnight without replacing media and next day used for further experiments.

3.2.8. Plasmid preparation

All plasmids were received as bacterial cultures. For preparation of stock plasmids, small amount of stock culture was used to prepare 2mL starter culture in LB media with appropriate antibiotic for 6h in shaking incubator with 250rpm at 37°C. After primary incubation, starter culture was transferred to 50mL LB media containing corresponding antibiotic, and incubated in shaking incubator with 250rpm at 37°C overnight. Plasmids were prepared with NucleoBond XTRA Midi kit as per manufacturer's instructions. Extracted plasmids were stored at -20°C for use.

3.2.9. Immunoprecipitation

Cells were lysed with RIPA and centrifuged at 11000g for 5min at 4°C to remove cell debris. Supernatant was collected in fresh tube and 1/5th portion of lysate was set aside as 'input' for total protein analysis. Remaining portion was equally divided in two tubes and primary antibody for targeted protein or isotype control was added (1µL of antibody :50µL of lysate) and gently rotated at 4°C overnight. Next day, lysate with antibodies was transferred to fresh tube and Dynabeads G (1µL of beads :8µL of lysate) were added, and gently rotated at 4°C overnight. Following day, beads were thoroughly washed on magnetic separator with RIPA for 5 times. For detection of proteins, beads were directly heated with 4x Laemmlli buffer at 95°C for 7min, beads were removed using magnetic separator and sample was loaded to gel followed by western blot detection. For RNA extraction, beads were mixed with 100µL TRIreagent and RNA was isolated as per manufacturer's protocol.

3.2.10. dsRNA analysis

HEK293 ADAR1 WT and ADAR1 KO cells were seeded in 24 well plates on glass coverslips and infected with HSV-1. At indicated timepoints, cells were fixed for 20min in 2% PFA, permeabilized for 5min and blocked in blocking buffer for 30min at 37°C. Cells were incubated for 1h in 1:200 J2 antibody in blocking buffer, followed by 1h in Alexa Fluor 555 fluorescent secondary antibody at 37°C and stained with DAPI for 1min. Coverslips were mounted on microscope slides using mounting medium. Immunofluorescent images were taken with Zeiss Axio Observer Z1 epifluorescent

microscope with A Plan-Apochromat 63x/1.40 Oil DIC M27 objective combined with 1.6x Tubelens optovar. Confocal images were captured from sequential z-stacks using the Zeiss LSM880 confocal microscope, equipped with an Argon-laser multiline (458/488/514 nm) and HeNe lasers (543 nm and 633 nm). A Plan-Apochromat 63×/1.40 oil DIC III objective was used for imaging.

For dot-blot analysis, 1µg of total RNA, 1µg/µl and 0.5 µg/µl of dsRNA positive control RNA, and 10µg/µl and 5µg/µl ssRNA negative control RNA were loaded onto a Nytran Supercharge membrane and UV-crosslinked at 1200mJ/cm2. To normalize the loading of the tested samples, the membranes were stained in 0.1 % solution of methylene blue. For the detection of dsRNA, the membranes were pretreated in a blocking solution according to the manufacturer's instructions and incubated overnight at room temperature in a solution of the J2 antibody. After incubation, the membranes were washed 3 times in TBS-T and incubated with an HRP-positive secondary antibody, RNAs were visualized with Supersignal West Femto Detection Reagent in Chemidoc and quantified with ImageJ.

3.2.11. Nascent protein synthesis assay

ADAR1 WT or ADAR1 KO cells were seeded in 12 well plates and infected with HSV-1 at indicated MOI as described earlier [106]. OPP was added to the culture medium at a concentration of 50 µM for 1h before collection and at 12hpi cells were fixed with ice-cold methanol for 2min at −20 °C. After fixation, cells were washed twice with Tris-buffered saline, permeabilized with TBS with 0.2% Triton X-100 for 7min, and washed twice with TBS. Fixed cells were incubated with staining solution for 30min, and 20 µM azide conjugated to Alexa Fluor 594, washed twice with TBS with 0.5% Triton X-100, and incubated with DAPI for 5min. Cells were rinsed four times with PBS and once with distilled H2O before inclusion in ProLong Gold antifade reagent. Images were taken with a Zeiss LSM700 confocal laser scanning microscope using a 40× Plan Apochromat objective, and results were analyzed with the ZEN imaging software package provided by Zeiss. Mean immunofluorescence signal intensity per cell was obtained by dividing the total fluorescence intensity (n = 8–10 fields with 1,500 cells) by the number of cells.

For Click-iT reaction, infected cells were processed 3h before collection, time of collection is labelled as hpi. Cells were placed in methionine free DMEM with dialysed FBS for 1h. After preincubation 25 µM Azide-homoalanine AHA was added for 2h. Cells were lysed in RIPA buffer and supernatant was labelled with biotin-alkyne with Click-iT Protein Reaction Buffer Kit as per manufacturers protocol. Western blot was performed as described earlier and blots were detected with Streptavidin linked HRP antibody.

3.2.12. Statistical analysis

All analysis was performed with Graphpad Prism 8. For comparison of differences between two groups was tested using unpaired Student's t test. Multiple samples in one group were compared with ordinary one-way ANOVA and multiple samples with multiple groups were compared with two-way ANOVA or mixed model. P-values are quantified against control in respective group and are denoted as $p>0.05$ as 'ns', $p\leq0.05$ as '*', $p<0.01$ as '**', $p<0.001$ as '***', and $p<0.0001$ as '****'. Unless otherwise indicated, error bar denotes mean ± SD for all figures.

4. RESULTS

PART I: Analysis of the Phenotype

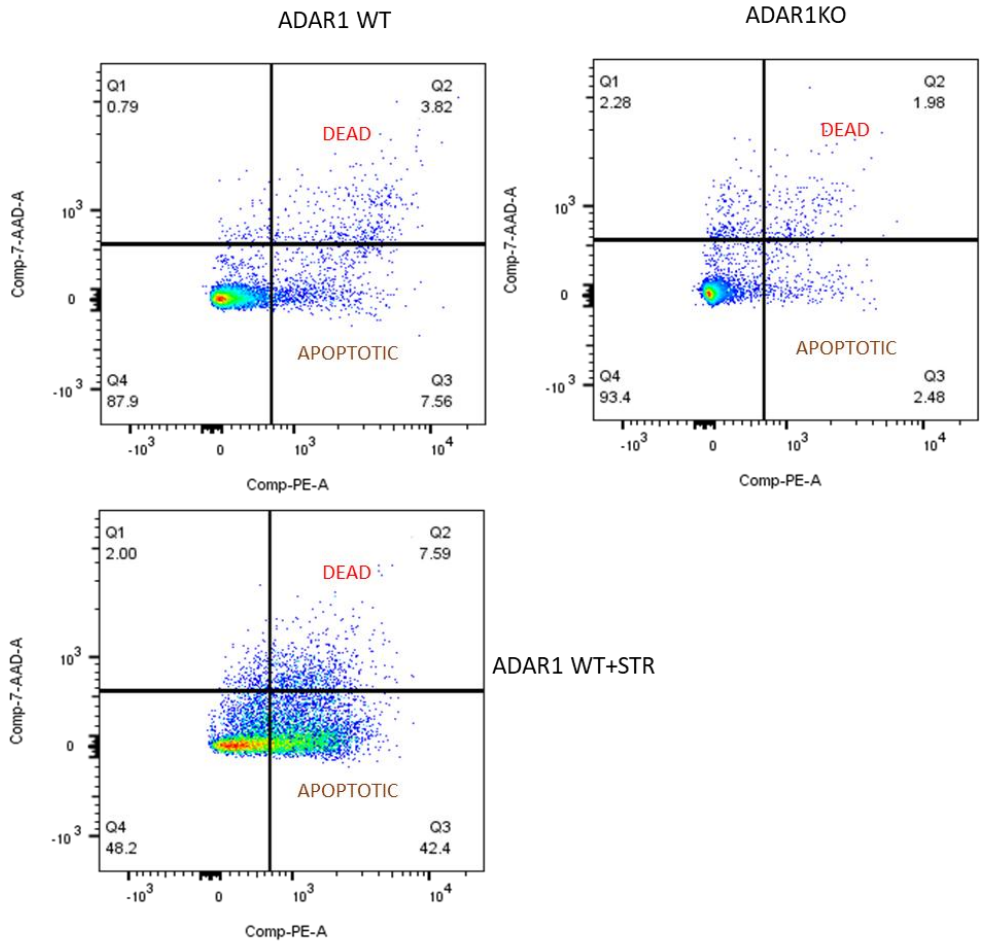
4.1. ADAR1 is required for efficient replication during HSV-1 productive infection

4.1.1. ADAR1 deficiency does not significantly change basal apoptotic properties in HEK293 cells

Previous studies have reported both proviral and antiviral functions of ADAR1. To understand the necessity of ADAR1 in HSV-1 replication, we decided to employ the knockout approach. HEK293 cells deficient in both isoforms of ADAR1, p110 and p150, were generated using the CRISPER/Cas9 technology by Maelfeit lab (CRIG, Ghent University) [96]. We did not observe any morphological differences between WT and ADAR1 KO cells and these cells duplicated in similar kinetics, indicating that ADAR1 deficiency does not significantly affect viability of HEK293 cells. Nonetheless, to investigate that the basal levels of apoptosis in ADAR1 knock-out (KO) cells were comparable with wild-type parental cells (WT), we tested both cell lines maintained in culture for apoptosis using annexin V/PI staining and Caspase Glo 3/7 assays. In short, both cell lines exhibit similar properties, yielding no significant difference between WT and KO (Fig. 14). The basal level of cell death observed in maintained cells using Ann.V/PI staining was less than 10% in both cell lines. Staurosporine treated WT cells were used as positive control, which was measured 42% cells as apoptotic upon 3h treatment. Whereas in Glo assay measuring caspases 3/7 with luminescence, there was no significant difference in WT vs KO apoptotic cells in culture. However, in both cases, basal level of apoptosis was measured bit higher rather in WT than KO cells. This led us to conclude that ADAR1 knockout had limited or no significant effect on generated KO cell line.

Together, these results indicate that ADAR1 knockout does not significantly affects basal level of apoptosis in HEK293 cell culture.

a.



b.

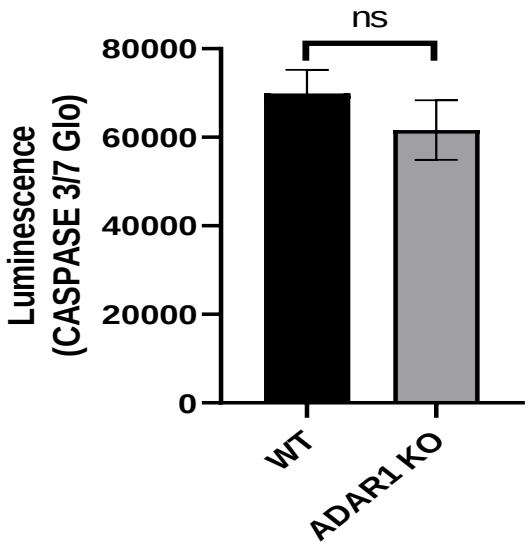


Figure 14. ADAR1 knockout does not significantly change basal apoptotic properties in HEK293 cells. a. Cells were stained with Annexin V/PI, acquired with flow cytometry within 1h. Acquisition

profiles of HEK293 derivatives ADAR1 WT (upper left) and ADAR1 KO (upper right) cells and positive control (lower) for induction of Apoptosis (WT+ 2μM staurosporine for 3h). **b.** Luminescence measured by Caspase 3/7 glow assay for WT and KO cells in culture. Data is shown as mean ± standard deviation (SD); ns – not statistically significant by Student's t test for (b).

4.1.2. Replication of HSV-1 is significantly impaired in ADAR1 deficient cells

Upon confirming that apoptosis due to ADAR1 knockout was not compromising factor in cell growth, to evaluate role of ADAR1 in viral replication, both WT and KO cells were infected with HSV-1 strain KOS at high (MOI 3) and low (MOI 0.01) multiplicities of infection. While high MOI ensures more synchronous infections, low MOI mimics more natural viral spread, highlighting the cumulative effect on replication in ADAR1 deficient conditions. Samples were collected to determine released virus in supernatant at the indicated timepoints. Viral replication was 10x more productive in high MOI, while in case of low MOI difference in virus production was 200x between WT and KO cells (Fig. 15). This indicated that deletion of ADAR1 was indeed a limiting factor in HSV-1 replication.

a. **b.**

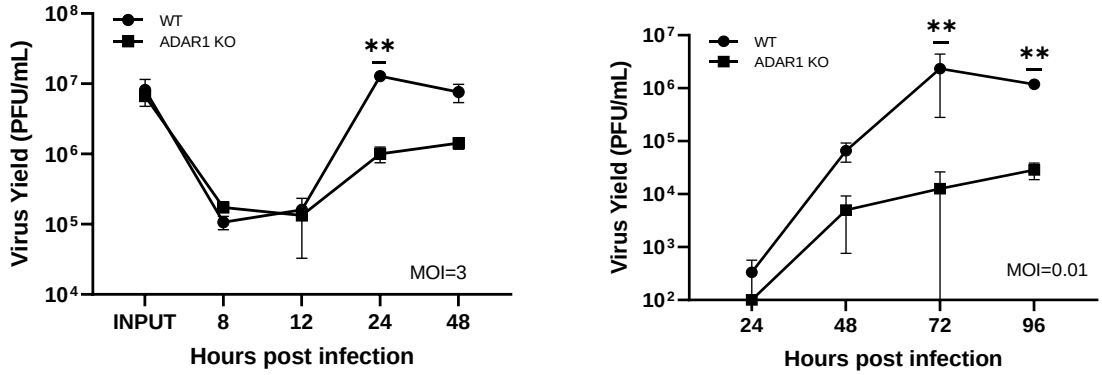


Figure 15. Replication of HSV-1 is significantly impaired in ADAR1 deficient cells. ADAR1 WT and knockout ADAR1 KO were infected with HSV-1 at **a.** high MOI (MOI 3) and **b.** low MOI (MOI 0.01). At given timepoint (hpi) supernatant was collected for the plaque assay. The data shown is representative of multiple (>3) independent experiments performed and shown as mean ± standard deviation (SD). 'ns' no statistical significance-not shown; **, p<0.01; by two-way ANOVA.

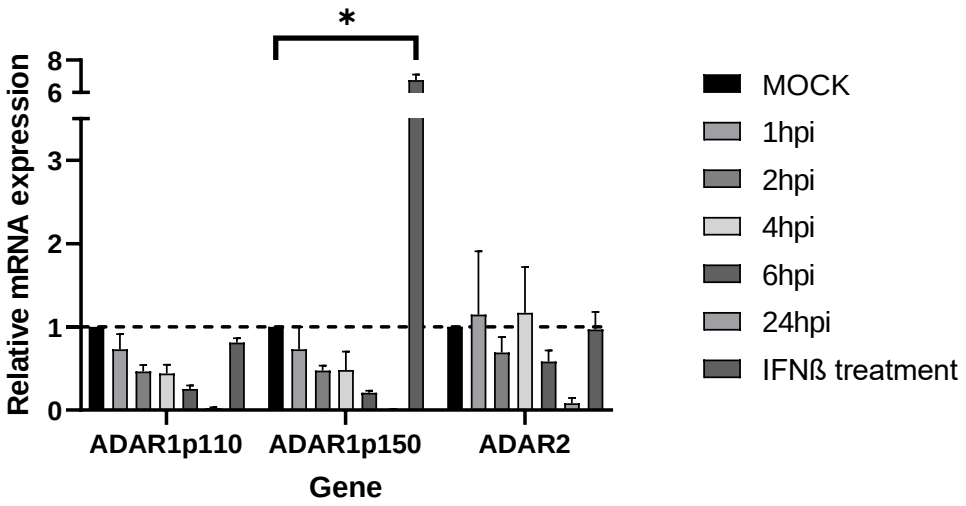
4.1.3. ADAR1 is not induced in WT cells during HSV-1 infection

We established a link between ADAR1 deficiency and HSV-1 replication. Subsequently, to check effect of infection and replication of virus on ADAR1 expression, we analysed transcripts and protein levels of ADAR1. We measured the transcript levels of ADAR1 (p110 and p150 isoforms) and ADAR2. Both isoforms of ADAR1 are depleted with the progression in infection. ADAR2 also exhibit deceasing trend as the infection progressed. To ensure reliability of the assay in measuring transcripts for different isoforms, RNA from cells treated with IFNβ (10ng/mL) for 24hpi were used as a positive control. In positive control we observed significant upregulation p150 transcripts as expected, while no change in p110 or ADAR2 were seen. To further support our observation, we analysed publicly available dataset from productively infected HFF cells by HSV-1 (S17 at MOI 10) [107]. Consistent with our result we observed decrease in transcripts of both ADAR1 (ENSG00000160710) and ADAR2 (ENSG00000197381) (Fig. 16). Decrease in transcripts of ADAR protein can be attributed to function of viral protein 'vhs' (virion host shutoff) clearing the host transcripts for efficient translation of viral proteins.

At level of proteins, both isoform of ADARs (p110 and Interferon-inducible p150) do not significantly vary throughout infection (Fig. 17). We also tested the expression of the two members of the ADAR protein family, ADAR2 and ADAR3. Both ADAR2 and ADAR3 were barely detectible in WT or KO cells. This is observation is consistent with previous reports as expression of ADAR2 and ADAR3 is limited to specific tissue types, and their functions have been frequently studied with ectopic expression [73], [108]. These observations suggest that though the transcripts are depleted in infection, at protein levels, ADAR1 has consistent expression pattern.

It is important to add that occasionally, we observed ADAR1 positive cells (WT like) in KO cultures, either in immunofluorescence or a faint band in some of the western blot experiments, which might be inefficient deletion of ADAR1 in cells, but it given that the total number of positive cells did not increased more than 1-2% in subsequent cultures, it had negligible impact on overall study.

a.



b.

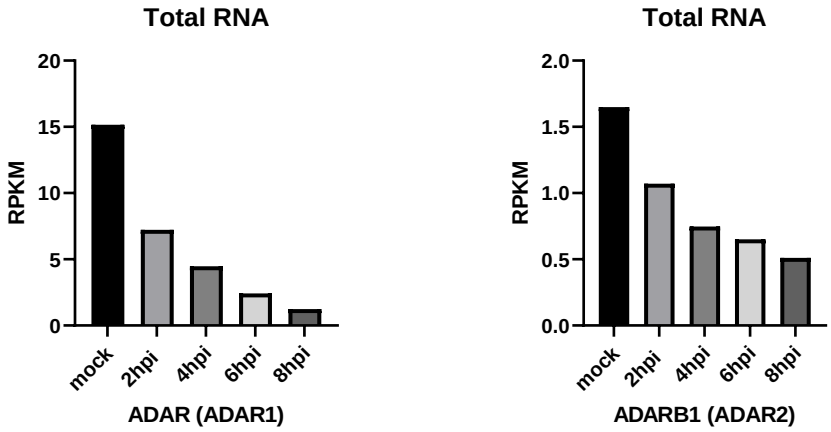


Figure 16. ADAR1 is not induced in WT cells are during HSV-1 infection. a. Relative mRNA expression in ADAR1 WT cells with MOCK infected, IFNβ (10ng/mL) for 24h or infected with HSV-1 (MOI 1) at indicated hpi. **b.** Transcripts ENSG00000160710 (ADAR1) and ENSG00000197381 (ADARB1/ADAR2) indicated as reads per kilobase per million mapped reads (RPKM) taken from total RNA of HFF infected with HSV-1 strain 17 at MOI 10 *[107]*. Data is shown as mean ± standard deviation (SD); *, p≤0.05 denoted only for relative expression >1, by One-Way ANNOVA for (a).

4.1.4. The expression levels of late viral proteins are reduced in ADAR1 KO cells

Since HSV-1 replication was impaired in ADAR1 KO cells, we investigated if the viral protein expression is affected. To address this, we analysed expression of key viral proteins in each stage of infection, i.e., immediate early (ICP0, ICP4 and ICP27), early

(ICP8 and TK) and late (gC and VP16). This analysis of proteins collected from infected ADAR1 WT and KO cells during the course of infection (1-24hpi) showed that at early time in infection, up to 7hpi, there is no significant difference in expression of immediate early proteins such as ICP0 and ICP4. However, at later stage, there is a distinct reduction in viral protein expression in KO cells. The IE protein ICP27 and E protein ICP8 were detected only later in the infection (12hpi and 24hpi respectively) which can be attributed to the sensitivity of antibody (Fig. 17). Taken together, these results indicate that in HEK293 cells ADAR1 is required for efficient viral replication. The defect in viral replication does not occur at initial stages of viral infection such as viral entry and initiation of immediate early gene expression, while expression of early and late genes is reduced or delayed in absence of ADAR1.

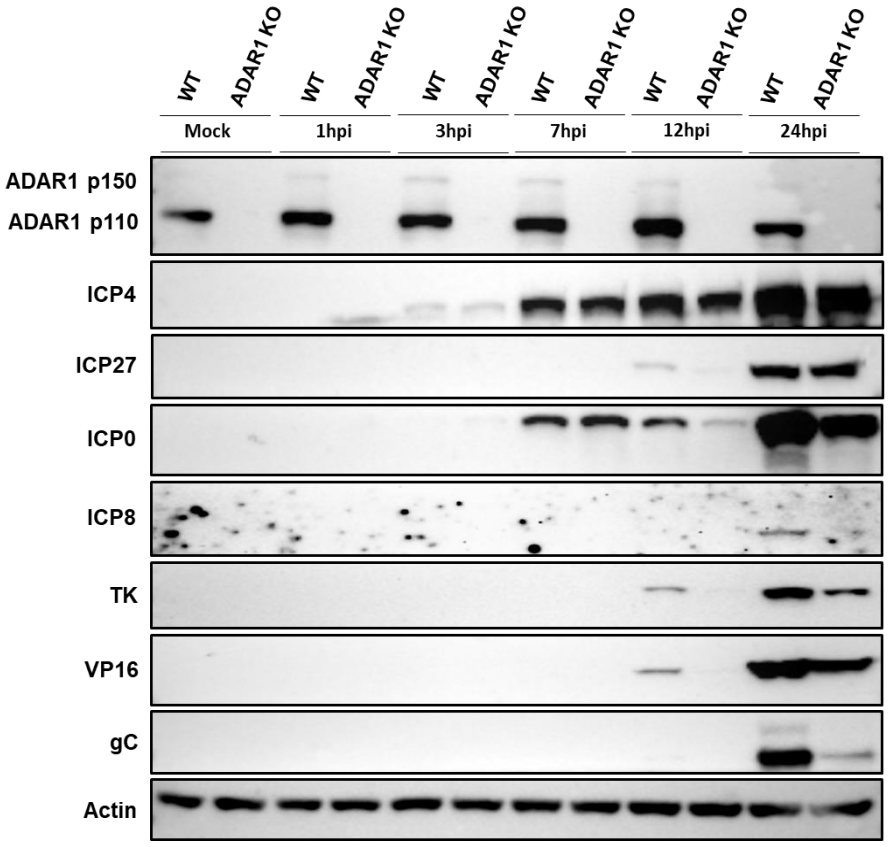


Figure 17. The expression levels of late viral proteins are reduced in ADAR1 KO cells. ADAR1 WT and KO were infected with HSV-1 at MOI 3. At given timepoint (hpi) cells were in RIPA buffer for Western blot. Expression of IE (ICP0, ICP4, ICP27), E (ICP8, TK) and L (VP16, gC) was detected throughout the course of infection.

