

Analysis of ADAR protein during productive HSV-1 Infection

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Mentor: Igor Jurak, PhD

SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
Diplomski sveučilišni studij
Biotehnološka istraživanja znanosti o životu

Justina Oluchi Echeta

Analyza ADAR proteina tijekom produktivne infekcije
HSV-1

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ABSTRACT

ADAR proteins are enzymes involved in the editing of dsRNA transcripts by deamination in which adenosine is changed to Inosine. This alteration ultimately affects protein translation. ADAR1 has two isoforms; ADAR1p110 and ADAR1p150, ADAR2 and ADAR3 make up the mammalian ADAR family. Both isoforms of ADAR1 and ADAR2 are catalytically active while ADAR3 is not. ADARs edit both self and non-self dsRNA. Several studies have shown the role of ADAR proteins mostly in RNA viruses, however, the role of these proteins in dsDNA viruses is largely unknown. During viral infection, they can play an antiviral role by forming part of the cell's innate immune response, a proviral role by blocking viral dsRNA from being recognized by the immune sensors, thereby facilitating viral replication in the host cell, or both roles as observed in Influenza A virus.

Based on in vitro experiments, we have established cell models to analyse the role of ADAR proteins during productive HSV-1 infection. ADAR protein and mRNA levels during productive HSV-1 infection were determined, showing upregulation of ADAR during the infection. We observed an upregulation of ADAR proteins in U2OS, HeLa, HEK293T, and HFF cells infected with wild-type HSV-1 using MOIs 5 and 10, but not with MOI of 1. Interestingly, we observed ADAR cells-specific response to infection with UV inactivated HSV-1, which we cannot explain at this point. To investigate the functional relevance of ADAR1 and ADAR2 proteins for virus replication, we transfected cells with ADAR1 and ADAR2 expressing plasmids to overexpress ADAR in cells. Surprisingly, we did not observe any effect on virus DNA replication, indicating that ADAR1 and ADAR2 might not play a role in productive HSV-1 infection. However further investigation is needed to answer this question.

Key words: ADAR, HSV-1, dsRNA, infection, innate immune response

SAŽETAK

ADAR protein su enzimi uključeni u uređivanje dsRNA transkripata deaminacijom u kojoj se adenzin mijenja u inozin. Ova promjena u konačnici može utjecati na stabilnost RNA te na translaciju proteina. Obitelj ADAR proteina čine ADAR1, koji ima dvije izoforme: ADAR1p110 i ADAR1p150, ADAR2 i ADAR3. ADAR1 i ADAR2 su katalitički aktivni dok ADAR3 nije. ADAR uređuju vlastite i brojne druge dsRNA. Nekoliko je studija pokazalo ulogu proteina ADAR većinom u RNA virusima, međutim njihova uloga kod infekcije s dsDNA virusima slabo je istražena. Tijekom virusne infekcije, oni mogu imati antivirusnu ulogu čineći dio urođenog imunološkog odgovora stanica, ali i provirusnu ulogu, blokirajući virusnu dsRNA da je prepozna imunološki senzor, čime se olakšava replikacija virusa u stanici domaćinu, ili obje uloge kao što je primijećeno kod Influenza A virus.

Na temelju in vitro eksperimenata, uspostavili smo stanične modele za analizu uloge ADAR proteina tijekom produktivne HSV-1 infekcije. Određene su razine ADAR proteina i mRNA tijekom produktivne HSV-1 infekcije. Uočili smo pojačanu regulaciju ADAR u U2OS, HeLa, HEK293T i HFF stanicama zaraženim divljim tipom HSV-1 te je takva pojačana ekspresija ovisila o količini virusa kojom se inficira stanica. Zanimljivo je da smo uočili različiti ADAR odgovor na infekciju s UV inaktiviranim virusom kod različitih vrsta stanica, što u ovom trenutku ne možemo objasniti. Nadalje, kako bismo istražili funkcionalnu relevantnost ADAR1 i ADAR2 proteina za replikaciju virusa, transfecirali smo stanice s ADAR1 i ADAR2 ekspresijskim plazmidima za prekomjernu ekspresiju ADAR-a. Naime, nismo primijetili nikakav učinak na replikaciju DNA virusa, što ukazuje da ADAR1 i ADAR2 možda nemaju ulogu u produktivnoj HSV-1 infekciji. Međutim, potrebna su daljnja istraživanja kako bi se odgovorilo na ova pitanja.

Ključne riječi: ADAR, HSV-1, dsRNA, infekcija, urođeni imunološki odgovor

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1.0 INTRODUCTION

Adenosine deaminases acting on RNA (ADAR) are enzymes discovered over 30 years ago by Brenda Bass and Harold Weintraub (1,2). The highly conserved RNA binding proteins are known to mediate the irreversible conversion of adenosines (A) to inosines (I) in double-stranded RNA (dsRNA) substrates, in a process known as RNA editing or A-to-I editing. The Adenosine to inosine (A-to-I) RNA editing involves the selective conversion of adenosines to inosine by hydrolytic C6 deamination leading to an inosine to uracil (I:U) base-pairing in a post-transcriptional modification process. The resulting I:U mismatch is less stable than the normal adenosine to uracil (A:U) base-pairing. During the RNA editing, ADAR proteins bind to messenger RNA (mRNA) transcripts and change the nucleotide content of the RNA. The inosine produced in this process possesses the same properties as guanosine (G) (3). For instance, inosine readily forms two hydrogen bonds with cytosine (C), hence it is detected as guanosine by the cellular translational machinery. Consequently, an inosine to cytosine (I:C) base-pairing is produced, giving rise to a change in the coding sequence. This switch in the nucleotide content of RNA alters their structure as a result of the unstable I:U mismatch, and effectively changes the coding sequence for proteins and their functions as a result of the I:C base-pairing.

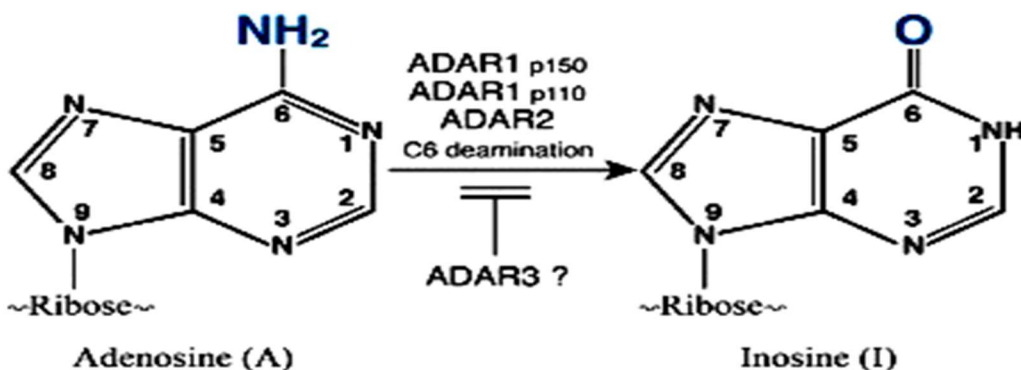


Figure 1: Deamination of adenosine (A) deamination to inosine (I) in double-stranded RNA. The deamination of A to form I is mediated by members of the ADAR family including an interferon-inducible ADAR1p150 and three constitutively expressed ADAR1p110, ADAR2, and ADAR3. ADAR1p150, ADAR1p110, and ADAR2 possess catalytic activity, while ADAR3 does not, but can inhibit active ADARs. Figure adapted from (4).

It has been discovered that ADAR proteins have splicing regulatory capabilities, due to their editing and RNA binding abilities. ADAR is believed to have emerged from ADAT (Adenosine Deaminase Acting on tRNA) protein which is present in eukaryotes at an early metazoan period through the addition of a dsRNA binding domain (5,6). The post-translational modification catalyzed by ADAR proteins is one of the conventional types of RNA editing that generates different RNA molecules and the resulting proteins. ADAR proteins have both selective and nonselective activity and can edit both protein-coding mRNAs and noncoding mRNA molecules such as microRNAs (miRNAs), resulting in a change in the specificity of the miRNA. Various miRNA precursors known as pre-miRNA which are formed from primary miRNA (pri-RNA), undergo RNA editing at precise adenosine sites (7). However, editing of these precursors could lead to an inhibition of further cleavage and processing of RNA, and ultimately the regulation of gene expression (8). miRNAs are a highly conserved class of small interfering RNAs (siRNAs) that are involved in gene silencing and the regulation of cellular processes such as cell development, differentiation, and apoptosis (6,9,10). They are encoded within the non-coding regions of the genome and they form pri-miRNAs structures which in their double-stranded state could serve as a substrate for ADAR. The pri-miRNAs are then processed in the nucleus by Drosha to form pre-miRNA which is then spliced to produce the mature miRNA by Dicer in the cytoplasm. Consequently, the adenosine deamination established in these noncoding regions affects the biogenesis as well as the required target recognition of siRNAs in the RNA interference (RNAi) pathway (6). A study conducted by Nishikura¹¹ suggests that there could be an interaction between the RNAi pathways and editing events which could disrupt the RNAi pathway. Additionally, adenosine deamination could change the entire structure of dsRNA substrates, thereby affecting Dicer processing and the resulting siRNA production. Also, editing of siRNAs could alter the base-pairing of the RNA-induced silencing complex (RISC) which

consists of the actual mRNA targets, thereby hampering the RNAi cleavage step (6,11).