4.1.5. Transcription of HSV-1 genes at early timepoints in infection is comparable between ADAR1 WT and KO cells

As seen earlier, there was minimal effect on expression of IE proteins in absence of ADAR1, but E and L proteins were largely affected, we hypothesised that ADAR1 deficiency may have effect on viral genes at transcriptional level. To answer this query, we analysed transcripts from all kinetic classes of genes during course of infection up to 12hpi. Initially, all transcripts were normalised to cellular 18S RNA in MOCK infected cells as an internal control. However, as MOCK infected samples lack detectable viral transcripts, relative mRNA expression was subsequently normalised to the viral transcripts detected at 1hpi in each experimental group.

Quantitative analysis revealed similar trends as seen in protein expression. Up to 3hpi, there was no significant difference in viral gene expression between ADAR1 WT to KO cells in all classes of genes. Though as expected, only transcripts of immediate early (IE) genes (ICP0, ICP4 and ICP27) are largely present at that timepoint, in overall progression of infection expression of IE genes remained comparable between the cell types. However, from 6hpi onwards, when early (E) class of genes (ICP8 and TK) as well as late (L) class of genes (gC and VP16) begun to be transcribed, levels of mRNA present in ADAR1 KO were significantly less (Fig. 18). In concurrence with observation at protein level, transcription analysis revealed that the viral replication defect in ADAR1 KO cells arises during the intermediated stages of HAV-1 infection, around the onset of viral DNA synthesis.

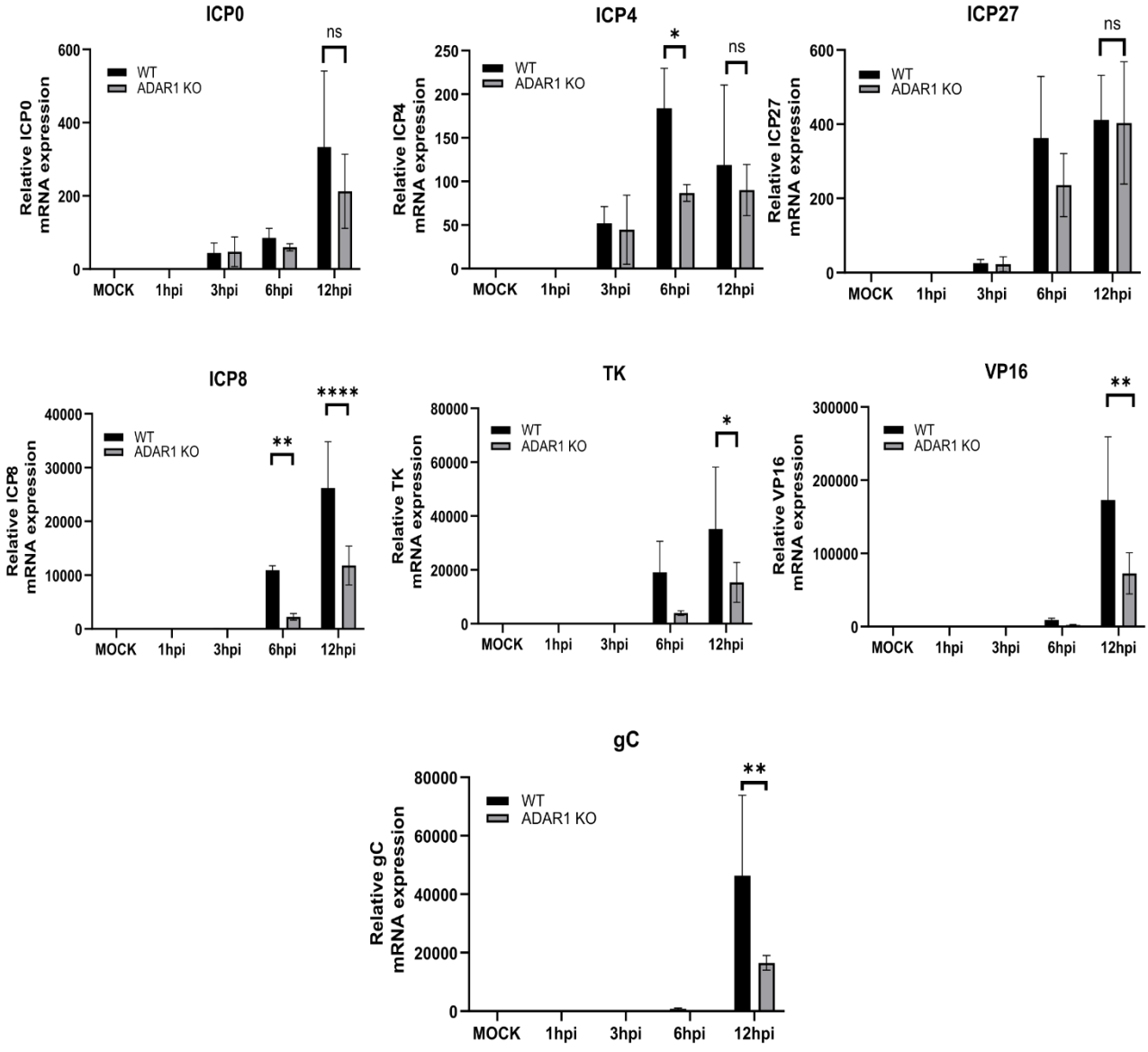


Figure 18. Transcription of HSV-1 genes at early timepoints in infection is comparable between ADAR1 WT and KO cells ADAR1 WT and KO were infected with HSV-1 at MOI 1 in triplicates. RNA was extracted from cells at indicated times after infection (hpi). Immediate early (ICP0, ICP4, and ICP27), early (ICP8 and TK), and late (VP16 and gC) transcripts were detected using RT-qPCR. The experiment was performed in triplicates. All samples were normalized to MOCK infected 18S followed by normalization to 1hpi for viral genes. Relative expression of viral genes is indicated in proportion to transcripts detected at 1hpi. Data are shown as mean ± standard deviation (SD), 'ns' not significant; * p≤0.05; **, p<0.01, **** p<0.0001 by Two-Way ANOVA.

4.1.6. Transient knockdown of ADAR1 impairs HSV-1 replication

Following up to the observed phenotype, we tested if ADAR1 is a general requirement for viral replication and not cell line specific property of HEK293 derivatives, i.e., ADAR1 WT and KO cells. To address this, we applied siRNA mediated knockdown

approach in different cells. HFF (Human Foreskin Fibroblast), a diploid primary cell line retains the intact innate immune pathways and it is physiologically relevant in studying virus host interaction. HFF are highly permissive to HSV-1 and with low level of basal ISG (interferon stimulated gene) expression, cell line is widely employed to study viral kinetics. In addition, HeLa is a transformed cell line by human papilloma virus genes which maintains functional components of innate immunity and widely used cells in virology. In all cell lines tested, both primary and immortalised, knocking down of ADAR was successfully achieved, albeit with different degrees of downregulation.

a. **b.**

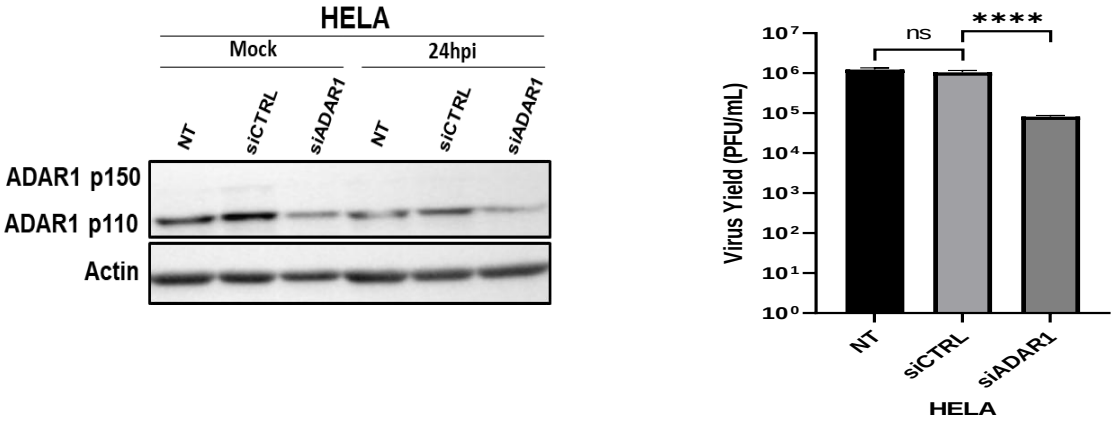


Figure 19. Transient knockdown of ADAR1 impairs HSV-1 replication in Hela cells. Hela cells were transfected with control or ADAR1 siRNA for 24 h and then infected with HSV-1 (MOI 1). **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay. The data shown is representative of multiple (>3) independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; ****, $p<0.0001$ by One-way ANNOVA for (b).

In case of Hela cells, upon knocking down ADAR1, HSV-1 replication was reduced by 12x (Fig. 19), while in HFF 1.5x less replication was observed (Fig. 20). The observed variation may reflect multiple factors such as degree by which ADAR1 is depleted in cells, permissiveness of cells for the infection and biology of the cell itself. Nevertheless, it can be concluded that ADAR1 is a general requirement for HSV-1 replication in vitro.

a. **b.**

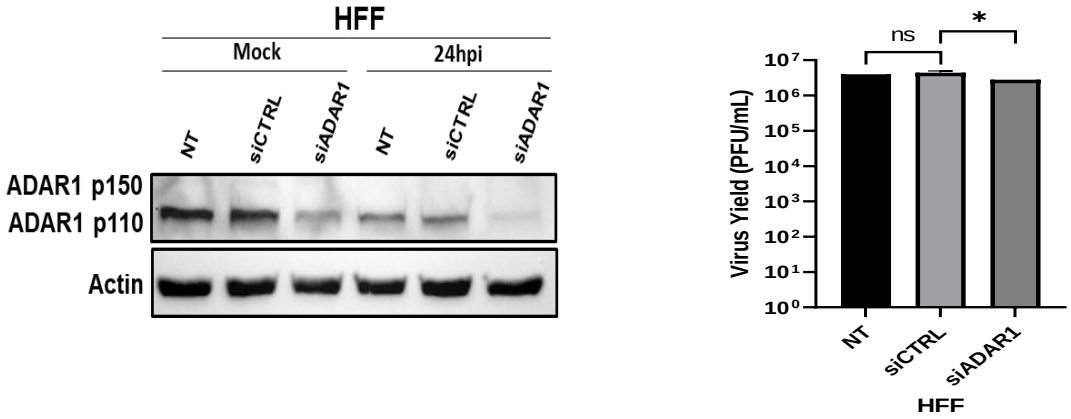


Figure 20. Transient knockdown of ADAR1 impairs HSV-1 replication in HFF cells. HFF cells were transfected with control or ADAR1 siRNA for 24 h and then infected with HSV-1 (MOI 1). **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay. The data shown is representative of multiple (>3) independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; *, p≤0.05 by One-way ANNOVA for (b).

4.1.7. ADAR1 is required for efficient HSV-2 replication

Having demonstrated that ADAR1 is a general requirement for efficient HSV-1 replication, we wondered whether this property can be applied to other related herpesviruses. HSV-2, a closely related *alphaherpesvirus* to HSV-1 was utilized for the study. Though exhibiting very similar properties with HSV-1, HSV-2 differs in several key points. HSV-2 is primarily associated with genital herpes and establishes latency in dorsal root ganglia. We infected WT and KO cells with HSV-2 at MOI of 0.1 and observed significant replication defect of approximately 100x, consistent with observations made in low MOI HSV-1 infection. Furthermore, in western blot analysis, late viral protein VP-16 was significantly reduced consistent with replication assay (Fig. 21). Collectively, these observations led us to conclude that, indeed, ADAR1 is required for productive replication of HSV-1 and HSV-2, and it acts as a proviral factor, i.e., promoting viral growth.

a. **b.**

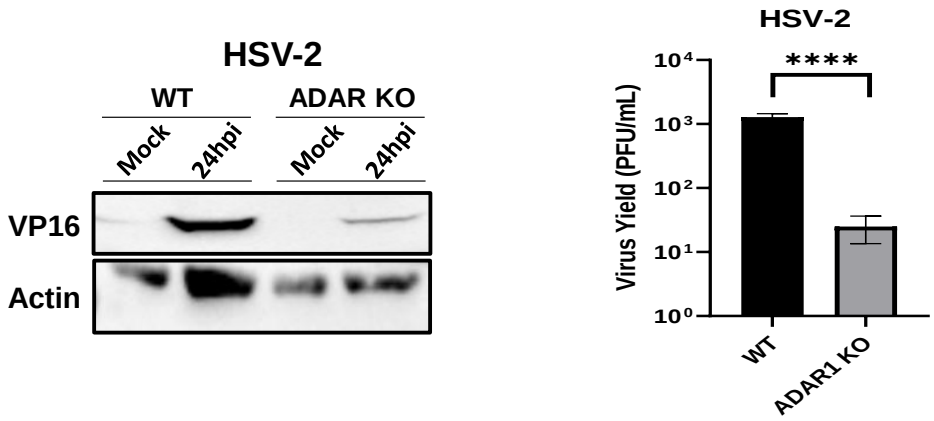


Figure 21. ADAR1 is required for efficient HSV-2 replication. ADAR1 WT and ADAR1 cells were infected with HSV-2 (MOI=0.1) **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay. The data shown is representative of multiple (>3) independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; ****, p≤0.0001 by Student's t test for (b).

4.2. Loss of ADAR1 results in enhanced PKR activation during productive HSV-1 infection

4.2.1. ADAR1 KO cells, compared to WT cells, are not more prone to apoptosis upon HSV-1 infection

Previous observations indicated that the basal level apoptosis in WT and KO cells is not significantly different between these cells. Since HSV-1 can induce apoptosis during early stages of infection, we investigated whether enhanced apoptosis in ADAR1 KO cells is contributing factor to the deficit in viral replication. To determine contribution of apoptosis, we utilized pharmacological modulators of apoptotic pathways, with apoptosis inhibitor, zVAD (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) and inducer, staurosporin. zVAD irreversibly inhibits both initiator and effector caspases of apoptosis, as well as partially inhibits pyroptosis and necrosptosis caspases. Whereas, staurosporin (originally isolated from bacterium *Streptomyces staurosporeus*) inhibits protein kinase C and induces release of cytochrome C activating intrinsic caspase cascade.

Firstly, we tested induction of apoptosis in both cells by assessing the response to apoptosis inducer staurosporin and reducing the effect in presence of zVAD. Our result show, that though the KO cells have bit higher susceptibility towards staurosporin, it does not significantly differ than WT cells (Fig. 22). Moreover, treatment with zVAD effectively reversed the induction by staurosporine in both WT and KO cells, reducing it near the basal levels. This suggests that apoptosis inducted by virus is unlikely to account for reduced HSV-1 replication in KO cells.

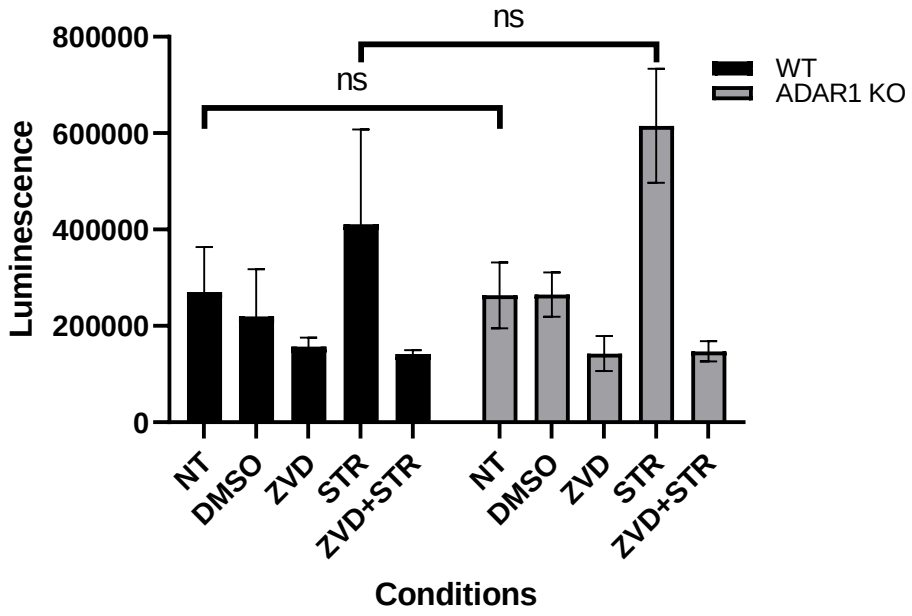


Figure 22. ADAR1 WT and KO cells respond comparably to induction and inhibition of apoptosis. Luminescence measured by Caspase-Glo 3/7 assay ADAR1 WT and KO cells treated with 2μM Staurosporin (STR) or 20μM zVAD (ZVD) or both for 3h, 0.5% DMSO were used as control. Data is shown as mean ± standard deviation (SD); ns – not statistically significant; Student's t test independently performed for each denoted pair.

Next, to determine whether increased sensitivity to HSV-1 induced apoptosis contributes to defect in viral replication in absence of ADAR1, we inhibited apoptosis during infection using zVAD in both WT and KO cells. If excessive apoptosis limits viral replication in KO cells, suppression of apoptotic pathways should, at least partially, rescue HSV-1 replication. WT and KO cells treated with zVAD prior to infection with HSV-1 (MOI 3), to ensure suppression of basal level of apoptosis, as well as induction during different stages of infection. Consistent with previous observation, our results show that HSV-1 replicated less in non-treated KO cells compared with WT, and treatment with zVAD failed to rescue viral replication (Fig. 23). Therefore, we concluded that apoptosis is not limiting factor for defective viral replication in KO cells.

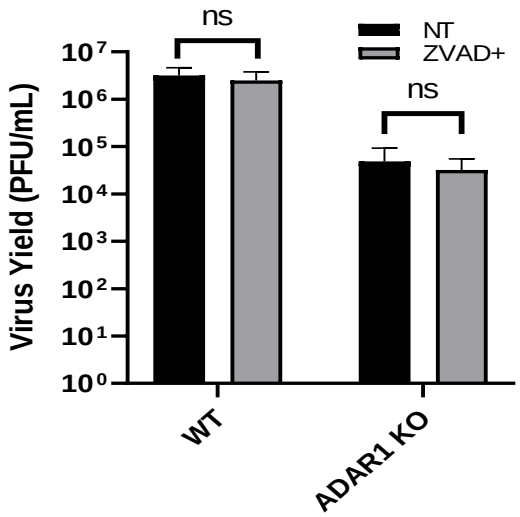


Figure 23. Apoptosis inhibitor zVAD does not rescue HSV-1 replication in ADAR1 KO cells. WT and ADAR1 KO cells were mock treated (NT) or treated with 20μM zVAD for 30min and infected with HSV-1 (MOI=3). One hour after infection, infectious media was removed and fresh media containing zVAD was added. Virus titres in the supernatants were determined 24hpi. Data are shown as mean ± standard deviation (SD); ns – not statistically significant by Student's t test.

4.2.2. Transient depletion of PKR rescues HSV-1 replication in ADAR1 KO cells

After demonstrating that apoptosis was not the primary factor limiting viral replication, we investigated whether that activation of innate immune pathway contributed to the observed phenotype.

Host cell detects viral infection through multiple pathogen-associated molecular patterns (PAMPs) produced during ongoing infection. Several cytosolic sensors recognise these structures and initiate antiviral response. As ADAR1 is known to regulate innate immune response through interactions with mostly dsRNA sensing pathways, we selected a panel of sensors and their downstream effectors, previously reported to have interactions with ADAR1, or functionally overlap with ADAR1 regulated pathways. These include, RIG-I (senses 5'-triphosphate ds or ssRNA), MDA-5 (detects long dsRNA), MAVS (downstream effector of RIG-I and MDA5 which activates downstream NF-κB pathway), PKR (cytosolic dsRNA sensor which induces translational arrest), LGP-2 (modulator of RIG-I MDA5 signalling pathway), OAS-1 (dsRNA sensor modulates RNAse L and viral RNA degradation), and ZBP1 (Z-form nucleic acid sensor and activator of necroptosis and inflammasome). cGAS (a cytosolic

dsDNA sensor activating STING pathway) included as a control due to known role during HSV-1 infection despite no established interaction with ADAR1.

In ADAR1 KO cells, each of these sensors were transiently depleted using siRNA to determine their contribution to antiviral phenotype. Based on previously reported studies, particularly in KSHV, we expected depletion of MDA5-MAVS to restore HSV-1 replication in absence of ADAR1. However, to our surprise, depletion of MDA5 or MAVS did not produce a significant rescuing effect on HSV-1 (Fig. 24).

In contrast, though depletion of RIG-I or LGP-2 resulted reproducible but non-significant increase in viral yield. However, depletion of PKR strongly complemented ADAR1 deficiency and significantly restored HSV-1 replication, increasing viral yield more than one log higher compared to control siRNA treated ADAR1 KO cells. These results identify PKR as the predominant pathway, restricting HSV-1 replication in ADAR1 deficient cells.

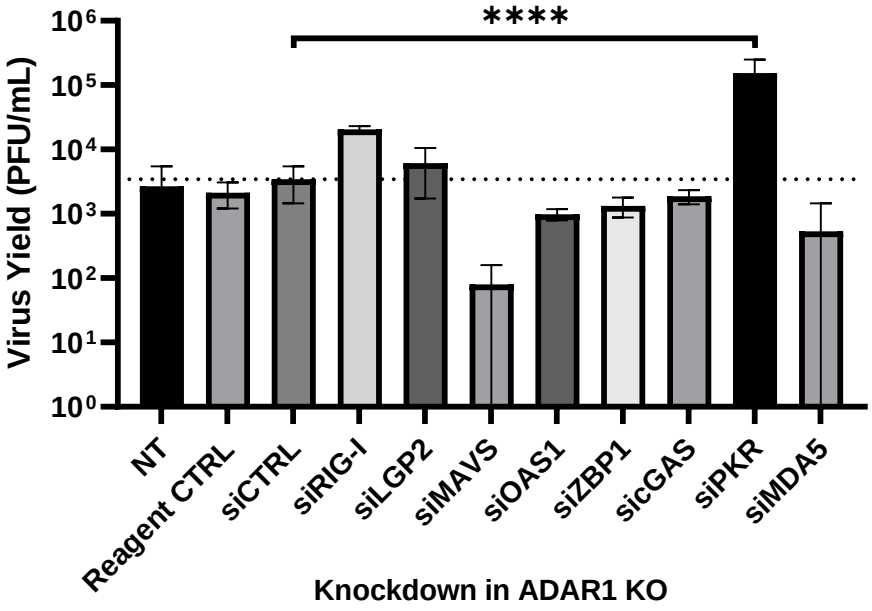


Figure 24. Transient depletion of PKR rescues HSV-1 replication in ADAR1 KO cells. ADAR1 KO cells were transfected with indicated siRNAs or Non-treated (NT), transfected transfection reagent only (Reagent CTRL), random scrambled siRNA control (siCTRL) for 24h and infected with HSV-1 (MOI 1). Virus titres in the supernatants were determined 24hpi. The results represent the mean values of two independent experiments performed in quadruplicates. Data are shown as mean ± standard deviation (SD)); ns – not statistically significant-not shown, **** p<0.0001 by One Way ANOVA.

4.2.3. PKR and downstream effector eIF2α are activated in KO cells upon infection

The siRNA screen in KO cells hinted that PKR might be a major innate immune pathway contributing in reducing the viral replication. Therefore, to further investigate pathway activation and complement the results from siRNA scree, we performed comparative analysis of broader set of target proteins between WT and KO cells with Western blot. This panel included the target proteins in the screen such as PKR, MDA5, RIG-I, LGP2, ZBP1, MAVS, OAS1 as well as the downstream pathway proteins including eIF2α, NF-κB, IRF3 and RIPK3.

Important to note that, under our experimental setup we could not reproducibly detect expression of RIG-I, LGP2, ZBP1, cGAS (proteins in siRNA screen experiment) and RIPK3 (downstream effector of ZBP1). This could indicate low expression or absence of these proteins in WT and KO cells. However, based on this model, we could not conclusively determine whether these proteins play any functional role in regulating HSV-1 replication.

In contrast, upon infection at early time between 6-9hpi, PKR was strongly activated in KO cells than WT cells which persisted until end of experiment at 24hpi. PKR activation was detected by phosphorylation at Thr446, a key autophosphorylation site associated with PKR activation following recognition of dsRNA or other substrates triggers. This observation was concurrent with increased phosphorylation of eIF2α at Ser51, a downstream target of activated PKR (Fig. 25). These results complement the siRNA screen suggesting enhanced PKR dependent signalling in absence of ADAR1.

No major difference observed in expression of other proteins screened such as MDA5, MAVS, OAS1 and TBK1. NF-κB is downstream to most of the proteins covered in siRNA screen and activation of antiviral immunity, however, no change in expression was seen. Although comparatively higher expression was observed in its activated form pNF-κB (phosphorylated NF-κB at Ser536) at 6-12hpi, its activation pattern was comparable between WT and KO cells suggesting similar activation pattern upstream. Together, these results support siRNA screen data and indicate PKR activation is the major antiviral pathway associated with impaired HSV-1 replication in ADAR1 KO cells.

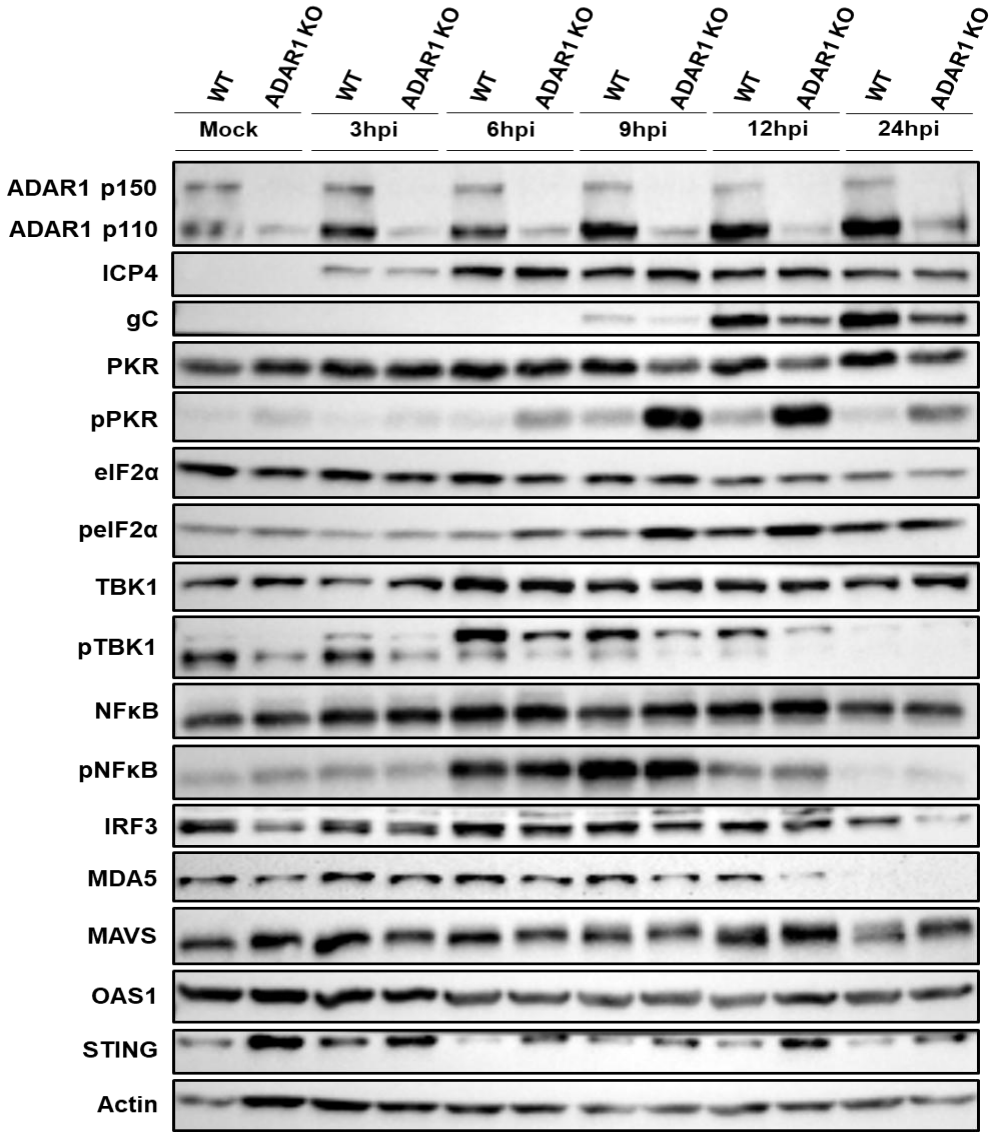


Figure 25. PKR and downstream effector eIF2α are activated in KO cells upon infection. ADAR1 WT and KO cells were infected with HSV-1 (MOI=3). At indicated times after infection (hpi) cells were collected for western blot analysis.

4.2.4. Transient depletion of ADAR1 enhances PKR activation during HSV-1 infection

To provide further evidence that observed ADAR1 mediated suppression of PKR is not a specific property of our HEK knockout (ADAR1 KO) model, we performed siRNA mediated transient knockdown of ADAR1 in additional systems. We utilized two models, HEK293 (ADAR1 WT cells) to assess that the similar results can be achieved without knockout and A549 (human lung epithelial carcinoma cells) with their functional innate immune pathways including RIG-I, MDA-5, PKR and NF-κB, making them ideal to assess further impact of ADAR1 in HSV-1 infection.

Efficient downregulation of ADAR1 was observed in siADAR1 treated WT cells. By 24hpi, eIF2α phosphorylation was higher in infected siADAR1 treated WT cells compared to NT (non-treated) or siCTRL (scrambled siRNA) treated cells. Consistent with PKR pathway activation, virus yield at 24hpi in siADAR1 treated cells were significantly lower than to NT or siCTRL, with no significant difference observed between controls. (Fig. 26)

a. **b.**

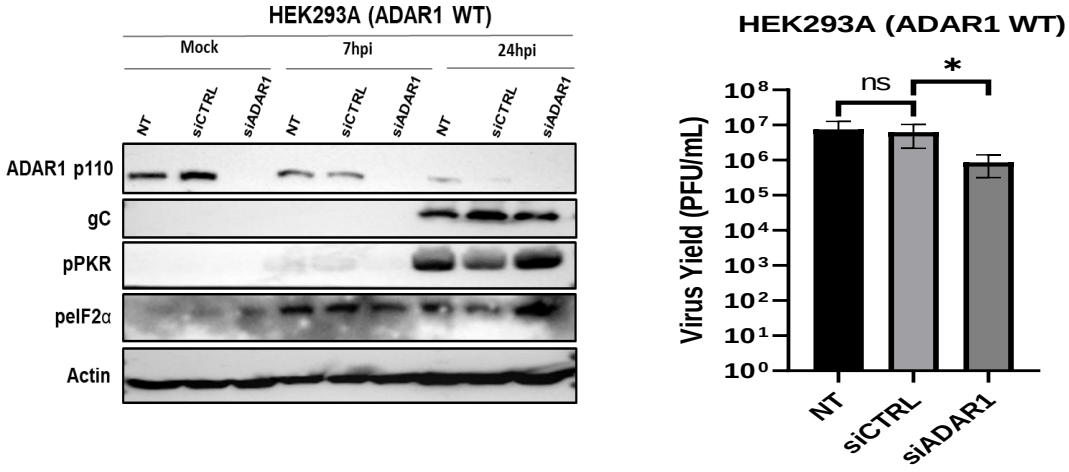


Figure 26. Transient depletion of ADAR1 enhances PKR activation in ADAR1 WT cells. ADAR1 WT cells were transfected with control or ADAR1 siRNA for 24 h and then infected with HSV-1 (MOI 1). **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay at 24hpi. The data shown is representative of multiple (>3) independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; *, p≤0.05 by One-way ANNOVA for (b).

Furthermore, in A549 the knockdown of ADAR1 was less efficient than in WT cell; however, strong phosphorylation of PKR (pPKR) was still observed upon siADAR1 treatment, compared to NT and siCTRL treated cells. At the same time, expression of late viral protein gC was lowered in siADAR1 treated cells (Fig. 27). Although, the viral yield was significantly lowered in siADAR1 treated A549, the effect was less pronounced than in WT cells. As mentioned earlier, this difference may reflect variation of siRNA treatment and response of the cells, and the biology of the cells in terms of viral permissiveness and overall metabolism.

a. **b.**

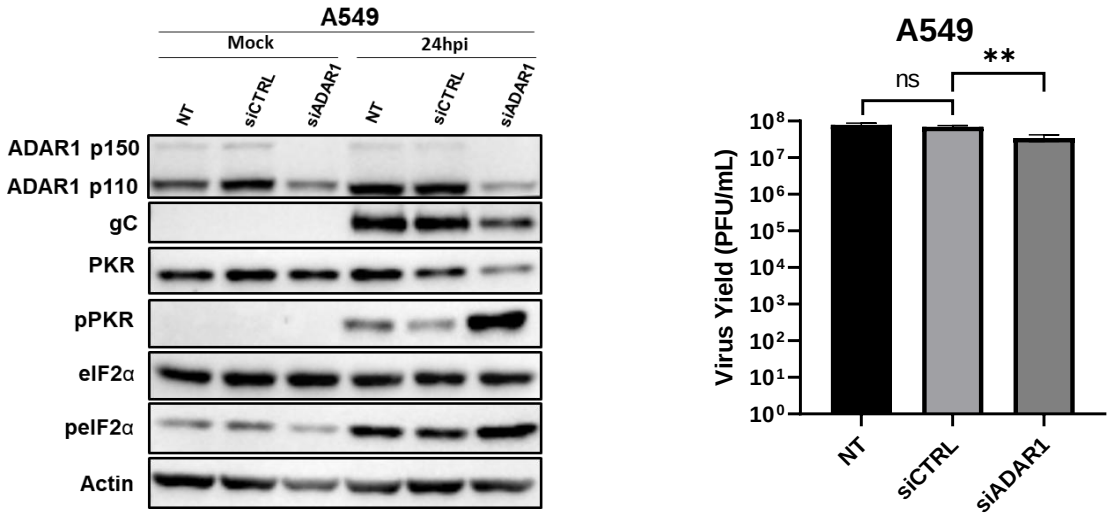
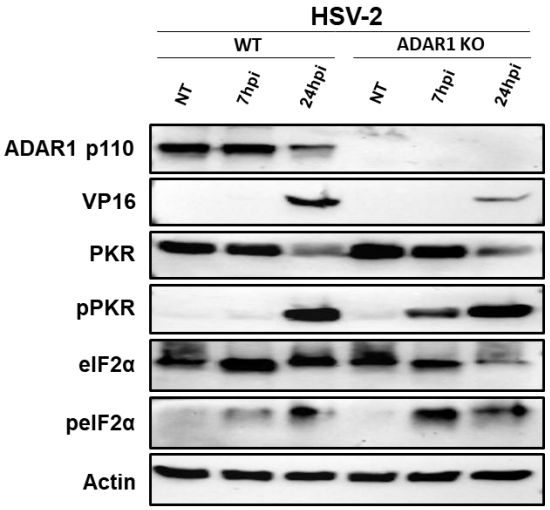


Figure 27. Transient depletion of ADAR1 enhances PKR activation in A549 cells. A549 cells were transfected with control or ADAR1 siRNA for 24 h and then infected with HSV-1 (MOI 1). **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay at 24hpi. The data shown is representative of multiple (>3) independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; **, p≤0.01 by One-way ANOVA for (b).

4.2.5. ADAR1 deficiency enhances PKR activation during HSV-2 infection

Earlier we observed the replication defect of HSV-2 in KO cells. It led to hypothesis that activation of PKR and its downstream effector eIF2α, in ADAR1 deficient system might be conserved molecular phenomenon in the *Simplexvirus* genus. To test this, we infected WT and KO cells with HSV-2, and assessed activation of PKR at early (7hpi) and late (24hpi), and virus yield at 24hpi. concurrent to our earlier observations, HSV-2 yield in WT cells was significantly higher than KO cells. In addition, expression of late viral protein VP16, used here as a marker of infection, was reduced in KO cells. ADAR1 KO cells showed increased phosphorylation of PKR and eIF2α at 7hpi, indicating enhanced activation of PKR pathway early in HSV-2 infection (Fig. 28). Together, these findings suggest that ADAR1 deficiency during HSV-2 infection initiates similar response as HSV-1 in KO cells, suggesting molecular mechanism might be conserved in the *Simplexvirus* genus.

a.



b.

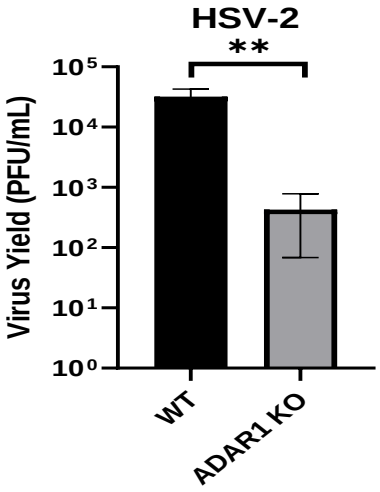


Figure 28. ADAR1 deficiency enhances PKR activation during HSV-2 infection. ADAR1 WT or KO cells were infected with HSV-2 (MOI 1). **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay at 24hpi. The data shown is representative of multiple (>3) independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; **, p≤0.01 by Student's t test for (b).

Overall, our results indicate that in absence of ADAR1, PKR mediated eIF2α phosphorylation is primary pathway activated during HSV-1 infection. Activation of this pathway is likely to promote translational arrest, thereby limiting HSV-1 replication in ADAR1 deficient cells. These findings collectively identify PKR- eIF2α signalling is a major innate immune mechanism impairing HSV-1 replication upon loss of ADAR1.

PART II: Functional Characterization

4.3. PKR/eIF2α-mediated translational arrest is responsible for the HSV-1 replication defect in ADAR1 KO cells

4.3.1. Depletion of PKR rescues HSV-1 replication in ADAR1 KO cells but has limited effect in WT cells

To determine whether PKR restricts HSV-1 replication specifically in ADAR1 KO, or it also limits the replication independent of ADAR1, we further expanded siRNA approach in reciprocity. We treated both ADAR1 WT and KO cells with siRNA targeting PKR, and tested PKR activation and HSV-1 replication. Western blot analysis confirmed efficient depletion of PKR expression. In ADAR1 KO cells, PKR knockdown restored expression of late viral protein gC comparable to WT cells, indicating recovery of viral protein synthesis. In contrast, PKR depletion had no visible effect on expression of gC in WT cells. Consistent with protein analysis, PKR depletion in ADAR1 KO cells significantly rescued HSV-1 replication, whereas in WT cells effect way minor and statistically not significant (Fig. 29). Although, contribution from ADAR1 independent of PKR cannot be completely ruled out, this result strongly suggests that impaired HSV-1 replication in ADAR1 KO cells is primarily driven by PKR.

a. **b.**

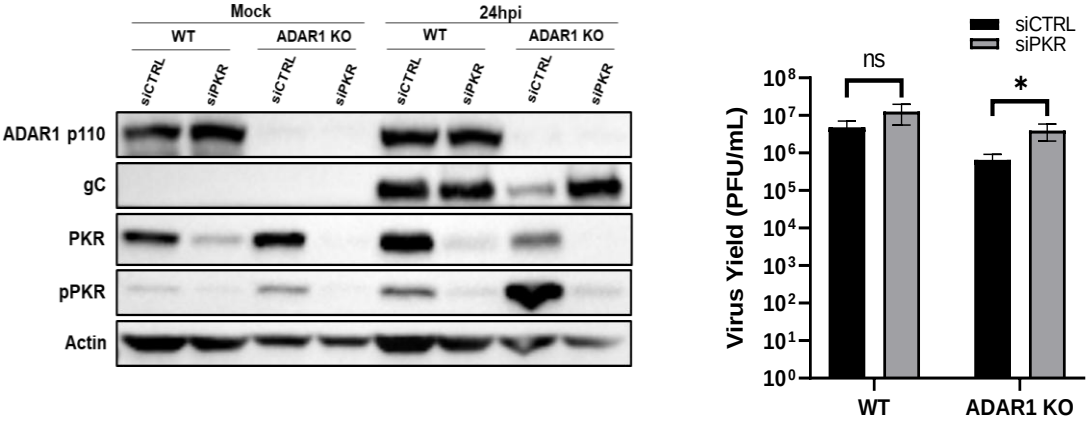


Figure 29. Depletion of PKR rescues HSV-1 replication in ADAR1 KO cells. ADAR1 WT and KO cells were transfected with control or PKR siRNA for 24h and then infected with HSV-1 (MOI 3). **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay at 24hpi. The

data shown is representative of multiple (>3) independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; *, p≤0.05 by Student's t test for (b).

4.3.2. PKR is the primary restricting factor limiting HSV-1 replication in ADAR1 depleted A549 cells

Having shown that PKR depletion rescues HSV-1 replication in ADAR1 KO cells and it is dependent of ADAR1 expression, we asked whether the same ADAR-PKR axis could be observed in an independent cell model. To further complement results obtained in the siRNA screen using ADAR1 KO cells, we employed simultaneous knocked down approach in A549 cells. ADAR1 was depleted across experimental conditions, together with individually targeted each one of the cytosolic sensors, providing combination of double knockdown conditions (i.e., ADAR1-/RIG-I-, ADAR1-/LGP2-, ADAR1-/MAVS-, ADAR1-/OAS1-, ADAR1-/ZBP1-, ADAR1-/cGAS-, ADAR1-/PKR-, ADAR1-/MDA5-). This reproduced similar screening strategy used for siRNA screen in ADAR1 KO cells.

Consistent with the observation made in ADAR1 KO cells, depletion of PKR together with ADAR1 was the only condition that significantly restored the HSV-1 replication to the levels of non-treated cells. Co-depletion of RIG-I, LGP2, MAVS, or MDA5 with ADAR1 produced modest but non-significant increase in viral replication (Fig. 30). These results support PKR as the primary antiviral factor responsible for restricting HSV-1 replication in ADAR1 deficient conditions.

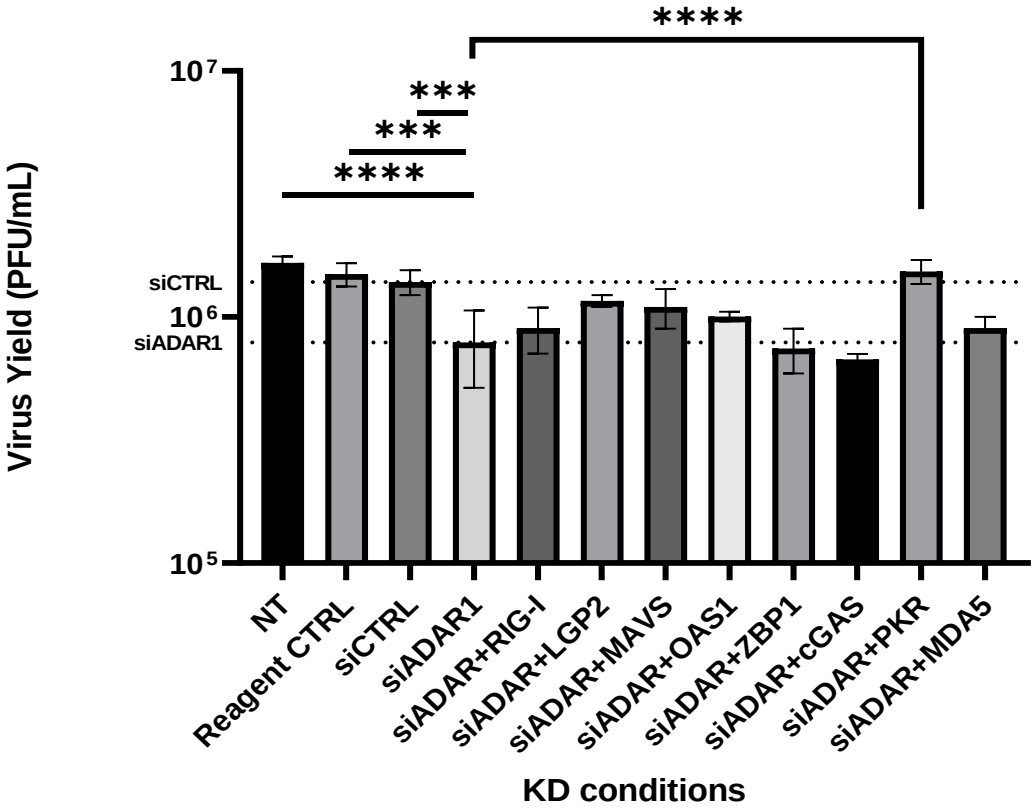


Figure 30. PKR is the primary restricting factor limiting HSV-1 replication in ADAR1 depleted A549 cells. A549 cells were transfected with indicated siRNAs or Non-treated (NT), transfected transfection reagent only (Reagent CTRL), random scrambled siRNA control (siCTRL) for 24h and infected with HSV-1 (MOI 1). Virus titres in the supernatants were determined 24hpi. The results represent the mean values of two independent experiments performed in triplicates. Data are shown as mean ± standard deviation (SD)); ns – not statistically significant-not shown, *** p<0.001, **** p<0.0001 by One Way ANOVA.

In concurrence with earlier results in HEK model, not all cytosolic sensors could be readily and reproducibly detected in A549 cells, however, ADAR1 knockdown was confirmed across all cases, albeit in varying degrees. Interestingly, in ADAR1 depleted cells infected with HSV-1, increased PKR phosphorylation was observed, irrespective of whether other sensors were retained or depleted. These results suggest the dominant role of PKR activation during HSV-1 infection in absence of ADAR1 across tested systems (Fig. 31).

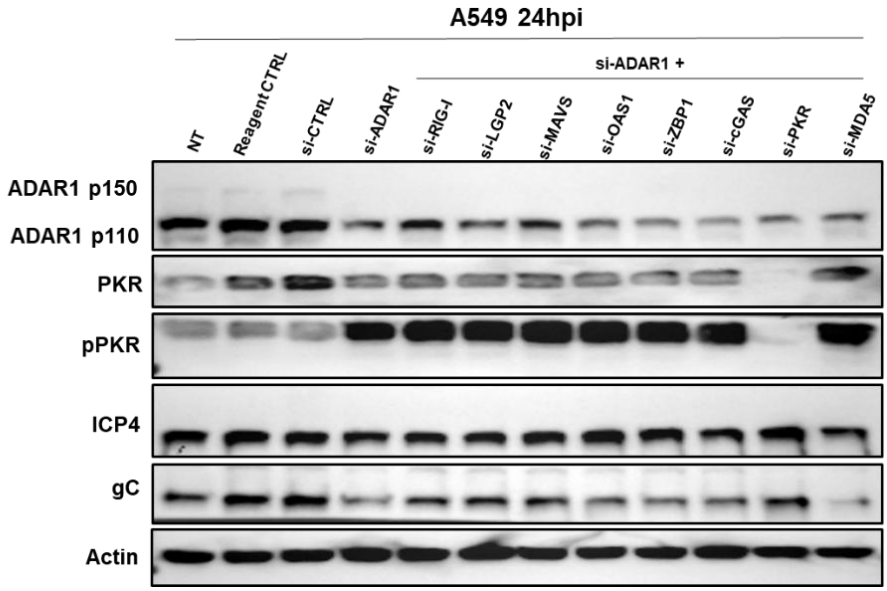


Figure 31. Transient knockdown of ADAR1 activates PKR in A549 cells. A549 cells were transfected with indicated siRNAs or Non-treated (NT), transfected transfection reagent only (Reagent CTRL), random scrambled siRNA control (siCTRL) for 24h and infected with HSV-1 (MOI 1). Cells were collected in RIPA buffer at 24hpi for western blot analysis.

4.3.3. ADAR1 regulates HSV-1 replication in A549 cells through the PKR axis

To further substantiate our finding from A549 siRNA screen and PKR depletion ADAR1 WT cells, we knocked down ADAR1 or ADAR1 and PKR simultaneously in A549 cells. Western blot analysis revealed that, in ADAR1 knockdown PKR was indeed activated reducing late viral protein gC. However, simultaneously depleting PKR with ADAR1 enhanced gC expression suggesting better viral replication. In agreement with protein level results, viral titre analysis showed that HSV-1 production was significantly reduced with ADAR1 depletion, however simultaneous depletion of ADAR1 and PKR rescued viral replication, slightly above control levels (Fig. 32). Though the extent of rescue was slightly greater than that observed in siRNA screen, this likely reflects differences in knockdown efficiency, and experimental variability. Importantly, both experiments consistently indicate that PKR depletion counteracts replication defect of HSV-1 due to loss of ADAR1.

a. **b.**

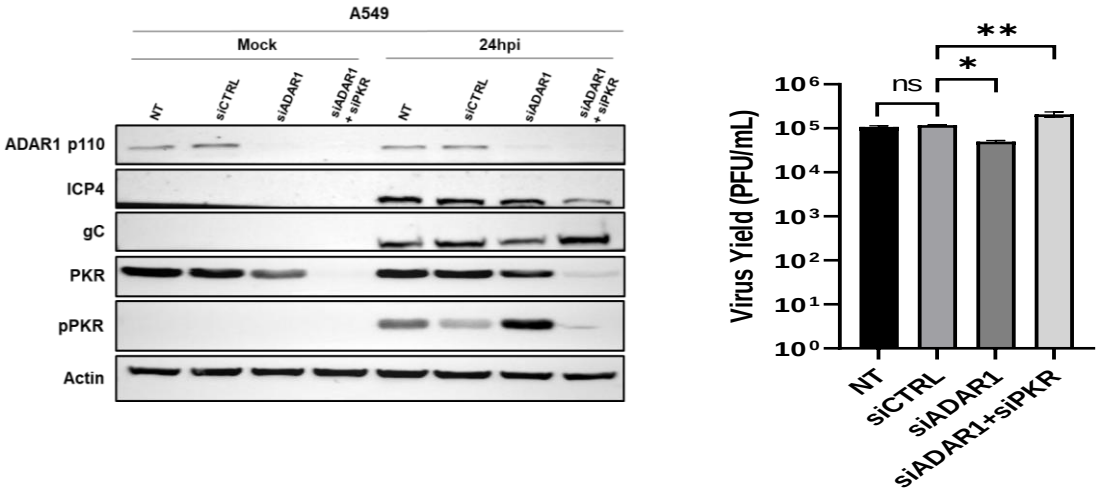


Figure 32. ADAR1 regulates HSV-1 replication in A549 cells through the PKR axis. A549 cells were transfected with control or ADAR1, PKR or ADAR1 and PKR siRNA for 24h and then infected with HSV-1 (MOI 3). **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay at 24hpi. The data shown is representative of three independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; *, p≤0.05, ** , p<0.01 by One Way ANOVA for (b).

4.3.4. ICP34.5 mediated reversal of eIF2α phosphorylation rescues HSV-1 replication in ADAR1 KO cells

The results described earlier indicated that PKR and its downstream effector eIF2α contribute to impaired viral replication in absence of ADAR1. Therefore, we hypothesized that reversing the downstream effect of PKR activation should have similar effect as depleting PKR. HSV-1 encodes various proteins that directly inhibits PKR and downstream effectors of PKR. A late viral protein ICP34.5 (Infected cell protein 34.5) is a critical virulence factor which reduces effect of PKR activation by dephosphorylating eIF2α by recruiting protein phosphatase 1 (PP1), enabling continued protein synthesis.

To test whether the ectopic expression of ICP34.5 could reverse the replication defect in ADAR1 KO cells, we transfected cells with FLAG-tagged ICP34.5 expression plasmid before HSV-1 infection. Usually, ICP34.5 is expressed at later stages of infection, after strong PKR/eIF2α activation in ADAR1 deficient cells, therefore transfection with expression plasmid allowed ICP34.5 to be present well before infection.

Expression of ICP34.5 was confirmed by detecting FLAG tag in Western blot. Upon infection in ADAR1 KO cells expressing ICP34.5, phosphorylation of eIF2α was reduced. PKR phosphorylation was also reduced, but it was less pronounced than eIF2α. Western blot analysis further confirmed that the transfection itself, even in mock infected cells, can contribute to activation of PKR, which provides plausible explanation to why we did not observe dramatic upregulation in expression of gC in ICP34.5 expressing cells compared to non-transfected (NT) cells, although gC expression is higher than control plasmid transfected (pcDNA) cells (Fig. 33).

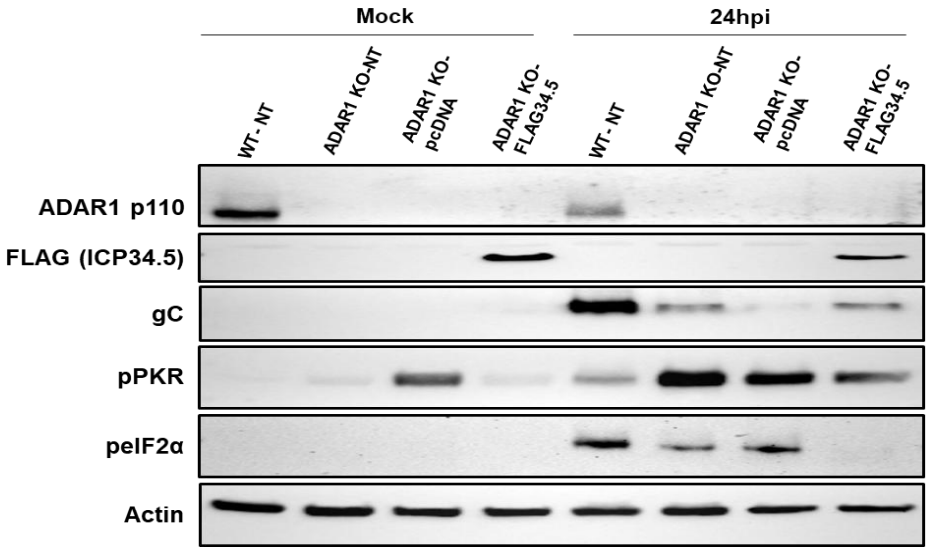


Figure 33. Early expression of ICP34.5 of can reverse the effect of PKR activation in HSV-1 infected ADAR1 KO cells. ADAR1 KO cells were transfected with a control plasmid (pcDNA) or plasmid expressing FLAG-ICP34.5 protein. At 24h transfected cells were infected with HSV-1 (MOI=3). Western blot analysis of proteins extracted from infected cells 24hpi.

Viral yield reflects a similar pattern, with significant increase observed in ICP34.5 expressing ADAR1 KO cells than both controls, however it was not restored to the levels of ADAR1 WT cells (Fig. 34). In addition to PKR activation upon transfection, limited efficiency of transfection likely contributed in incomplete phenotype. Here, we estimate that below 30% of the total cells were transfected, which underestimated real capacity of ICP34.5 in inhibiting this pathway. Nevertheless, this approach successfully delivered that, reversal of downstream effects of PKR/eIF2α activation indeed rescues viral replication in absence of ADAR1.

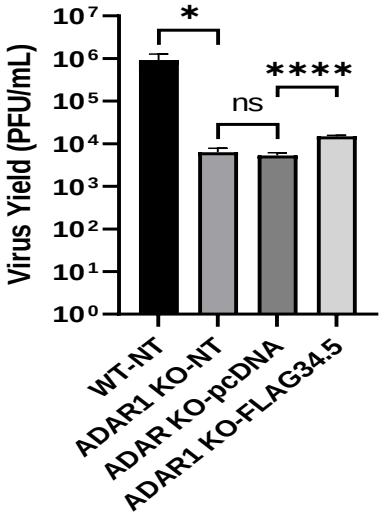


Figure 34. Expression of ICP34.5 rescues HSV-1 replication in ADAR1 KO cells. ADAR1 KO cells were transfected with a control plasmid (pcDNA) or plasmid expressing FLAG-ICP34.5 protein. At 24h transfected cells were infected with HSV-1 (MOI=3). Virus titre was determined in supernatants collected at 24hpi. The data shown is representative of multiple (>3) independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; *, p≤0.05, ** , p<0.01 by One Way ANOVA.

4.3.5. Inhibition of translational arrest using pharmacological agent effectively rescues HSV-1 replication in ADAR1 KO cells

The results described above demonstrated that PKR mediated translational arrest was key mechanism in limiting viral production in absence of ADAR1. To further substantiate this conclusion, we utilized pharmacological approach. In contrast with transfection and overexpression of protein, where efficiency and cell response could add additional layer of complexity, this approach allows more uniform and consistent exposure to achieve maximum effect.

ISRIB (Integrated Stress Response Inhibitor, chemically [(2R)-2-[(4S)-4-(3,5-dimethylphenyl)-4,5-dihydro-1H-imidazol-2-yl]-1-hydroxyethyl]benzamide) is a small molecule, mechanistically does not act on kinases themselves. It selectively reverses effect of eIF2α phosphorylation by binding to and stabilizing active confirmation of eIF2B, thereby restoring global translation in cell, despite presence of activated kinases and phosphorylated eIF2α.

ADAR1 WT and KO cells were infected with HSV-1 at high (3) and low (0.01) MOI cells in presence of ISRIB. Under high MOI conditions, ISRIB completely rescued viral replication in ADAR1 KO cells to the levels of WT cells. It also increased viral load

more than 10 times under low MOI by 48hpi. In either high or low MOI conditions, ISRIB had no significant effect on viral replication in ADAR1 WT cells, indicating its direct action only in context of PKR activation in ADAR1 KO cells (Fig. 35).

a. **b.**

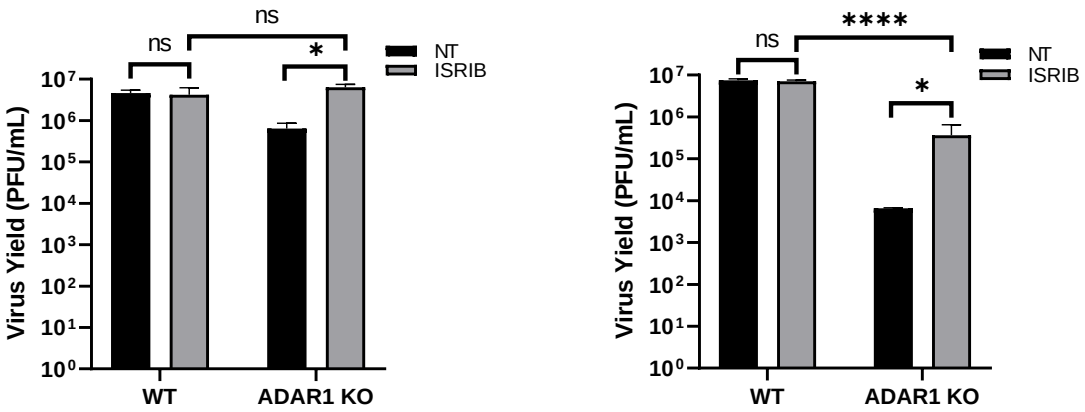


Figure 35. Inhibition of translational arrest using ISRIB effectively rescues HSV-1 replication in ADAR1 KO cells. ADAR1 WT and KO cells were pre-treated with 0.5µM Integrated Stress Response Inhibitor (ISRIB) for 30min, and infected with HSV-1 (a. MOI 3, b. MOI 0.01). Virus yield was determined by plaque assay at 24hpi for (a) and 48hpi for (b). The data shown is representative of three independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; *, p≤0.05, **** , p<0.0001 by Student's t test for each denoted pair.

Protein analysis of ISRIB treatment was performed under high MOI conditions with two timepoints, 7hpi where we observe strong PKR activation and 24hpi a later timepoint in the infection. At 24hpi, in both ADAR1 WT and KO cells infected in presence of ISRIB, we observed slight decrease in PKR phosphorylation, but did not affect levels of PKR or phosphorylation of eIF2α. Additional analysis confirmed that ISRIB treatment alone did not have effect on PKR or eIF2α levels when compared to non-treated cells. Although ISRIB rescued HSV-1 replication in ADAR1 KO cells, the reduction in PKR phosphorylation without a corresponding decrease in eIF2α phosphorylation suggests that additional mechanisms may contribute to the rescue effect. Nonetheless, consistent to viral titres, in ISRIB treated and infected ADAR1 KO cells, levels of late protein gC were restored similar to ADAR1 WT (Fig. 36). These results support the conclusion that ISRIB efficiently counteracts effect of ADAR1 deficiency, PKR activation and subsequent translation block during HSV-1 infection.

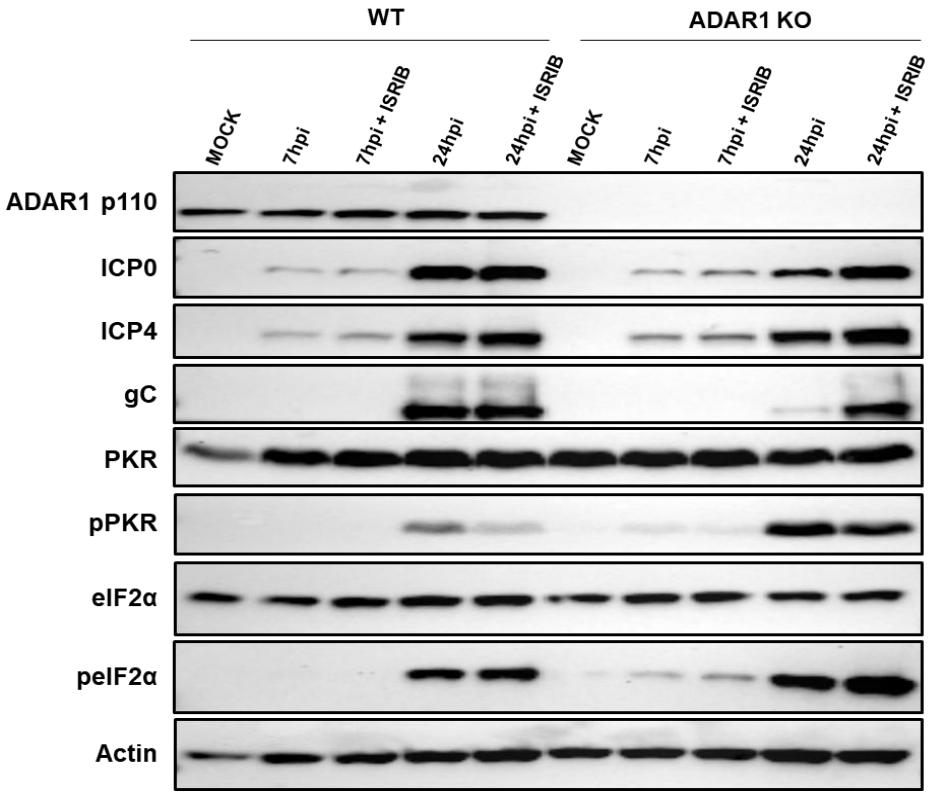


Figure 36. Pharmacological inhibitor of translational arrest reverses effects of PKR/eIF2α activation. ADAR1 WT and KO cells were pre-treated with 0.5μM Integrated Stress Response Inhibitor (ISRIB) for 30min, and infected with HSV-1 MOI 3. Cells were collected in RIPA buffer for Western blot analysis at indicated timepoints.

4.3.6. Increased ATF-4 protein expression indicates translational arrest in ADAR1 KO cells during HSV-1 infection

In our earlier experiments we provided evidence that in the absence of ADAR1, PKR phosphorylates eIF2α potentially leading to translational arrest. In addition, we show that, viral or pharmacological inhibitors were able to reverse the affected viral production. However, it remained unclear that whether activation of the PKR/eIF2α pathway functionally indeed resulted in translational arrest in ADAR1 deficient cells.

To address this question, we hypothesized that in cells undergoing certain level translational arrest, we should observe increased levels of ATF-4 (Activating Transcription Factor 4). ATF-4 is a key transcription factor undergoes selective translation in cellular stress conditions. Usually, ATF-4 is poorly translated in cells due to presence of upstream open reading frames (uORFs) in 5’ untranslated region (5’-UTR), thus inhibiting translation of main coding sequence. In contrast during stressed condition caused due to eIF2α phosphorylation, ribosomes bypass these uORFs,

allowing ATF-4 expression. This paradoxical conditions in cell during stress response caused by phosphorylated eIF2α serve ATF-4 as functional indicator of translational arrest.

We first assessed if ATF-4 transcript levels differed in ADAR1 WT vs KO cells during HSV-1 infection. Quantitative detection showed that levels of ATF-4 mRNA in both cell types was comparable upon normalizing to levels of 18S. It retained the trend of comparable levels of expression in both cell lines throughout the infection, although gradually decreasing as infection progressed, consistent with the previous transcriptome analysis (Fig. 37). This indicated infection did not specifically increased ATF-4 transcription in ADAR1 KO cells.

a. **b.**

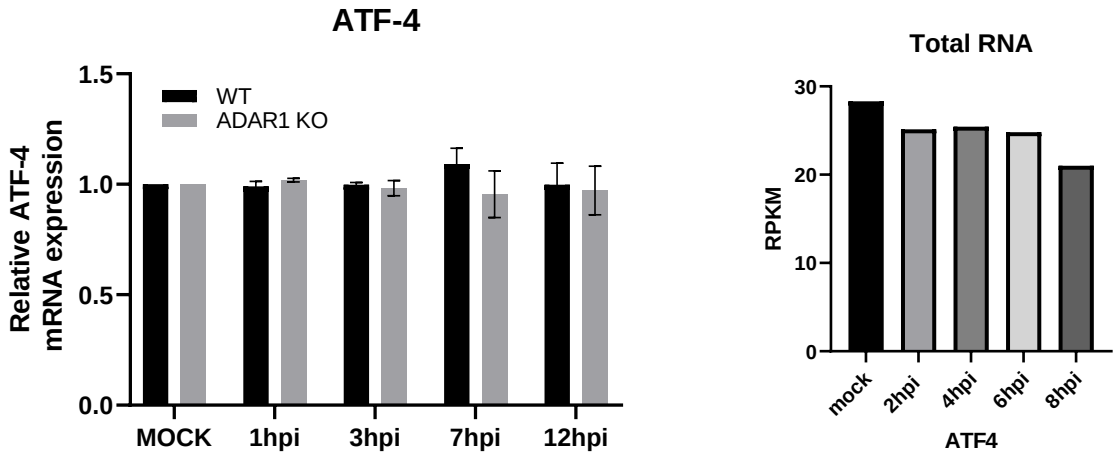


Figure 37. Transcription of ATF-4 does not differ in ADAR1 WT and KO cells during HSV-1 infection. a. ADAR1 WT and KO cells were infected with HSV-1 at MOI 1. RNA was extracted and RT-qPCR was performed on ATF-4. Experiment was performed in two independent replicates. **b.** Transcripts ENSG00000128272 (ATF-4) and is indicated as reads per kilobase per million mapped reads (RPKM) taken from total RNA of HFF cells infected with HSV-1 strain 17 at MOI 10 *[107]*.

Followed to transcription we examined protein levels by Western blot. In unstressed conditions, detection of ATF-4 protein can be challenging, which could result in missed or incorrect interpretation of expression of ATF-4 in stressed conditions. Therefore, we included thapsigargin, a widely used ER stress inducer as positive control. Thapsigargin is a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, which causes ER Ca^{2+} depletion and misfolding of ER proteins, which leads to activation of unfolded protein response (UPR) pathway resulting in translational block and increase in ATF-4 expression through PERK/eIF2α activation.

Upon HSV-1 infection, we detected high levels of ATF-4 protein by 24hpi in ADAR1 KO cells, whereas no substantial increase was seen in WT cells. Importantly, there was no difference in induction of stress response in ADAR1 WT vs KO cells upon thapsigargin treatment, indicating both cell lines were capable of activating ATF-4 translation through stress response pathways other than HSV-1 mediated PKR activation. Therefore, this selective increase in ATF-4 during HSV-1 infection in ADAR1 KO cells suggests that loss of ADAR1 promotes eIF2α dependent translational arrest during infection (Fig. 38).

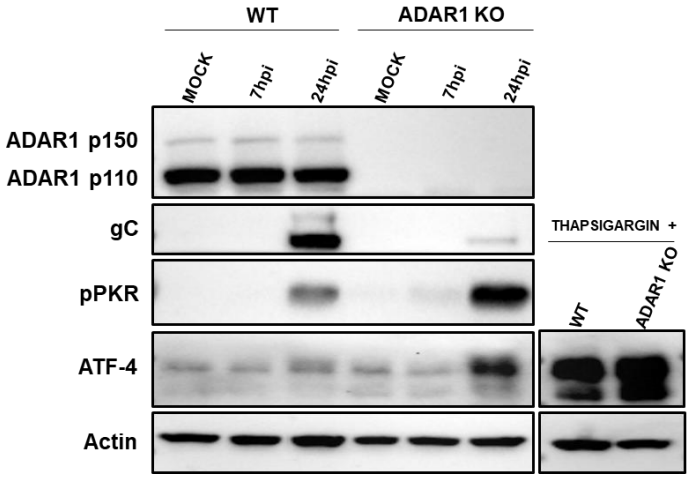


Figure 38. Increased level of ATF-4 expression in ADAR1 KO cells during HSV-1 infection ADAR WT and ADAR1 KO were infected with HSV-1 at MOI 1 or treated with thapsigargin for 7h. At the indicated timepoint cells were collected in RIPA buffer for Western blot analysis.

4.3.7. Translational difference between ADAR1 WT and KO cells is below detection limit of global protein synthesis assays

Our results strongly indicated the PKR/eIF2α mediated translational arrest in HSV-1 infected ADAR1 KO cells. In an effort to determine level of translational arrest in infected ADAR1 WT and KO cells, we decided to measure nascent protein synthesis with non-radioactive labelling methods. OPP (O-Propargyl-Puromycin) is synthetic analogue of puromycin containing alkyne functional group. Similar to Puromycin, OPP gets incorporated into elongating polypeptide chains at the ribosome's A-site, causing premature chain termination. Because of the presence of alkyne group, subsequent detection of OPP incorporated protein molecules by click chemistry via CuAAC (copper(I)-catalyzed azide-alkyne cycloaddition) is possible.

Initially, with fluorescent microscopy, we detected OPP incorporation in MOCK infected cells, with no difference in basal level global protein synthesis in ADAR1 WT vs KO cells. Following HSV-1 infection, synthesis of new proteins appeared to reduced insignificantly. This reduction was comparable between both WT and KO cells and no significant difference could be detected with this approach (Fig. 39).

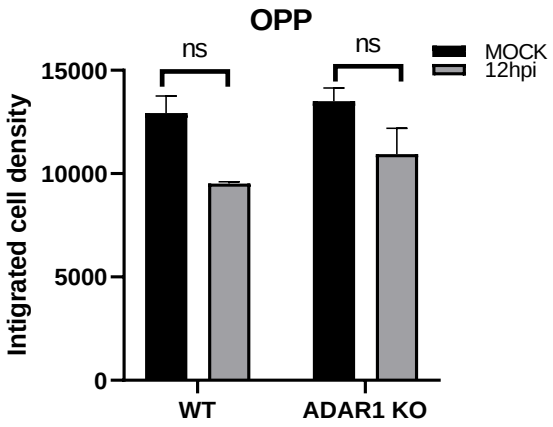


Figure 39. Translational difference between ADAR1 WT and KO cells is below detection limit of global protein synthesis assays. ADAR1 WT and KO cells were infected with HSV-1 MOI 1. At 11hpi cells were treated with OPP and analysed using Zeiss LSM700 confocal laser scanning microscope (Carl Zeiss). Mean immunofluorescence signal intensity per cell was obtained by dividing the total fluorescence intensity (n = 8–10 fields with 1,500 cells) by the number of cells. Data are shown as mean ± standard deviation (SD), 'ns' not significant by Student's t test for each denoted pair.

Though, overall results are very intriguing and one can hypothesize as to specific translational arrest limited mostly to late viral transcripts, collectively with certain host transcripts, we exercise scientific caution before over-interpreting. Specially, given the conditions of technical challenges, relatively high MOI use for synchronised infection, high metabolic activity of cell model, resolution of these methods might not have been sufficient to detect time constrained subtle differences in nascent protein synthesis.

To resolve technical challenges, we also adapted detection method to Western blot using biotin labelling. However, results were not informative due to poor detection in MOCK infected cells as well, rendering this approach unsuitable for detecting translational difference between ADAR1 WT and KO cells.

Nonetheless, collectively considered series of experiments including PKR depletion, ICP34.5 complementation, ISRIB treatment and induction of ATF-4, confirmed early PKR activation and PKR/eIF2α mediated translational arrest is the primary pathway

responsible for reducing viral replication in ADAR1 deficient cells, indicating role of ADAR1 and PKR interaction.

4.4. ADAR1p150 is required to supress PKR activation during productive HSV-1 infection

4.4.1. Complementation with p150, but not p110, prevents PKR activation and rescues HSV-1 replication in ADAR1 KO cells

Having established that ADAR1 suppresses PKR/eIF2α mediated translational arrest during HSV-1 infection, we next asked whether this function is isoform specific or shared by both isoforms. ADAR1 in humans is expressed in mainly two isoforms, the constitutively expressed nuclear p110 isoform, and predominately cytoplasmic interferon inducible p150. We hypothesised that given its cytoplasmic localization, p150 isoform may be more directly involved in suppressing PKR activation, as PKR is cytoplasmic dsRNA sensor. However, p110 is abundantly expressed in basal condition and being enzymatically active, it is highly likely that PKR suppression could have been standalone or combined phenotype.

To address this, firstly we adopted complementation by overexpression approach. ADAR1 KO cells were transfected with plasmids expressing either p110GFP or p150GFP isoforms. Typical transfection efficiency was estimated around 25% to 30% based on GFP expression detected in flow cytometry across multiple experiments. However, despite moderate transfection efficiency, the levels of ADAR1GFP protein expressed in ADAR1 KO cells were much higher than endogenous expression in WT cells. Upon microscopic imaging, we also confirmed localization patterns, with most of the cells transfected with p110GFP showed nuclear GFP expression, and cells transfected with p150GFP had cytoplasmic GFP expression (Fig. 40). Though, both transfection constructs expressed additional isoforms other than expected sizes, with p150GFP construct also producing low amount of p110GFP like signal possibly due to cellular post- transcriptional/translational processes (Fig. 41a). However, intensity of additional bands was lesser than transfected isoform in concurrence with imaging observations suggested that observed phenotypical effects in experiments were primarily due to plasmid originated protein.

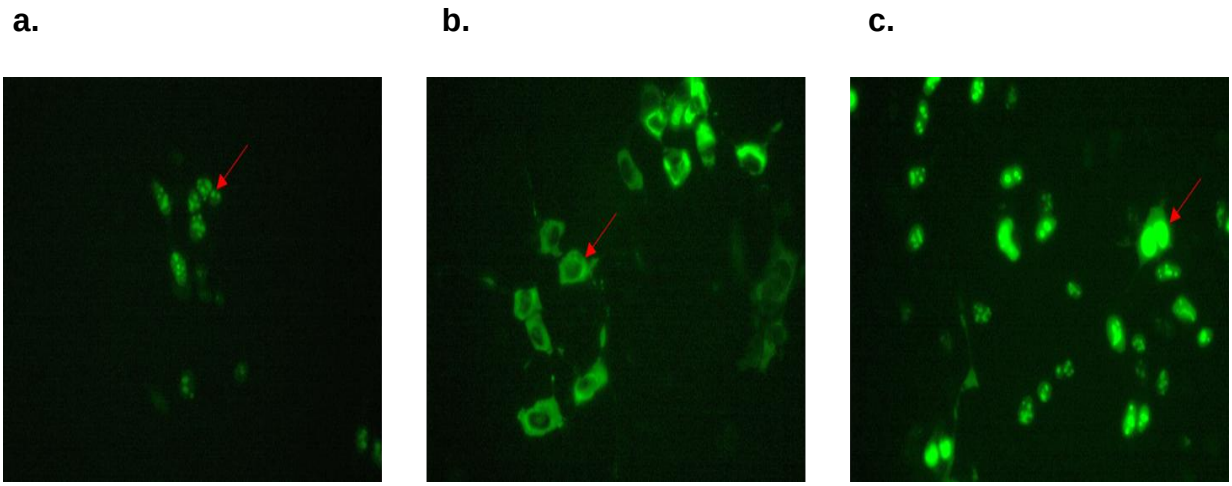
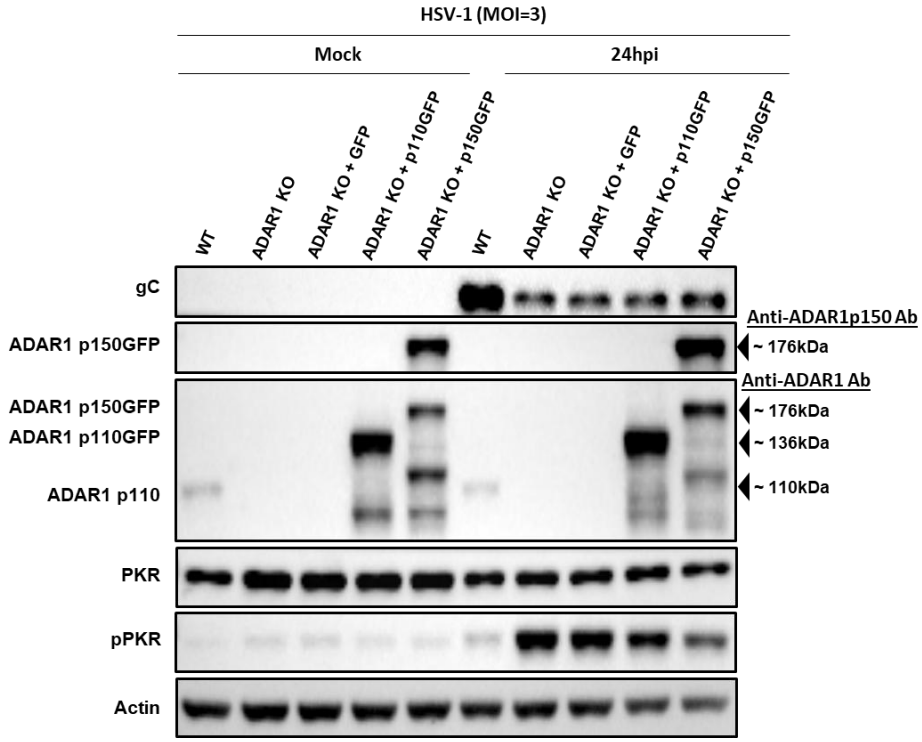


Figure 40. Ectopic expression of ADARs in ADAR1 KO cells. ADAR1 KO cells were transfected with plasmid expressing **a.** ADAR1p110GFP **b.** ADAR1p150GFP or **c.** ADAR2GFP (refer to section 4.4.5.). After 24h images of the transfected cells were captured with Olympus microscope for GFP.

ADAR1 KO cells transfected with constructs were infected with high (3) and low (0.01) MOI. Under high MOI conditions with synchronised infection, cells transfected with p150GFP significantly increased viral production, doubling than non-transfected or GFP transfected or p110GFP controls by 24hpi. Western blot analysis further revealed that phosphorylation of PKR was lowered in p150GFP than non-transfected, GFP transfected or p110GFP transfected cells, without affecting PKR levels. Late viral protein gC was also seen to be increased (Fig. 41). These results suggest that ADAR1p150, but not p110, reduces PKR activation and rescues viral replication under high MOI conditions.

Infections with low MOI successfully reproduced results from high MOI. Extending the infection timing up to 72hpi amplified the rescue phenotype, yielding 10-fold higher viral load in cells with p150GFP transfection than all other transfection conditions. Protein analysis also showed similar trends in late viral protein gC, though it did not restore the replication to levels of ADAR1 WT cells (Fig. 42). This was anticipated outcome due to efficiency of transfection and potential effects of transfection itself altering cell biology.

a.



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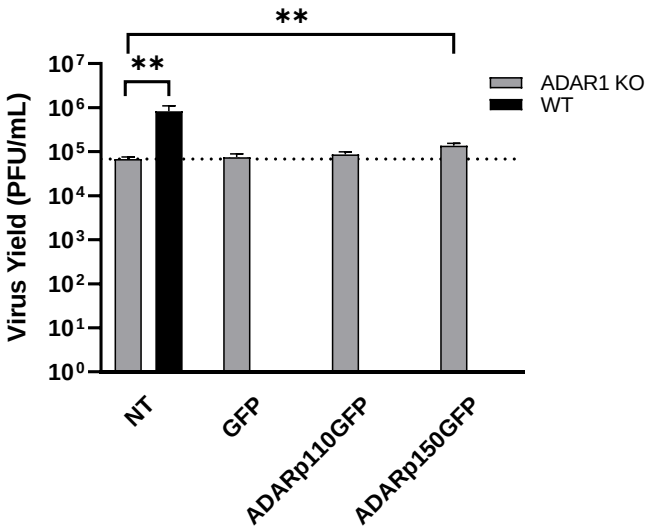
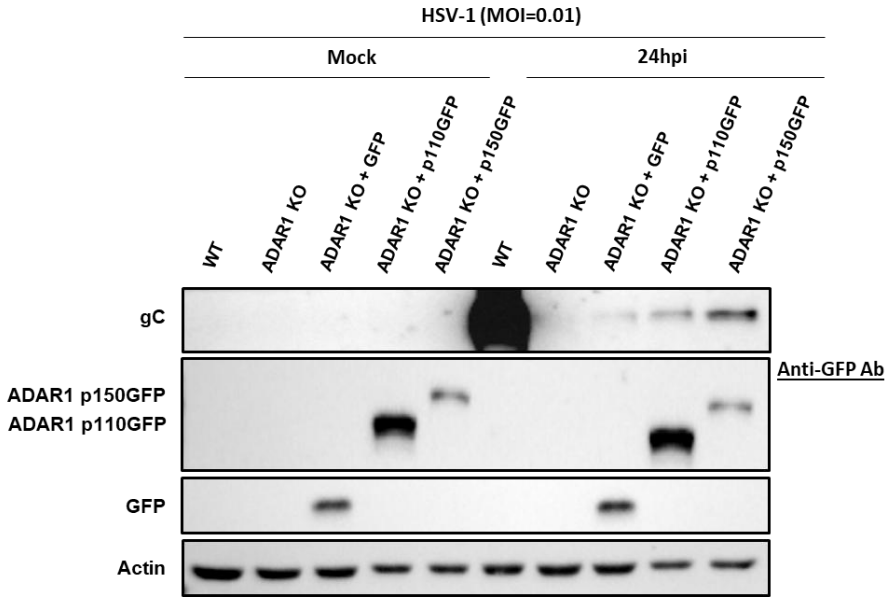


Figure 41. ADAR1p150 Complementation in ADAR1 KO cells rescues HSV-1 replication. ADAR1 KO cells were transfected with control plasmid pEGFP-N1 expressing EGFP, and plasmids ADAR1p110GFP or ADAR1p150GFP. At 24h cells were infected with HSV-1 MOI 3. **a.** Protein analysed by Western blot 24hpi. **b.** Virus yield was determined by plaque assay at 24hpi. Data are shown as mean ± standard deviation (SD). 'ns' not significant-not showed; ** $p<0.01$; by Student's t test for independent comparison of WT-NT and ADAR1 KO-NT and One-Way ANNOVA for all KO samples.

a.



b.

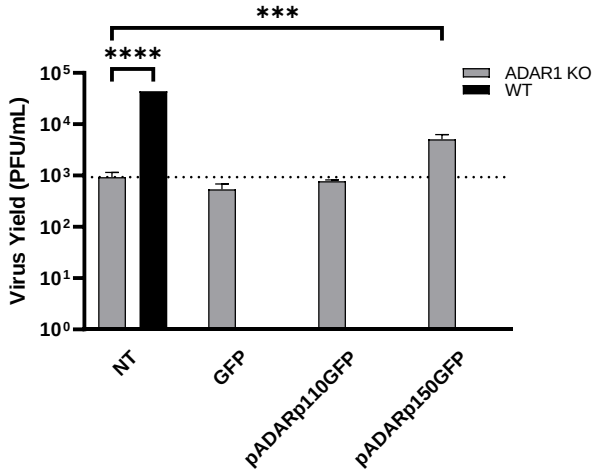


Figure 42. ADAR1p150 Complementation in ADAR1 KO cells rescues HSV-1 replication at low MOI. ADAR1 KO cells were transfected with control plasmid pEGFP-N1 expressing EGFP, and plasmids ADAR1p110GFP or ADAR1p150GFP. At 24h cells were infected with HSV-1 MOI 0.01. **a.** Protein analysed by Western blot 72hpi. **b.** Virus yield was determined by plaque assay at 72hpi. Data are shown as mean ± standard deviation (SD). 'ns' not significant-not showed; ** p<0.01; by Student's t test for independent comparison of WT-NT and ADAR1 KO-NT and One-Way ANNOVA for all KO samples.

Together, the complementation under high as well as low MOI demonstrated that ADAR1p150, but not p110, suppresses PKR activation and recues HSV-1 replication. This indicates that the proviral functions of ADAR1 during productive HSV-1 infection are isoform specific and primarily mediated by the cytoplasmic p150 isoform.

4.4.2. ADAR1p150 specific knockdown reduces viral replication in ADAR1 WT cells

Given the technical complexity involving complementation by transfection in ADAR1 KO cells, we next tested whether isoform specific depletion of ADAR1 could further validate earlier results. Until now, we were working with commercially available ADAR1 siRNA, which was responsible for targeting exon 2 shared by both p110 and p150 isoforms. However, with in-house (kindly provided by Jonathan Maelfeit (CRIG, Ghent University) designed siRNA targeting exon 1A unique to p150, we could target p150 isoform alone, although efficiency was much lower than commercial pan-ADAR1 siRNA.

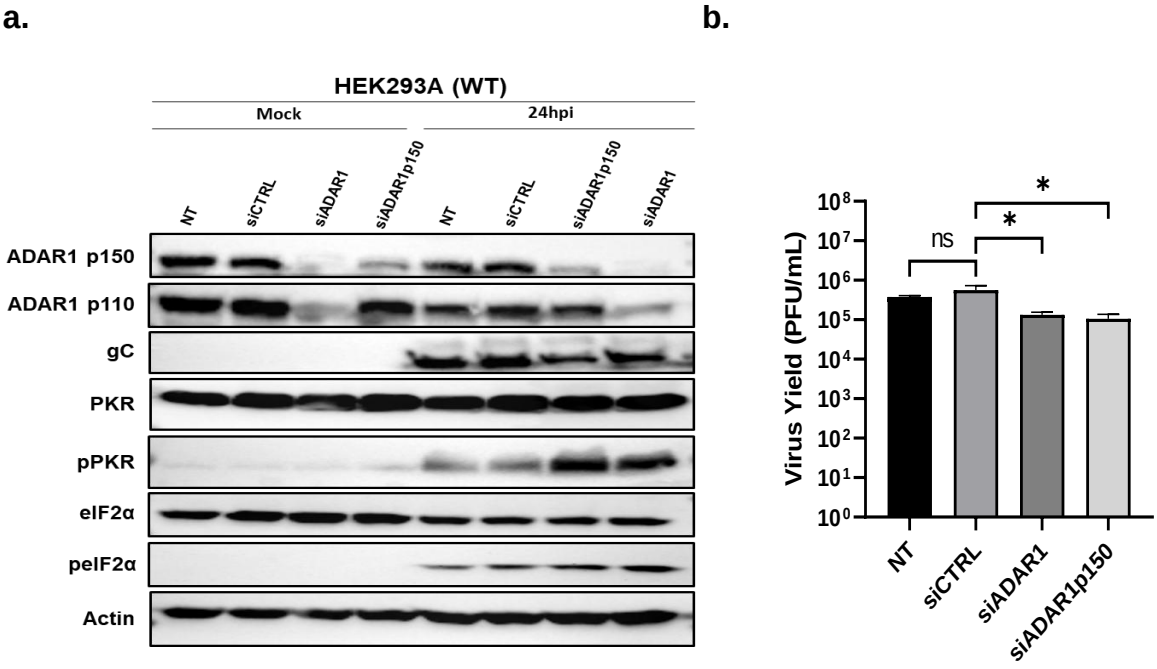


Figure 43. ADAR1p150 specific knockdown reduces viral replication in ADAR1 WT cells. ADAR1 WT cells were transfected with control or ADAR1 or ADAR1p150 siRNA for 24h and then infected with HSV-1 (MOI 3). **a.** Cells were collected in RIPA buffer at 24hpi and proteins were analysed by Western blot. **b.** Virus yield was determined by plaque assay at 24hpi. The data shown is representative of three independent experiments performed in triplicates are shown as mean ± standard deviation (SD). 'ns' no statistical significance; *, p≤0.05 by One Way ANOVA for (b).

Western blot analysis confirmed successful depletion of ADAR1 or ADAR1p150 specifically based on siRNA transfection. Following infection up to 24hpi with high (3) MOI, ADAR1 p150 specific knockdown activated PKR to the levels comparable with total ADAR1 (p110 and p150 together) depletion. Similarly, viral production in p150

specific depletion observed to be significantly reducing from non-transfected or control transfected cells reaching levels of total ADAR1 knockdown (Fig. 30). These findings reinforce the conclusion that ADAR1p150 is the major isoform required to supress PKR activation and support efficient HSV-1 replication.

4.4.3. Depletion of ADAR1p150 in A549 cells activates PKR during HSV-1 infection

To confirm isoform specificity of ADAR1p150 is not property of ADAR1 WT cells, we knocked down ADAR1 both isoforms, or specifically p150 isoform in A549 cells. Reflecting trends in ADAR WT cells, upon infection with HSV-1 high (3) MOI, activation of PKR and decreasing trend of late viral protein gC in isoform specific p150 depletion similar to complete ADAR1 knockdown, was observed (Fig. 44). These findings further support that ADAR1p150 is predominant isoform required to supress PKR activation during productive HSV-1 infection across cell systems.

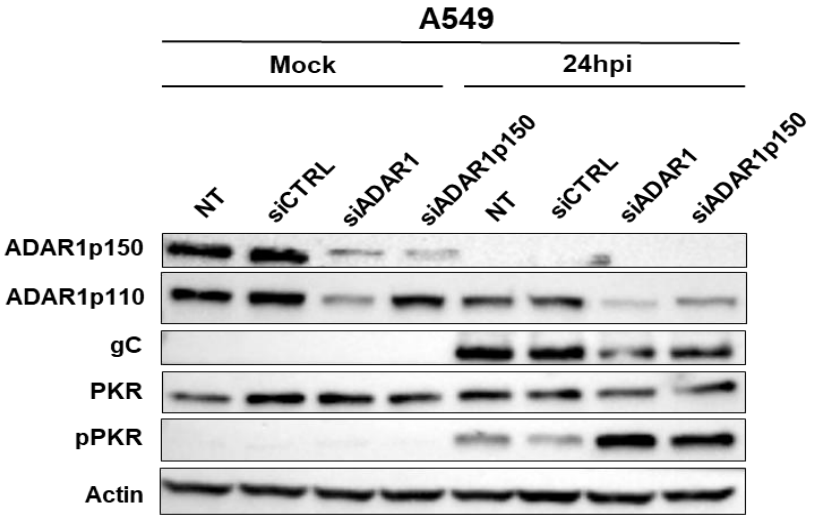


Figure 44. Depletion of ADAR1p150 in A549 cells activates PKR during HSV-1 infection. A549 cells were transfected with control or ADAR1 or ADAR1p150 siRNA for 24h and then infected with HSV-1 (MOI 3). Cells were collected in RIPA buffer at 24hpi and proteins were analysed by Western blot.

4.4.4. Early PKR activation in ADAR1 KO cells occurs independently of IFNβ induction

Having identified ADAR1p150 as the major isoform supressing PKR activation during HSV-1 infection, we asked whether this effect was directly linked to ADAR1-PKR regulation or driven indirectly by altered IFN I signalling. Our earlier experiments ruled

out immediate role of other cytosolic DNA and RNA sensors such as cGAS, RIG-I and MDA5, as well as downstream activation of NF-κB, which triggers IFN I transcription. Since p150 is induced by IFN signalling and appears to have major role in countering PKR activation during HSV-1 infection, we asked if enhanced PKR activation in ADAR1 KO cells could be answered by altered IFN signalling. Specifically, we considered that ADAR1 KO cells might respond more strongly to IFN or produce higher levels of IFN upon HSV-1 infection, therefore priming activation of PKR in absence of compensatory ADAR1p150, which is present in WT cells, and therefore affect HSV-1 replication.

To assess whether ADAR1 KO cells respond to IFNβ, similar to ADAR1 WT cells, we pre-treated both cell types with recombinant IFNβ (20ng/mL) overnight. In WT cells, IFNβ treatment increased ADAR1p150 expression, confirming expected IFN response. As expected, IFNβ treatment increased basal PKR phosphorylation in KO cells, whereas in WT cells signal was below the detection limit. However, in similarly treated and infected cells, IFNβ treated WT cells showed reduced viral protein expression, including decrease in IE protein ICP4 and complete loss of L protein gC, resembling to untreated KO cells. On the other hand, in ADAR1 KO cells, IFNβ treatment further enhanced PKR activation and resulted in complete loss of detectable viral protein expression (Fig. 45). These results confirmed that both ADAR1 WT and KO cells respond to IFNβ and its priming can restrict HSV-1 replication. However, this experiment alone could not confirm whether INFβ signalling was responsible for early activation of PKR in ADAR1 KO cells during infection.

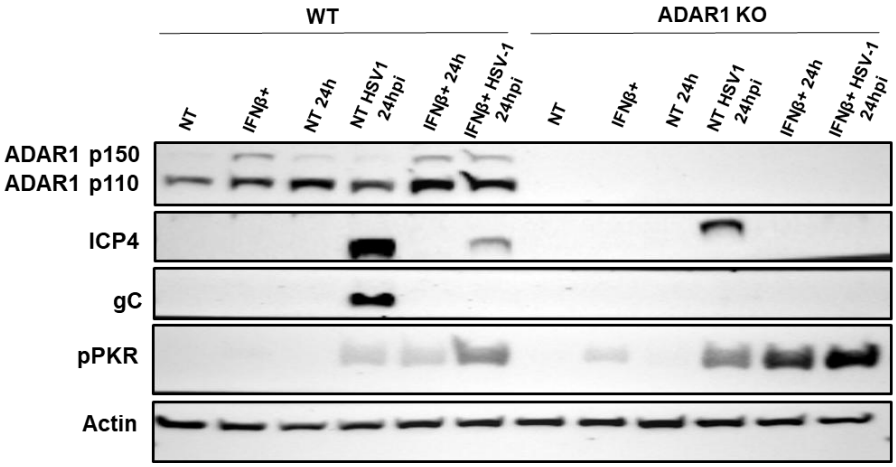


Figure 45. ADAR1 WT and KO showed reduced replication of HSV-1 upon IFNβ treatment. ADAR1 WT and KO cells were treated with 20ng/mL IFNβ for 24h. Cells were infected with HSV-1 (MOI 3) and collected in RIPA buffer at 24hpi. Proteins were analysed by Western blot.

Therefore, we measured IFNβ transcripts levels during HSV-1 infection to determine if IFNβ was induced before or coincided with PKR activation in KO cells. Measuring relative IFNβ mRNA expression showed that in both ADAR1 WT and KO cells there was no significant difference IFNβ transcription up to 7hpi, by which PKR is already activated in KO cells. Surprisingly, by 12hpi, IFNβ transcript levels were significantly higher in WT cells than in KO cells (Fig. 46). These results indicate that PKR activation in HSV-1 infected ADAR1 KO cells occurs before detectable IFNβ induction and therefore unlikely to be driven by IFN feedback. Collectively, this data supports a model in which, ADAR1p150 intrinsically limits PKR activation during HSV-1 infection, independent of IFNβ induction.

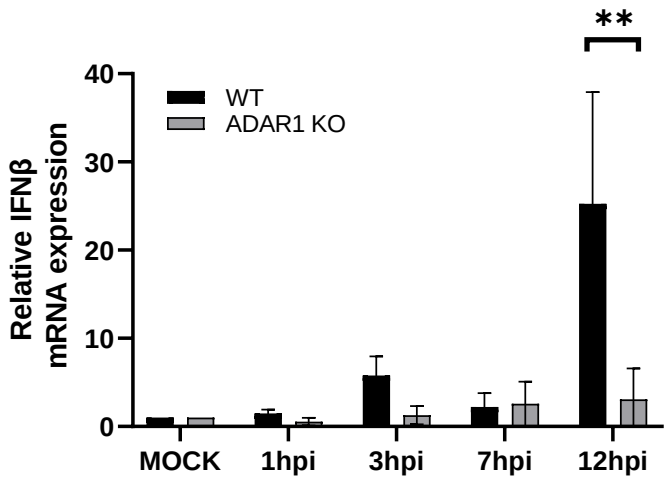


Figure 46. IFNβ does not precede early PKR activation in ADAR1 KO cells. ADAR1 WT and KO were infected with HSV-1 at MOI 1 in triplicates. RNA was extracted from cells at indicated times after infection (hpi). IFNβ transcripts were detected using RT-qPCR. The experiment was performed in triplicates. All samples were normalized to MOCK infected 18S. Data are shown as mean ± standard deviation (SD), 'ns' not significant-not shown; **, p<0.01, by Two-Way ANOVA.

4.4.5. ADAR2 does not complement loss of ADAR1 function during HSV-1 infection in ADAR1 KO cells

ADAR2, encoded by ADARB1 gene, is another catalytically active enzyme from ADAR family and, like ADAR1 contains dsRNA binding and deaminase domain. However, it lacks Z-DNA binding domain, and predominantly expressed in neuronal cells. Subcellular localization of ADAR2 is nuclear and resembles more to ADAR1p110. Given close association of HSV-1 neuronal infection and latency, we asked whether ADAR2 could compensate for loss of ADAR1 during HSV-1 infection.

We transfected ADAR1 KO cells with plasmids expressing ADAR1p110GFP, ADAR1p150GFP and ADAR2GFP. Consistent with previous complementation experiments ADAR1p150 and not p110 complementation significantly improved viral replication. Expression of ADAR2 did not have any significant effect on viral replication (Fig. 47). Although this experiment was performed in non-neuronal cell model, it suggests ADAR2 cannot functionally compensate for loss of ADAR1 during HSV-1 infection.

The inability of ADAR2 to recue HSV-1 replication may reflect functional differences in ADAR1 and ADAR2 or given the fact ADAR2 localizes in nucleus, similar to ADAR1 p110, may limit its access to PKR activation complex in cytoplasm. It remains possible that alteration in localization of ADAR1p110 or ADAR2 could redefine their ability to supress PKR, as suggested in earlier studies, although these observations were not made in context of viral infections [109]. However, to investigate this possibility was beyond the scope of the present study.

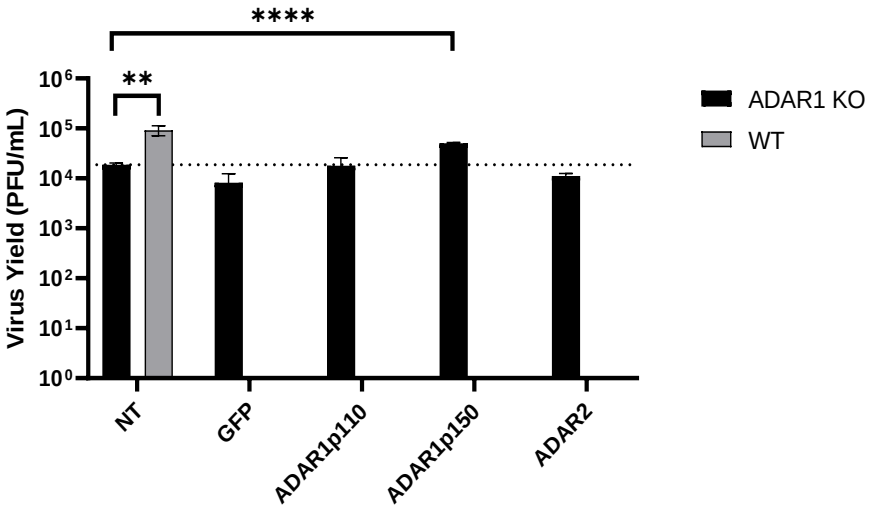


Figure 47. ADAR2 does not complement loss of ADAR1 function during HSV-1 infection in ADAR1 KO cells. ADAR1 KO cells were transfected with control plasmid pEGFP-N1 expressing EGFP, and plasmids ADAR1p110GFP, ADAR1p150GFP or ADAR2GFP. At 24h cells were infected with HSV-1 MOI 3. Virus yield was determined by plaque assay at 24hpi. Data are shown as mean ± standard deviation (SD). 'ns' not significant-not showed; ** $p<0.01$; **** $p<0.0001$ by Student's t test for independent comparison of WT-NT and ADAR1 KO-NT and One-Way ANNOVA for all KO samples.

Throughout part of this series of investigations, we demonstrated that ADAR1p150 plays a dominant proviral role during productive HSV-1 infection. ADAR1p150 supresses PKR/eIF2α pathway activation, thereby preventing translational arrest and enabling efficient viral replication. In contrast, ADAR1p110 and ADAR2 were unable to compensate, highlighting isoform specificity of this function.

PART III: Investigations into Molecular Mechanism

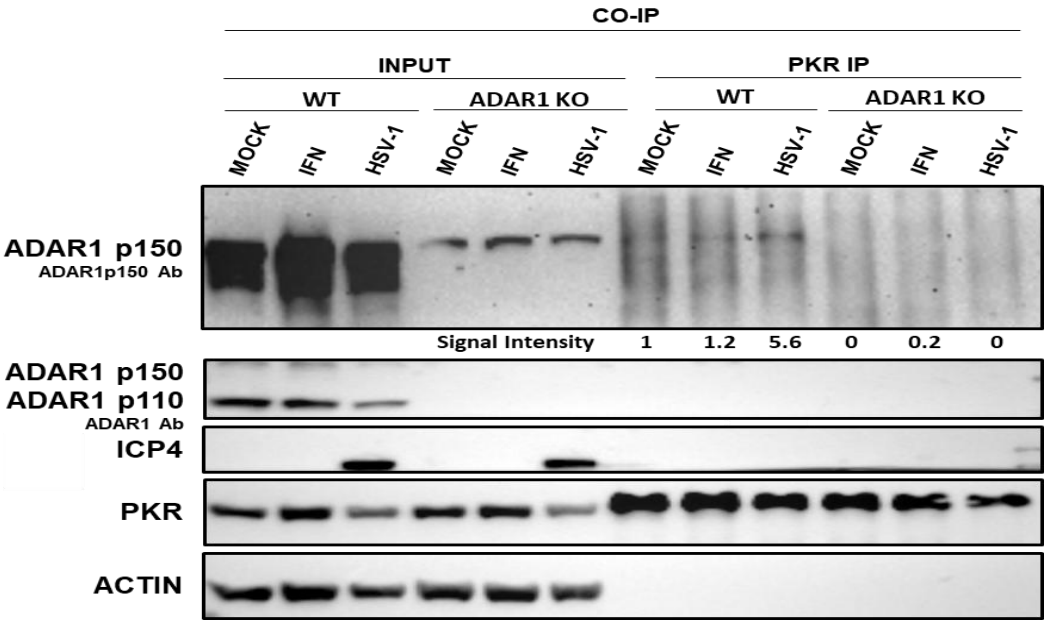
4.5. HSV-1 infection triggers ADAR1-PKR interaction

4.5.1. ADAR1p150 co-immunoprecipitates with PKR during HSV-1 infection

Our previous results described the proviral role of ADAR1p150 in HSV-1 infection by supressing PKR activation and subsequent translational arrest. Next, we wanted to address the molecular mechanism of this function. Previous studies have reported regulation of PKR activation by ADAR1 in viral infections. Recent report also suggested that ADAR1 supresses PKR activation upon interferon stimuli by protein-protein interaction. Therefore, we hypothesised that ADAR1p150 may supress PKR activation during HSV-1 infection through direct interaction with PKR.

To test this, we immunoprecipitated PKR from mock infected, infected with high (3) MOI or IFNβ treated ADAR1 WT and KO cells at 7h post treatment, an approximate timeframe of PKR activation in KO cells. PKR was successfully immunoprecipitated from all samples and the specificity was confirmed by using isotype IgG control. ADAR1p150 co-immunoprecipitated in all WT samples, but the association between the two was significantly higher, by 5 to 7 folds, during active HSV-1 infection in WT cells compared to non-infected cells. Interestingly, treatment with IFNβ only slightly increased co-immunoprecipitated ADAR1, compared to non-treated cells (Fig.48). ADAR1 KO cells served as negative control for ADAR1 detection in the PKR IP. Although PKR was efficiently pulled down comparable to the levels of WT, ADAR1p150 was not detected. Interestingly, despite being abundantly present, ADARp110 was not detected in immunoprecipitated samples, further strengthening our earlier observations that p150 is the key proviral isoform regulating PKR during productive HSV-1 infection.

a.



b.

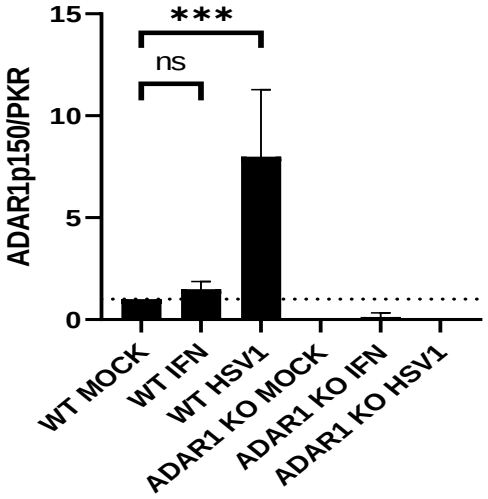


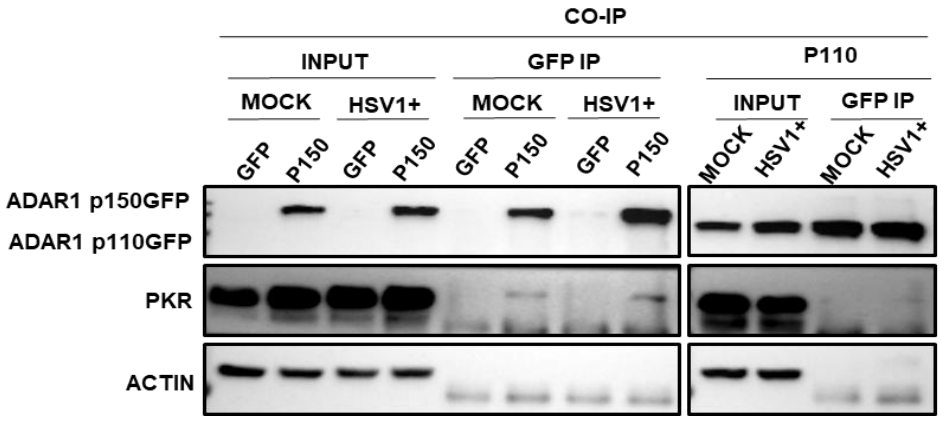
Figure 48. ADAR1p150 co-immunoprecipitates with PKR during HSV-1 infection. ADAR1 WT and KO cells were infected with HSV-1 (MOI 3) or treated with 10ng/mL IFNβ and at 7hpi cells were collected for protein co-immunoprecipitation (co-IP) using anti-PKR antibody. **a.** Representative western blot analysis of the input protein sample and PKR-co-immunoprecipitated proteins. Proteins detected with specific antibodies (indicated in brackets) are shown to the right left of the panel. ADAR1 isoforms were detected with ADAR1 Ab or ADAR1p150 Ab. The signal intensities determined with ImageJ are shown below the panel **b.** ImageJ quantification of co-immunoprecipitated ADARp150 normalized to the PKR signal. Quantification represents three individual experiments (n=3). Data is shown as mean ± standard deviation (SD); 'ns' not statistically significant; ***, $p<0.001$, by One-Way ANOVA for (b).

4.5.2. PKR co-immunoprecipitates with ectopically expressed ADAR1p150 during HSV-1 infection

To further confirm the isoform specific association, we performed reciprocal co-immunoprecipitation in ADAR1 KO cells complemented with plasmids expressing specific isoform of ADAR1, p110GFP or p150GFP. Plasmid expressing GFP included as transfection as well as IP control. Upon microscopic confirmation of GFP expression in all transfected samples, cells were infected with HSV-1 high (3) MOI. At 7hpi, GFP was immunoprecipitated from all lysates, with isotype IgG used as IP control.

We observed that PKR levels present in p110GFP immunoprecipitation were not significantly different than GFP immunoprecipitation in MOCK infected or infected cells. In contrast, PKR co-immunoprecipitated with p150GFP. The p150GFP also co-immunoprecipitated PKR in MOCK infected cells, but levels were 2-3 folds lower than HSV-1 infected cells (Fig. 49). This basal level association may reflect effect of transfection process, however infection of cells with HSV-1 significantly increased this association. In line with endogenous PKR immunoprecipitation results, reciprocal co-IP with ectopic ADAR1 expression confirmed that ADAR1p150 interacts with PKR during HSV-1 infection.

a.



b.

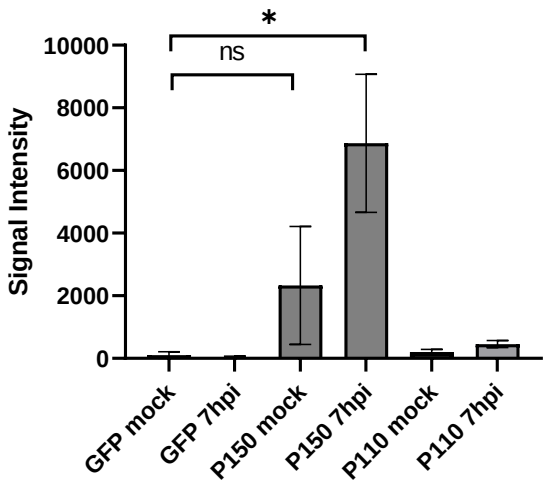


Figure 49. PKR co-immunoprecipitates with ectopically expressed ADARp150 during HSV-1 infection. ADAR1 KO cells were transfected with control plasmid pEGFP-N1 expressing EGFP, and plasmids ADAR1p110GFP or ADAR1p150GFP expressing p110 and p150 forms of ADAR1, respectively, and infected after 24h with HSV-1 at MOI 3. At 7hpi, cells were collected in RIPA buffer. The lysates were split for different IP conditions, antibody against GFP or isotype IgG (control) using Dynabeads G. **a.** Western blot on total protein extract (input) and IP samples. **b.** ImageJ quantification of PKR-IP samples (n=2).Data is shown as mean ± standard deviation (SD); 'ns' not statistically significant; *, p≤0.05, by One-Way ANOVA for (b).

4.5.3. ADAR1p150-PKR interaction is largely RNA dependent

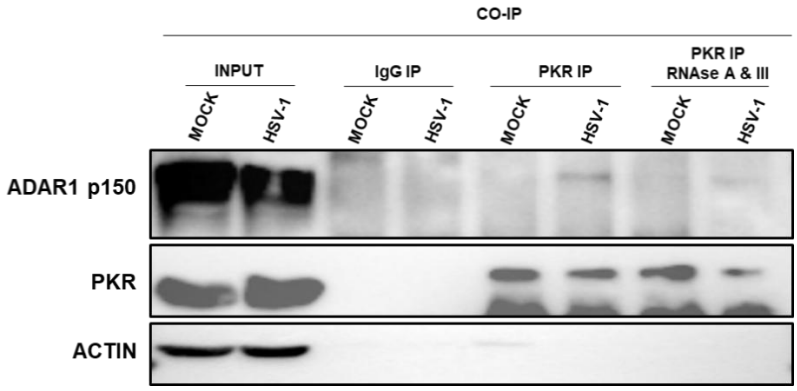
Both ADAR1 and PKR are RNA binding proteins containing dsRNA binding domain. Previous studies have reported that ADAR1 and PKR can bind dsRNA in both competitive and cooperative manner. Since our results showed that ADAR1p150 co-immunoprecipitated with PKR, we asked if this interaction depends on RNA.

To answer this, PKR immunoprecipitation was performed in the presence of RNase A and RNase III. RNase A belonging to Ribonuclease A superfamily usually targets single-stranded RNA (ssRNA), and does not target highly structured RNA. However, under denaturing conditions, such as prolonged extracellular processing in IP experiment, RNase A can degrade accessible dsRNAs. On the other hand, RNase III is dsRNA specific endonuclease.

PKR immunoprecipitation in ADAR1 WT cells infected with HSV-1 and treated with RNase A and III during IP procedure yielded 4 to 5 folds less ADAR1p150 than non-treated PKR immunoprecipitation. To further determine type of RNA involved, IP

samples were treated separately with either RNase A or RNase III. However, this approach did not resolve RNA species involved, possibly due to high RNA degradation post cell lysis or limited resolution of the assay, as no significant difference was seen in standalone or combined RNases treatment (Fig. 50).

a.



b.

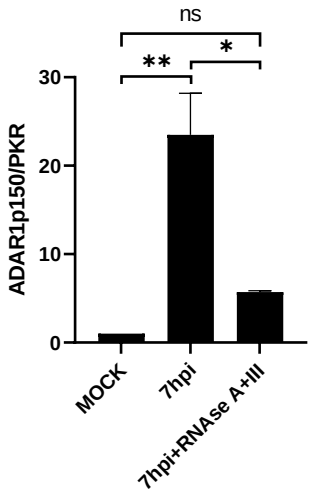


Figure 50. ADAR1p150-PKR interaction is largely dependent on RNA. ADAR1 WT cells were infected with HSV-1 (MOI 3). At 7hpi, cells were collected in RIPA buffer. The lysates were split for different IP conditions, including IP with antibody against PKR or Isotype isotype IgG (control) with or without treatment of RNase A (20ug/mL) and RNase III (2U/mL) using Dynabeads G. **a.** Western blot on total protein extract (input) and IP (isotype and PKR antibody) samples. **b.** ImageJ quantification of PKR-IP samples (n=2).Data is shown as mean ± standard deviation (SD); 'ns' not statistically significant; *, p≤0.05, **, p<0.01,by One-Way ANOVA for (b).

Nevertheless, these findings indicate that, during HSV-1 infection in ADAR1 WT cells, ADAR1-PKR interaction is largely dependent on RNA. Although this interaction is sensitive to RNase treatment, these results do not completely rule out possibility of direct protein-protein interaction between ADAR1p150 and PKR.

Taken together, our results strongly demonstrate that infection with HSV-1 promotes largely RNA dependent ADAR1-PKR association.

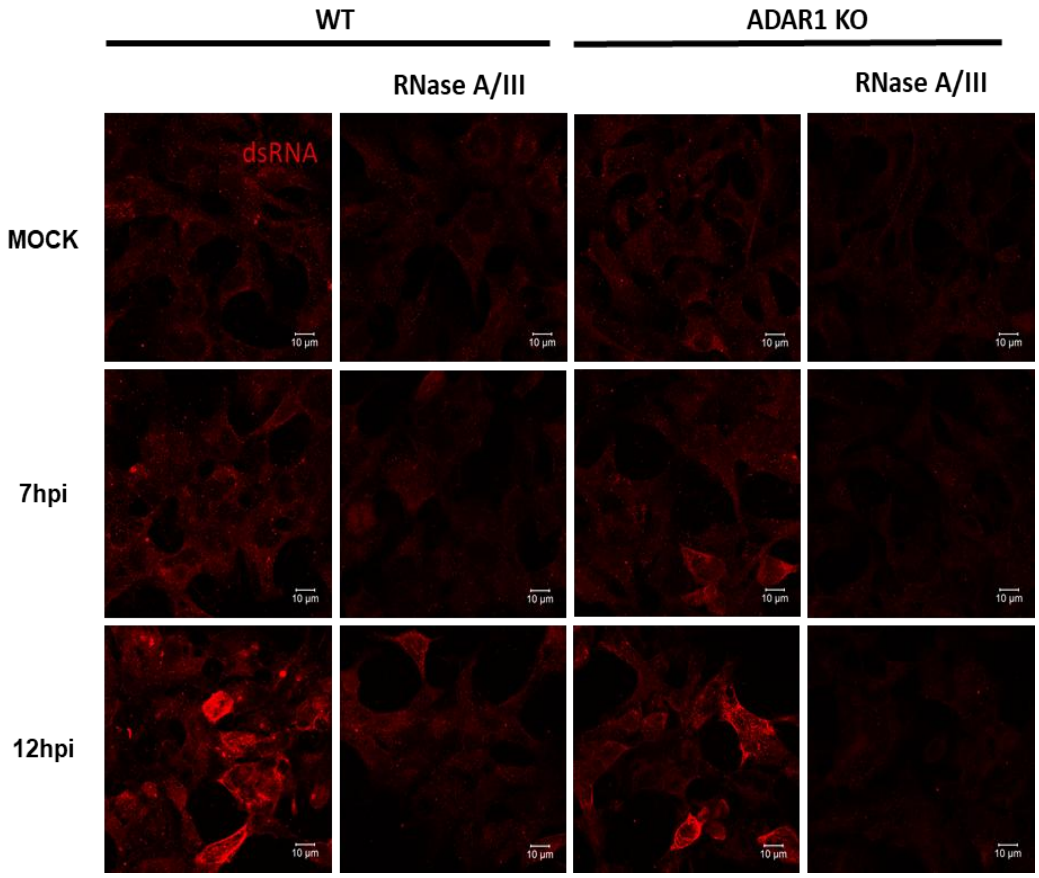
4.6. Immediate early and / or early transcripts contribute to PKR activation

4.6.1. ADAR1 WT and KO cells show comparable levels of dsRNA upon infection

Since dsRNA is a well-established trigger of PKR activation, we first asked whether enhanced PKR activation in ADAR1 KO cells is result of increased accumulation of dsRNA during HSV-1 infection. Nevertheless, we did not exclude that the possibility of host transcripts induced upon infection contributing to this phenomenon, based on the recent demonstration of host pseudogene transcripts playing role in activating RIG-I pathway in HSV-1 infection [110]. However, before defining origin of transcripts, it was necessary to test whether the difference between levels of dsRNA produced in ADAR1 WT and KO cells is acting as leading factor for early PKR induction. Therefore, we measured the levels of dsRNA in infected ADAR1 WT and KO cells with dsRNA specific antibody J2, utilizing two detection methods, immunofluorescence and dot blot assay.

ADAR1 WT and KO cells were MOCK infected with HSV-1 (MOI 3). Additionally, to confirm the signal obtained from immunofluorescence staining was indeed from dsRNA, each condition was additionally treated with combination of RNase A and RNase III. Mean fluorescent intensity (MFI) obtained over area showed that there was no significant difference between dsRNA levels between ADAR1 WT and KO cells in any conditions. At 7hpi, we did not observe significant increase in dsRNA levels. However, by 12hpi, dsRNA levels increased but remained comparable between WT and KO cells. RNase treatment confirmed the signal was indeed from J2 (dsRNA detecting antibody) (Fig. 51).

a.


WT
ADAR1 KO
RNase A/III
RNase A/III
dsRNA
MOCK
7hpi
12hpi
10 µm

b.

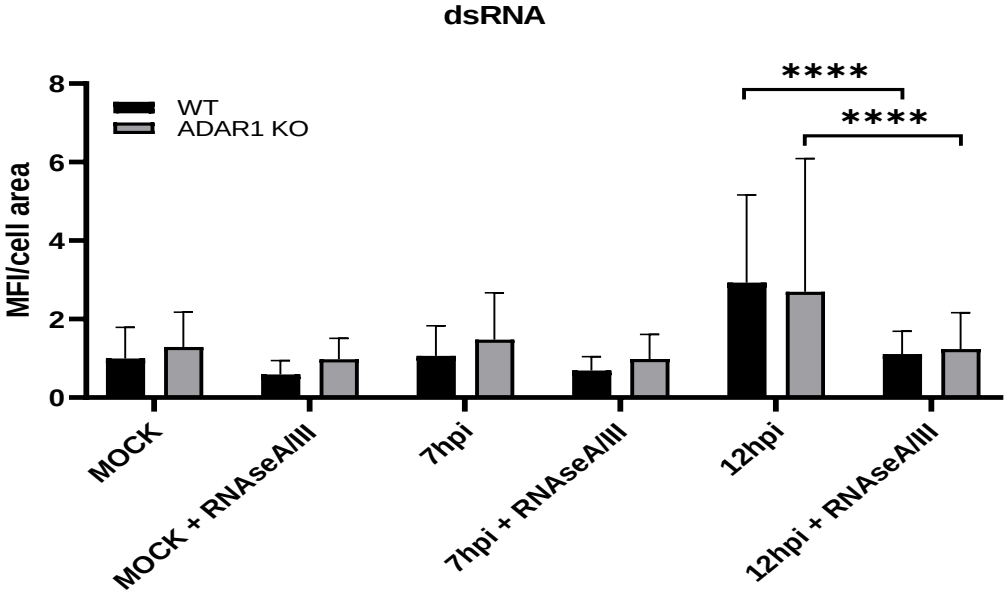
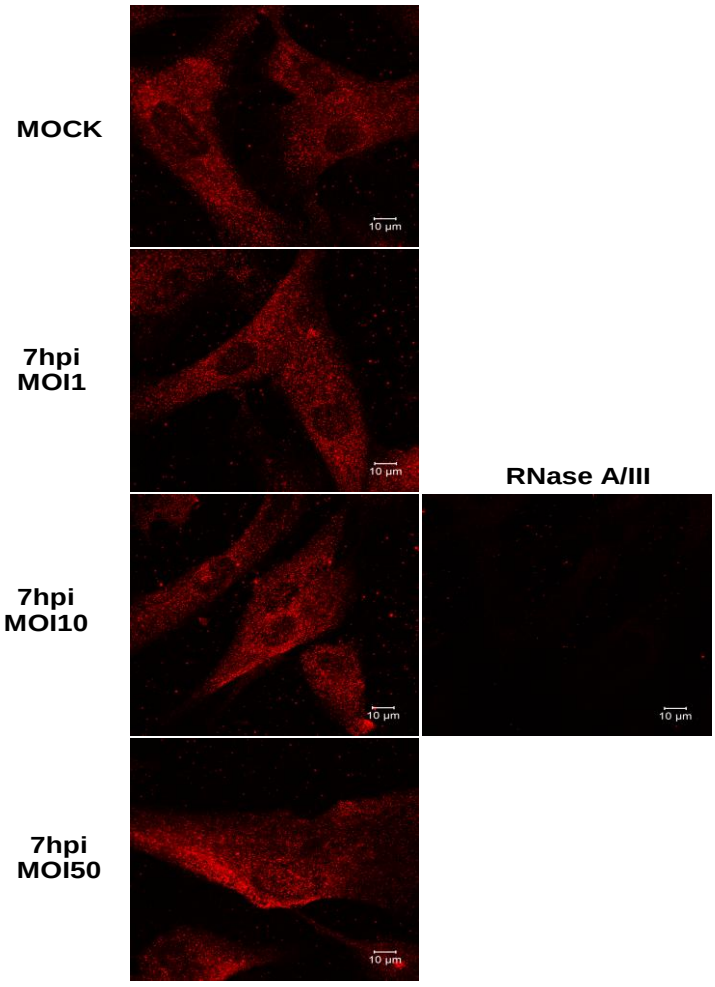

dsRNA
WT
ADAR1 KO
MFI/cell area
8
6
4
2
0
****
****
MOCK
MOCK + RNAseA/III
7hpi
7hpi + RNAseA/III
12hpi
12hpi + RNAseA/III

Figure 51. Similar levels of dsRNA are detected in HSV-1 infected ADAR1 WT and KO cells. ADAR1 WT and KO cells were seeded in infected with MOI 3 for 7 and 12h and were treated with RNase A (Promega) (20ug/mL) and Shortcut RNase III (NEB) (2U/mL) each for 1 hour. At indicated hpi cells were fixed, stained with J2 antibody and DAPI. **a.** Cells were imaged using Axio Observer Z1 fluorescence microscope. Mean fluorescence intensity (MFI) of 33 to 49 cells per group was measured using ZEN software (Carl Zeiss). **b.** Confocal images were captured from the microscopy slides in (a) using the LSM880 confocal microscope (Carl Zeiss). Maximum intensity projection images made by overlap of sequential z-stacks in ZEN software (Carl Zeiss) are shown. Data are shown as mean ± standard deviation (SD); 'ns', not statistically significant-not show; ****, p≤0.0001, by One-Way ANOVA for (a).

This observation raised a possibility that the assay may not be sufficiently sensitive to detect infection induced dsRNA differences in HEK derived ADAR1 WT and KO cells. This was particularly relevant, as similar results were seen in nascent protein synthesis assay showing no observable difference in cells infected as MOCK to the cells infected with HSV-1. In addition, lack of clear dsRNA increase at 7hpi appears contradictory to earlier reports, though those studies were performed in HFF cell line. Hence, we repeated similar setup in HFF, with addition of different MOIs to confirm the sensitivity of our assay in detecting dsRNA.

a.



b.

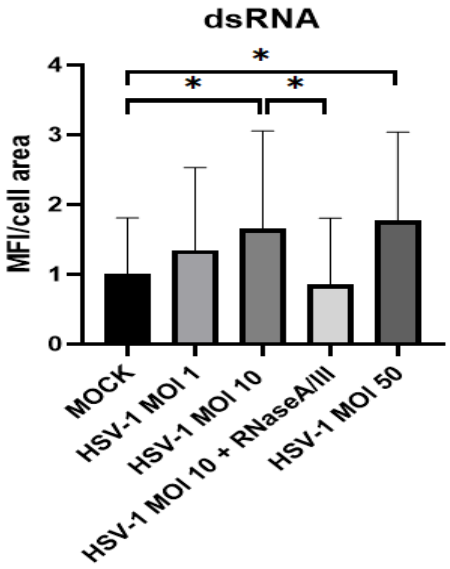


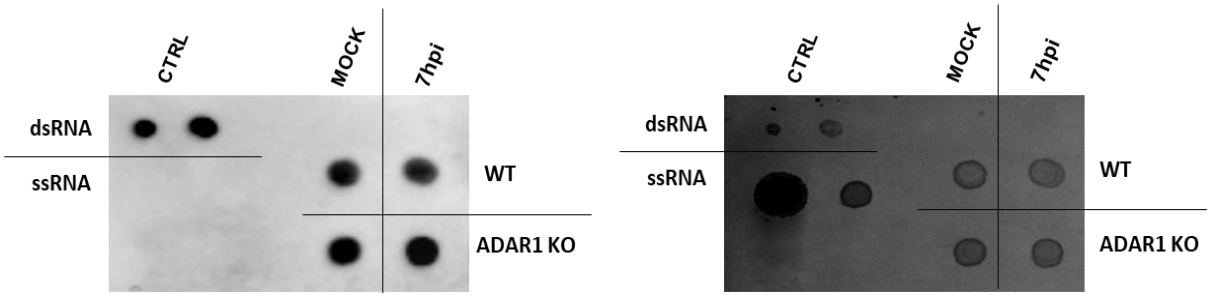
Figure 52. Levels of dsRNA are detected in HFF cells during HSV-1 infection. HFF cells infected with indicated MOI. Additionally, the cells infected with MOI 10 were treated with RNase A (Promega) (20ug/mL) and Shortcut RNase III (NEB) (2U/mL) each for 1 hour. At 7hpi cells were fixed, stained with J2 antibody and DAPI. **a.** Cells were imaged using Axio Observer Z1 fluorescence microscope. Mean fluorescence intensity (MFI) of 21 to 26 cells per group was measured using ZEN software (Carl Zeiss). **b.** Confocal images were captured from the microscopy slides in (a). Using the LSM880 confocal microscope (Carl Zeiss). Maximum intensity projection images made by overlap of sequential z-stacks in ZEN software (Carl Zeiss) are shown. Data are shown as mean ± standard deviation (SD); 'ns', not statistically significant-not show; *, p≤0.05, by One-Way ANOVA for (a).

HFF infected with HSV-1 MOI 1, 10 and 50 showed significantly higher amounts of dsRNA than MOCK infected, confirming we could reproducibly detect dsRNA at 7hpi with lower amount of infection (MOI 1), with negative control (RNase A and III treatment) showing significant reduction in levels of dsRNA further strengthening the results (Fig. 52). Therefore, we speculate that ADAR1 WT and KO cells (generated in HEK293) may have relatively high metabolic activity, evidenced by generally higher levels of basal dsRNA detected (i.e. high background signal), contributed in lowering sensitivity of J2 labelling, making difficult to differentiate between metabolic dsRNA with viral dsRNA signal at 7hpi. However, as HFF are relatively low in metabolism, increase in dsRNA due to infection was efficiently detectable by 7hpi.

In second approach, RNA extracted from ADAR1 WT and KO, MOCK and HSV-1 infected (MOI 3) cells up to 7hpi were spotted on a charged membrane, together with synthetic dsRNA and ssRNA as positive and negative controls. Amounts of ssRNA

spotted were kept excessive as shown in colorimetric image, to test specificity of J2 antibody on extended levels. Upon blotting the membrane, we only detected dsRNA from the positive controls, and also from MOCK and infected cells. Importantly, upon quantification, we did not observe any difference in levels of dsRNA between ADAR1 WT and KO cells, consistent with immunofluorescence results (Fig. 53).

a.



b.

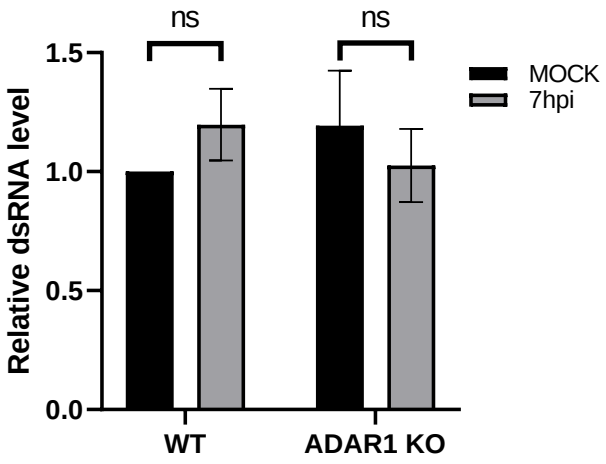


Figure 53. dsRNA detection in ADAR1 WT and KO cells by dot-blot. ADAR1 WT and KO were infected with HSV-1 (MOI 3) and at 7hpi total RNA was extracted. **a.** Total RNA blotted on charged membrane with positive and negative control. (LEFT) Image captured with HRP linked secondary. (RIGHT) Colorimetric image. **b.** ImageJ quantification of dot blot (n=18). Data are shown as mean ± standard deviation (SD); 'ns' not statistically significant, by One-Way ANOVA for (b)

These results suggested that dsRNA levels are comparable between ADAR1 WT and KO cells early in HSV-1 infection. Therefore, enhanced PKR activation in KO cells is unlikely to be explained by increase in dsRNA levels. Instead, this led us to believe that trigger for PKR activation is present in both WT and KO cell types, however,

absences of ADAR1 shifts the balance towards enhanced PKR activation and further downstream limits the viral replication.

4.6.2. Viral gene expression is required for PKR activation during HSV-1 infection

We established that dsRNA levels are comparable between ADAR1 WT and KO cells, we asked which stage of HSV-1 infection provides trigger for PKR activation. The earlier analysis of viral transcript revealed that the dynamics of viral gene expression is fairly comparable between ADAR1 WT and KO cells up to 6-7hpi in infection. However, PKR activation and downstream translational arrest in ADAR1 KO cell stalls expression of viral genes and proteins later in infection. These results indicate that events occurring before or around the time of viral DNA replication may trigger PKR activation, which subsequently has detrimental effect on virus. Since dsRNAs are widely reported to activate PKR, which is assumed to be the case here, firstly we wanted to understand the clear viral dynamics, from entry in host cells until DNA replication, at which PKR becomes activated.

To address this question, we used to stepwise inhibition of HSV-1 infection using small molecule approach. Actinomycin D (ACT) binds to GC rich double stranded DNA molecule, effectively inhibiting RNA polymerase progression, useful in dissecting transcription dependent and independent activities in cell. Cycloheximide (CHX) is a potent inhibitor of ribosomal translocation terminating translation elongation, therefore protein synthesis. On the other hand, Acyclovir (ACV) is guanosine analogue which gets phosphorylated by viral thymidine kinase, and incorporates in replicative viral genome terminating viral DNA replication. Thus, with use of these molecules at each stage during infection as ACT for transcription inhibition, CHX for translation inhibition and eventually ACV to inhibit viral DNA replication during early to late stages of infection, we can dissect stage dependency of PKR activation. Additionally, we used UV inactivated virus for mimicking virion entry. Since UV treatment causes genomic damage in virion, it is able to enter the cell but cannot initiate infection.

ADAR1 WT and KO cells were pre-treated with either ACT (100ug/mL), CHX (1ug/mL) or ACV (100uM) and pre-treated HSV-1 virus with UV (5000J) for inactivation. Pre-treated as well non-treated cells were infected with HSV-1 (MOI 3) and another set of

non-treated cells were infected with UV inactivated HSV-1 (MOI 3). Upon analysing protein profile at 24hpi, we observed higher phosphorylated PKR and lesser late viral protein gC in non-treated ADAR1 KO cells than WT , consistent with translational arrest phenotype described earlier. In cells infected with UV-inactivated virus, ACT and CHX pre-treated infections, we did not observe any viral protein, neither detectable activation of PKR (Fig. 54). This indicates that virion entry alone is not sufficient to trigger PKR, viral transcription and/or translation are required.

In ACV treated cells, we observed similar amounts of immediate-early viral protein ICP4, but absence of late viral protein gC, indicating ACV terminated viral DNA replication in both cell lines. Under these conditions, PKR phosphorylation in ADAR1 KO cells was reduced compared to untreated cells, but was not completely abolished. In contrast, no comparable increase in PKR phosphorylation was observed in WT cells (Fig. 54). These observations suggest that events occurring before viral DNA replication, likely involving IE and/or E gene expression processes, are sufficient to trigger PKR activation in KO cells, while progression beyond DNA replication further enhances this response.

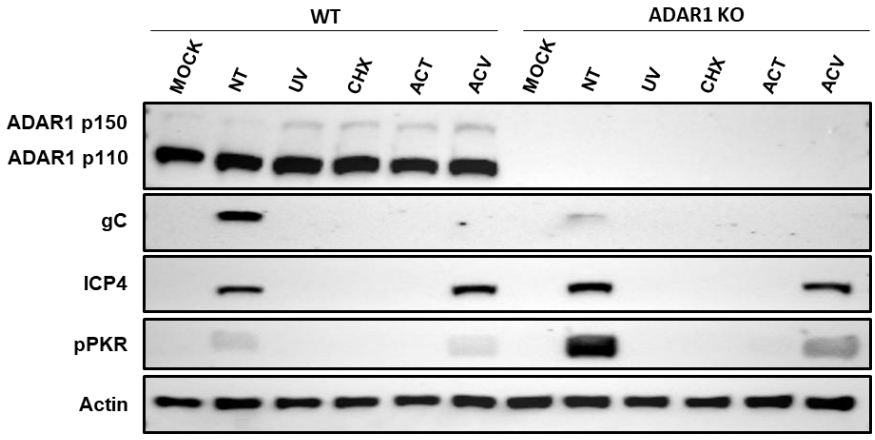


Figure 54. IE and/or E viral gene expression is required for PKR activation during HSV-1 infection. ADAR1 WT and KO cells were pre-treated with 100ug/mL Cycloheximide (CHX) or 1ug/mL Actinomycin D (ACT) or 100uM Acyclovir (ACV) for 1 hr. In absence of noted inhibitors, cells were infected with wild type or 5000J UV inactivated HSV-1 at indicated MOI. After 1 hr infectious replaced with fresh media and inhibitors were added at previously indicated concentrations. At 24hpi cells were colledted in RIPA. Protein analysis by Western blot.

Collectively, it can be concluded that, entry of HSV-1 virion is not sufficient to activate PKR. Additionally, absence of activated PKR in ACT as well as CHX treated samples

indicate, presence of both immediate early / early transcripts and proteins might be required to reach threshold for initiation of PKR activation. ACV treatment did not completely inhibit PKR phosphorylation in ADAR1 KO cells, indicating, IE / E transcripts and/or proteins are sufficient to initiate PKR activation and progression of infection beyond DNA replication unequivocally enhances this effect further. Nevertheless, though we cannot definitely dissect between requirement of IE or E, transcripts or proteins, we could conclude again that events taking place early in infection are certainly responsible for early PKR activation.

4.6.3. Viral transcripts bind to PKR during HSV-1 infection

The inhibitor experiment indicated that viral gene expression was required for PKR activation in ADAR1 KO cells during HSV-1infection. Since blocking viral transcription and translation prevented detectable PKR phosphorylation, IE and/or E gene transcripts/proteins may contribute to PKR activation. Therefore, to further support this possibility, we hypothesised that HSV-1 transcripts associate with PKR during infection.

Therefore, we tested for all classes of viral genes (viz., ICP0, ICP4 and ICP27 in immediate early or IE class, ICP8 and TK in early or E class and gC and VP16 in late or L class) in RNA precipitated with PKR immunoprecipitation (RIP: RNA co-immunoprecipitation) assay in ADAR1 WT and KO cells infected up to 7h with HSV-1 MOI 3. Transcripts were quantified with qPCR against quantity of 18S RNA present which was considered as 1. Results show that All classes of transcripts immunoprecipitated with PKR, with ICP0 and ICP4 from IE class being more abundant, however, in control immunoprecipitation with IgG, no transcripts were detected (Fig. 55). This strengthen our earlier findings, suggesting viral transcripts indeed associate with PKR and contribute to its activation during HSV-1 infection. Again, given the limits of the experiment performed, we certainly cannot exclude role of host transcripts, nonetheless known effect of viral protein vhs (virion host shutoff) in degrading majority of host transcripts in initial phases of HSV-1 infection, targeted investigation on viral transcripts underlines central role in activating PKR.

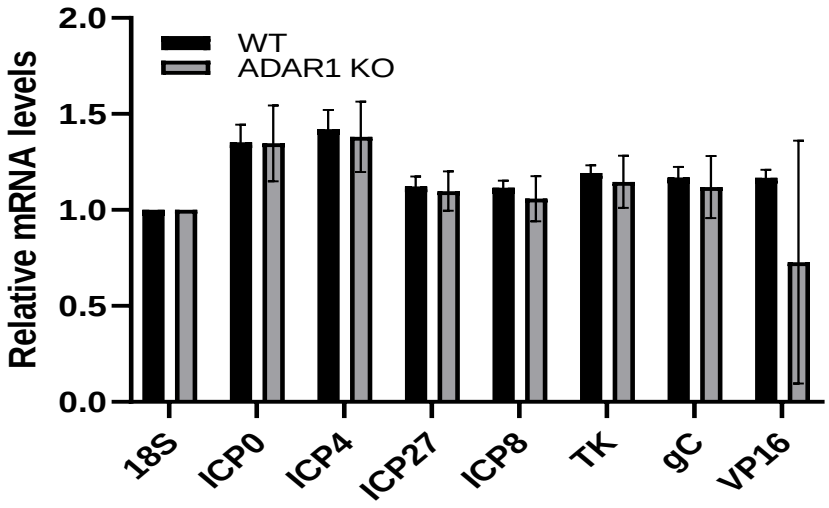


Figure 55. Viral transcripts co-immunoprecipitate with PKR in ADAR1 WT and KO cells. ADAR1 WT and KO cells were infected with HSV-1 (MOI 3) and 7h.p.i. PKR was immunoprecipitated using PKR or IgG control antibody and RNA was extracted. Indicated target genes were detected using RT-qPCR and normalized to 18S of corresponding cell type. (n=4; Corresponding IgG is below detection limit.) Data is shown as mean ± standard deviation (SD); ns – not statistically significant-not shown; by Student's t test for each pair.

Together, these findings support the conclusion that viral transcripts associate with PKR during HSV-1 infection. In combination with earlier inhibitor experiments, we can report that immediate early and/or early transcripts and protein are necessary for PKR activation in absence of ADAR1 during productive HSV-1 infection, while subsequent progression of infection may further enhance this response.

In summary, ADAR1 acts as a proviral factor during productive HSV-1 infection by supressing PKR/eIF2α mediated translational arrest. The function is specific to isoform p150 of ADAR1, which associates with PKR in RNA dependent manner. In absence of ADAR1, PKR is activated by immediate early/early transcripts and proteins, which results in impaired viral replication.

5. DISCUSSION

This study identifies ADAR1, particularly cytoplasmic p150 isoform, as a key regulator of modulating PKR activation therefore preventing translational arrest and facilitating efficient HSV-1 replication.

Unlike observations in other herpesviruses, such as HCMV, where upregulation of specific isoform of ADAR1 protein, p110, was reported, levels of ADAR1 did not change with progression of HSV-1 infection (Fig. 17) [95]. This lack of modulation of ADAR1 expression during progression of HSV-1 infection suggests that virus may engage distinct mechanisms in utilizing ADAR1 function as immune modulator, further highlighting differences in virus strategies for interacting with host antiviral response.

In previous studies among herpesviruses, ADAR1 promotes KSHV reactivation from latency by modulating RLR signalling pathway, particularly RIG-I mediated dsRNA sensing [94]. However, to our surprise, sensors and effectors from RLR signalling pathway, RIG-I, MDA5 and MAVS played very limited role in rescuing HSV-1 replication from ADAR1 deficient cells, whereas depletion of PKR restored viral replication comparable to wild type conditions (Fig. 24, Fig. 30). Similarly, depletion of individual PRR or effector from distinct sensing pathways (except PKR) in combination with ADAR1, did not prevent PKR activation during HSV-1 infection (Result Fig. 31). This consistent activation of PKR, despite depletion of other sensors suggest that PKR activation occurs independently of upstream signalling, strengthening PKR as a primary sensor during HSV-1 productive infection. This observation further highlights the fundamental differences in virus specific virus-host interaction justifying the rationale for investigating ADAR1 function in HSV-1 productive infection.

The immune modulation by ADAR1 is classically defined by A-to-I RNA editing, which reduces immunogenicity and recognition of dsRNA structures by pattern recognition receptors or other cytosolic sensors. However, in several virus systems such as Influenza A virus (IAV) or KSHV, suppression of immune system is mainly attributed to editing independent activity of ADAR1, where binding to dsRNA was sufficient to supress RLR pathway [88], [94]. In case of HSV-1 we report similar observations. Despite productive infection being characterised by massive transcription, A-to-I editing, at least in case of miRNAs is very limited compared to latency [12]. Therefore, it can be speculated that editing of viral transcripts during productive infection has

limited role in preventing the recognition by cytosolic sensors. Whereas, in latency editing seems to have more direct role.

Further observation supporting limited role of editing is that the viral replication in ADAR1 deficient cells can be efficiently rescued by depleting PKR (Fig. 32), pharmacological inhibition of downstream effector eIF2α (Fig. 36), and ectopic expression of viral antagonist protein IP34.5 (Fig. 33). Additionally, both being catalytically active, complementing only with ADAR1p150, but not p110 restores viral replication (Fig. 41). These findings collectively suggest A-to-I editing plays limited role in regulating PKR activation during productive HSV-1 infection.

The editing independent effects of ADAR1 in HSV-1 infection also highlight isoform specificity. Though both p110 and p150 isoforms are catalytically active, complementation with only cytoplasmic p150 isoform suppressed PKR activation in ADAR1 deficient cells (Fig. 41, Fig. 42). Notably, complementation by ADAR2, another catalytically active ADAR family member with nuclear localization, also failed to rescue viral replication in absence of ADAR1 (Res Fig. 47). Additionally, selectively depleting ADAR1p150 isoform also reduces HSV-1 replication (Res Fig. 43). These observations suggest that rather than editing capacity alone, subcellular localization, access to cytosolic substrate as well as sensors are key determinants of ADAR function. In this context cytoplasmic p150 isoform of ADAR1 effectively modulates PKR activation thereby facilitating HSV-1 replication.

The productive HSV-1 infection is characterised by extensive transcriptional activity, including generation of overlapping antisense transcripts that can form dsRNA structures (Fig. 51, Fig. 52) [8]. Multiple cytosolic RNA sensors such as MDA5 or PKR are capable of recognising these structures; however, PKR appears to have predominant antiviral effect in absence of ADAR1. These sensors usually target distinct structures; therefore, it is possible that transcript generated by HSV-1 preferentially engage PKR than other sensors. Consistent to this, a recent study reported that during later timepoints in HSV-1 infection, rather host transcripts were enriched in association with RIG-I, showing different RNA sensors function in non-redundant and complementary manner [110].

In case of PKR, it could be activated by broad range of RNA structures resembling dsRNA such as bulges, loops and short dsRNA with single stranded overhangs [29],

[30], [31]. Interestingly, all HSV-1 transcripts tested were predicted to make dsRNA structures compatible for recognition by PKR. Furthermore, all three classes (IE, E and L) of viral transcripts we tested were co-immunoprecipitated with PKR confirming their potential to interact (Fig. 55). Therefore, together these observations suggest that dsRNA structures generated during HSV-1 infection can engage with PKR initiating the activation. Additionally, PKR activation was consistently observed in ADAR1 deficient cells upon HSV-1 infection, regardless of depletion of other cytosolic sensors. This suggests that threshold for PKR activation, at least in our tested models, was much lower than other cytosolic sensors, therefore making PKR as primary mediator of antiviral response in loss of ADAR1 function.

Tegument proteins such as US11, which directly inhibit PKR [54], or vhs, which degrades mainly host transcripts and limits accumulation of dsRNA [59], contribute to limiting PKR activation. However, their functions alone are insufficient to compensate for loss of ADAR1 during HSV-1 infection, highlighting importance of host mediated regulation. Notably, several viral proteins that inhibit PKR or its downstream effectors including US11, ICP34.5, and vhs, are expressed as late genes, gives further insights into evolutionary reliance of HSV-1 on host mediated control of innate immune responses during early stages of infection.

Furthermore, although all classes of viral transcripts are associated with PKR, its activation appears to occur during early stages of infection, particularly during immediate early (IE) and early (E) stages (Fig. 25). Viral entry alone was insufficient to trigger PKR; instead both transcription and translation was required to initiate PKR activation, indicating newly synthesised viral RNA and/or proteins contribute to this mechanism. Contrastingly, inhibitor of viral DNA synthesis using acyclovir (ACV) reduced extent of PKR activation, but could not completely abolish it. This observation can be interpreted as IE and E transcripts along with proteins trigger PKR activation which is further amplified during later stages of infection (Fig. 54). However, further studies are required to precisely identify the structures triggering PKR activation.

In absence of ADAR1, increased availability of immunogenic RNA likely enhances activation of PKR. Given that both ADAR1 and PKR are dsRNA binding protein, it is plausible that ADAR1 binds and sequesters dsRNA, thereby reducing its accessibility to PKR. Consistent with this, co-immunoprecipitation experiments demonstrated association of ADAR1p150 and PKR during HSV-1 infection (Fig. 48). Notably, ectopic

expression of ADAR1p150 alone could co-immunoprecipitate PKR, but p110 could not, further highlighting isoform specificity (Fig. 49).

This interaction of ADAR1p150 and PKR appears largely RNA dependant, as treatment of PKR co-immunoprecipitation complex with RNases reduced the association (Fig. 50). While previous studies have reported more direct protein-protein interaction of ADAR1 and PKR [43], at least in context of HSV-1 infection, it is largely RNA dependant. The residual interaction of ADAR1p150 and PKR observed upon RNase treatment can be attributed to insufficient activity of RNases, limited accessibility of RNases to preformed ADAR1p150-RNA-PKR complex. However, we cannot fully exclude direct ADAR1p150-PKR association, where further investigations are warranted.

Activation of PKR leads to phosphorylation of eIF2α resulting in inhibition of translation initiation and subsequent global translational arrest. However, our assay measuring global nascent protein synthesis did not report any significant difference in protein synthesis between ADAR1 wild type and ADAR1 deficient models (Fig. 39). This result can be attributed to limited resolution of assay and cell system used with relatively high MOI with smaller difference in viral replication. However, ATF-4, a well-established marker of selective translation during eIF2α mediated translational arrest, was upregulated in ADAR1 deficient cells upon infection (Fig. 38).

Furthermore, viral protein synthesis progresses comparably in both ADAR1 wild type and deficient cells until PKR activation around ~6-7hpi (Fig. 25). However, major difference arises especially in late protein levels at later timepoints (Fig. 17). Similarly, up to activation of PKR around, viral RNA levels in ADAR1 deficient cells is comparable to wild type (Fig. 18). However, even RNA levels for most of late proteins are significantly reduced. Reduction in both RNA and protein levels of mainly late genes, coincidence between PKR shows a stall of HSV-1 replication in ADAR1 deficient environment. The observation in reduction of RNA as well as proteins could be explained by interdependence of transcription and translation during infection. Efficient progression of viral transcription and translation cascade relies on continuous synthesis of viral proteins, particularly those required for phase transitions. PKR induced translational arrest likely disrupt this cascade resulting in broader stall of HSV-1 infection in ADAR1 deficient environment.

Interestingly, we also observed differential effect on IE protein expression at later stages of infection. While levels of ICP0 were decreased in ADAR1 deficient cells, ICP4 expression remained largely unaffected. This gives rise to intriguing hypothesis that some viral transcripts might be less sensitive to translational arrest and selectively translated such as ATF-4, however additional studies are clearly required to determine this mechanism. Overall, the activation of PKR and phosphorylation of eIF2α, the stalling of HSV-1 replication, and upregulation of ATF-4 in ADAR1 deficient cells collectively provide strong evidences for PKR mediated translational arrest.

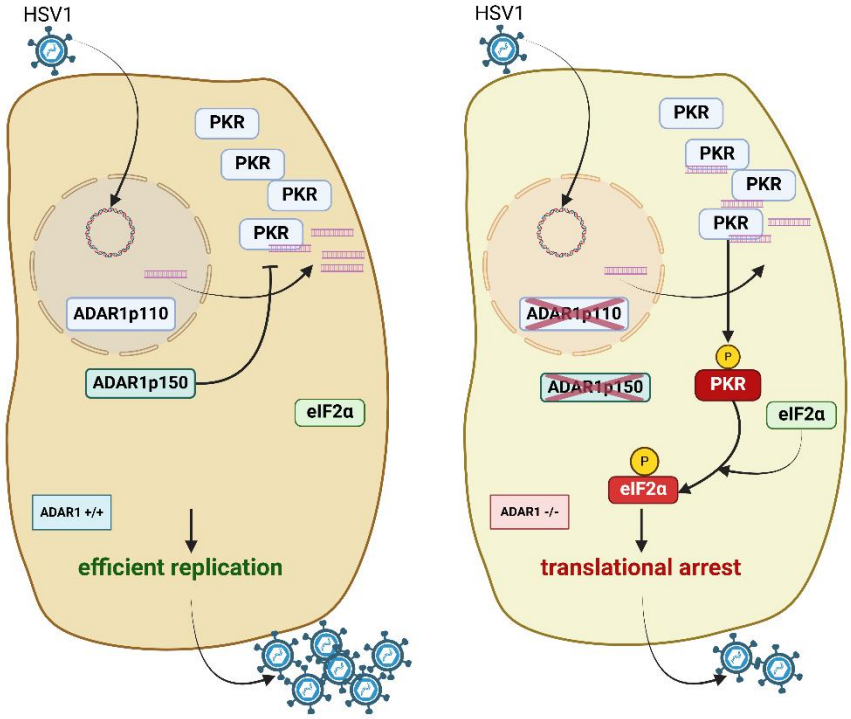


Figure 56. ADAR1p150 prevents PKR activation during productive HSV-1 infection. Upon HSV-1 infection, immunogenic dsRNAs are recognised by PKR. In ADAR1 deficient cells this leads to PKR mediated translational arrest limiting viral replication. However, in wild type cells ADAR1p150 supress PKR activation resulting in efficient viral replication. This schematic representation was created in BioRender by Parchure A. for publication, adapted from *[111]*.

In summary, this study identifies ADAR1 as a proviral host factor during productive HSV-1 infection. Specifically, the p150 isoform, suppresses PKR activation, thereby preventing translational arrest and facilitating efficient viral replication (Fig. 56).

6. CONCLUSIONS

In this study, we report that ADAR1p150 prevents HSV-1 from triggering PKR- eIF2α mediated translational arrest and it is required for efficient viral replication. Accordingly, from the results and discussion, following conclusions can be drawn,

- ADAR1 is required for efficient HSV-1 replication. ADAR1 is proviral factor in HSV-1 productive infection.
- Loss of ADAR1 during viral replication predominantly leads to activation of PKR, which restricts HSV-1 replication.
- PKR is the primary dsRNA sensor responsible for antiviral effect, while other sensors have minimal or later contributions
- Activation of PKR in ADAR1 deficiency leads to phosphorylation of downstream effector eIF2α, thereby inducting translational arrest leading to stalling of HSV-1 replication.
- The cytoplasmic p150 isoform od ADAR1 is responsible for supressing PKR activation and facilitating viral replication. The nuclear p110 isoform of ADAR1 or ADAR2 do not rescue viral replication.
- The role of ADAR1 in HSV-1 infection is largly A-to-I editing independant and depends on its binding activity to dsRNA sunstrate and interaction with PKR
- ADAR1 regulates PKR activation by interacting primarily in RNA depedent manner, likely by sequestering immunogenicity of dsRNA structures generated during viral replication
- PKR activation during HSV-1 infection is initiated at early stages of infection, immediate early (IE) and early (E) transcripts and/or proteins contribute to this activation.

7. CONTRIBUTIONS FROM THIS STUDY TO THE FIELD

This study provides novel insights into the role of ADAR1 in HSV-1 infection. While ADAR1 has been widely studies for its role in regulating immune sensors through A-to-I editing, this work demonstrates its proviral role during HSV-1 infection largely independent of its catalytic activity. ADAR1p150 acts as a key modulator of supressing PKR activation through RNA dependent manner. These findings contribute to a broader understanding of host mediated immune regulation mechanism and evolutionary strategies by which viruses modulates host environment in the favour of their replication.

In addition, this study identifies PKR as a dominant antiviral sensor restricting HSV-1 replication in absence of ADAR1. This refines the current understanding of dsRNA sensing during herpesvirus replication. The identification of isoform specific function of ADAR1 along with demonstration of its interaction with PKR provide mechanistic understanding about dynamics of virus-host interaction. Furthermore, this work expands our understanding in relatively underexplored field of ADAR1 mediated regulation of innate immunity in DNA viruses, particularly herpesviruses.

Importantly, this study also highlights ADAR1 as a potential target for host directed antiviral strategies. Targeting ADAR1 functions in dsRNA sensing rather than its catalytic activity, may enhance innate immune response and complement existing therapies. These approaches are more relevant in the context of antiviral resistance, as targeting host could reduce likelihood of rapid viral evolution and escape, though this remains to be further explored.

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Appendix

1. List of figures

2. List of tables

CURRICULUM VITAE

ADWAIT ANAND PARCHURE

Laboratory for Molecular Virology,
Faculty of Biotechnology and Drug Development, University of Rijeka
adwait.parchure@uniri.hr

Education

2022 – today **PhD student**, PhD program Medicinal chemistry,
Faculty of Biotechnology and Drug Development, University of Rijeka

2014 – 2016 **Masters of Science (Bioinformatics and Applied Biotechnology)**
IBAB, Bangalore (KA), University of Mysore (KA)

2011 – 2014 **Bachelor of Science (Biotechnology)**
University of Mumbai (MH)

Career

2022 – 2025 **Graduate Research Assistant**
Researcher in Laboratory for Molecular Virology, Faculty of Biotechnology and Drug Development, University of Rijeka
Principal investigator: Professor Igor Jurak, PhD

Teaching Assistance: Microbiology, Recombinant DNA technology

2019 – 2021 **Senior Research Associate**
Bristol Myers Squibb RD Center (BBRC), Syngene, Bangalore (KA)

2018 – 2019 **Design and Development Officer**
PMC Pvt. Ltd., Vapi (GJ)

2017 – 2018 **Research Assistant**
Project: Derivation of Functional Hepatocytes from Human Induced Pluripotent Stem Cells
Funded by: Indian Council of Medical Research, New Delhi (DL)
National Centre for Cell Science, Pune (MH)

2016 – 2017 **Junior Research Fellow**
Project: Innate lymphocyte responses in EBV associated Pediatric Hodgkin's Lymphoma
Kidwai Cancer Institute, Bangalore (KA)

Selected trainings

2024 – 2025 Doctoral Mobility Internship at Department of Virus-Host-Interaction, Leibniz Institute of Virology (LIV), Hamburg, Germany

2022 FluoMicro@ICGEB' practical course on fluorescence microscopy and high throughput imaging, ICGEB-Trieste, Italy

2022	Short term course on 'Whole transcriptomic Data Analysis', EMBL-Heidelberg, Germany
2022	NGS Library preparation hands on, In collaboration with Laboratory of Advanced Genomics, Ruđer Bošković Institute (IRB), Zagreb, Croatia

Languages

Native language	Marathi
Other languages	English (C2), German (A2), Croatian (A1), Hindi (C1 equivalent), Sanskrit (B1 equivalent)

Selected scholarships and awards

2024	**MOBDOK Doctoral Mobility Fellowship** by Croatian Science Foundation (HRZZ) for attending training at LIV, Hamburg, Germany
2023	**FEBS-IUBMB-ENABLE European Travel Grant** for attending conference in Cologne, Germany
2023	**HDBMB support grant** for attending conference in Cologne, Germany
2022	**ICGEB support grant** for attending course 'FluoMicro@ICGEB'
2022	**EMBL corporate partner fellowship (Registration fee waiver)** for attending course on 'Whole transcriptomic Data Analysis'

Memberships

2022 – today	Croatian Society of Biochemistry and Molecular Biology (HDBMB)
2022 – today	Croatian Microbiology Society (HMD)

Selected conferences

2025	**Talk** at **9th European Congress of Virology**, Dubrovnik, Croatia, titled 'Different roles of ADAR1 during productive and latent herpesvirus infection'
2025	**Talk** at **34th Annual Meeting of the Society of Virology (GfV),** Hamburg, Germany, titled 'ADAR1 p150 prevents HSV-1 from triggering PKR/eIF2α-mediated translational arrest and is required for efficient viral replication'
2023	**Poster presentation** at **2nd Intl. FEBS-IUBMB-ENABLE PhD and Post-Doc Conference,** Cologne, Germany, titled 'Investigating the molecular basis of the pro-viral role of ADAR1 in productive HSV-1 infection'
2023	**Poster presentation** at **HDBMB-22: Science to knowledge**, Brela, Croatia, titled 'Elucidating post-transcriptional modifications of viral transcripts in latently infected human trigeminal ganglia with herpes simplex virus 1'

Publications

2025 **Adwait Parchure**, Mia Cesarec, Antonija Braut, Robert Kolman, Vlatka Ivanišević, Marina Čunko, Slađana Bursać, Richard de Reuver, Antonija J. Begonja, Umberto Rosani, Siniša Volarević, Jonathan Maelfait, Igor Jurak. ADAR1 p150 prevents HSV-1 from triggering PKR/eIF2α-mediated translational arrest and is required for efficient viral replication, **PLoS Pathogens.**

2023 Andreja Zubković, Cristina Gomes, **Adwait Parchure**, Mia Cesarec, Antun Ferenčić, Filip Rokić, Hrvoje Jakovac, Abigail L. Whitford, Sara A. Dochnal, Anna R. Cliffe, Dražen Cuculić, Angela Gallo, Oliver Vugrek, Michael Hackenberg, Igor Jurak. HSV-1 miRNAs are post-transcriptionally edited in latently infected human ganglia, **Journal of Virology.**

2020 Alka M, Chatterjee S, **Parchure A**, Mahantesh S, Sravanthi Davuluri, Arun Kumar Ar, Avinash T, Padma M, Premalata Cs, Mahua Sinha, Anup Chugani, Vishnu Priya R, Acharya Kk, Jayshree R S. Natural Killer cell transcriptome during primary EBV infection and EBV associated Hodgkin Lymphoma in children-A preliminary observation, **Immunobiology.**

2018 Sreenivas Adurthi, Mahesh M. Kumar, H. S. Vinodkumar, Geetashree Mukherjee, H. Krishnamurthy, K. Kshitish Acharya, U. D. Bafna, Devi K. Uma, B. Abhishekh, Sudhir Krishna, **A. Parchure**, Murali Alka & R. S. Jayshree. Oestrogen Receptor-α binds the FOXP3 promoter and modulates regulatory T-cell function in human cervical cancer, **Scientific Reports**.

Selected Extracurricular Activities

2025 Science popularization on Faculty of Biotechnology and Drug Development, University of Rijeka with activities: Open doors of the Laboratory for Molecular Virology

2023 Mentor and Jury member at Stemgames-2023, Umag, Croatia