Editing by ADAR proteins can be both antiviral and proviral. That is, the RNA editing process could either initiate or suppress miRNA/target interaction by modifying the pri-miRNA at necessary complementary positions for proper miRNA targeting. Suggestions have been made that ADARs may edit viral RNA in a way that the outcome of the viral infection is affected by acting directly on the virus, or indirectly by editing the cellular RNA in a way that the cellular product is altered, thereby affecting the interaction of the virus with the host cell. On the other hand, ADAR proteins may possibly display an editing independent approach, where the target protein or nucleic acid binding interactions are altered leading to an eventual change in the viral infection consequence. ADAR1 and ADAR2 however, have common substrate specificity (12). More so, an abnormal or lack of editing by ADAR has been associated with epilepsy (13,14), schizophrenia (15,16), amyotrophic lateral sclerosis (ALS) (17), and suicidal depression (6,18,19). Genetic studies associate ADAR1 mutation with Aicardi-Goutières syndrome (AGS) (20) and Dyschromatosis symmetrica hereditaria (DSH) (21). Therefore, more studies of ADAR proteins will provide an understanding of their functional properties and possible therapeutic strategies for these diseases.

1.1 MEMBERS OF THE ADAR FAMILY

There are three types of ADAR in mammals, namely; ADAR1, ADAR2, and ADAR3. Although they share many similarities, they also have significant differences in their regulation, subcellular localization, protein structure, and function (12). ADAR1 and ADAR2 are found in many tissues of the body and are known to be catalytically active (12) while ADAR3 is found only in the brain and is known to be catalytically inactive (22).

1.1.1 ADAR1

Adenosine deaminases acting on RNA 1 (ADAR1) has two isoforms known as ADAR1p110 and ADAR1p150, named by the size of proteins in kilodaltons (kDa). The full length (ADAR1-p150) and an amino-terminally truncated (ADAR1-p110) isoforms are generated by an alternative promoter usage within the ADAR1 transcript and consist of three dsRNA binding domains as well as the deaminase domain. ADAR1p150 is interferon (IFN) inducible and is an N-terminal extension of the ADAR1p110. The ADAR1p110 is primarily found in the nucleus while the ADAR1p150 is found in the nucleus and cytoplasm and shuffles between the cellular compartments, but is predominantly in the cytoplasm. The cytoplasmic localization of ADAR1p150 could mean that a selected class of dsRNAs is targeted in the cytoplasm as mature messenger RNA (mRNA). ADAR1 Expression is driven by alternative promoters (P_A and P_B) (23,24). The P_B promoter identifies the constitutively and ubiquitously expressed ADAR1p110, while the IFN-activated P_A promoter, produces transcripts that encode the inducible ADAR1p150 protein. The P_A promoter possesses an interferon-stimulated response element (ISRE) which is a feature of type I IFN ($IFN\alpha/\beta$) inducible genes (23,24). Following the canonical $IFN\alpha/\beta$ signalling response, the binding of type I IFNs to their allied cell receptor (Interferon alpha/beta receptor), initiates the activation of Jak-STAT signaling (25) which leads to the phosphorylation of STAT1 and STAT2 transcription factors. Phosphorylated STAT1 and STAT2 dimerize and associate with transcription factor IRF9 (Interferon regulatory factor 9) to form the heterotrimeric ISGF3 (Interferon stimulated gene factor 3) complex which then migrates to the nucleus and binds DNA at the ISRE element of type I interferon-stimulated genes.

The p150 isoform initiates from AUG1 which is present in exon 1A of the IFN-inducible human transcript, while the constitutively expressed p110 isoform initiates from AUG 296 present in exon 2 since the exon 1B alternative lacks

an AUG (26). Exon 7a is also found in the ADAR1 transcripts that specify the p110 isoform which is predicted to have 931 amino acids in humans and 903 amino acids in mice. Accordingly, the smaller exon 7b is found in the IFN-inducible human ADAR1 transcripts that specify the p150 isoform which is predicted to have 1200 amino acids in humans and 1152 amino acids in mice (4,12,26). However, the exon organization and expression of ADAR1 mice involves the use of alternative promoters as well as alternative splicing just like the human ADAR1 protein (26–28).

Both p110 and p150 ADAR1 isoforms actively catalyse adenosine deamination in dsRNA (29–31). They both possess a C-terminal region which represents the catalytic domain and three copies (R_I , R_{II} and R_{III}) of the dsRNA binding domain present in the central regions. (29,30,32,33). The p150 isoform is extended in the N-terminal region compared with the p110 isoform, with an additional Z-DNA binding domain (12,34). The p150 isoform has $Z\alpha$ and $Z\beta$ -DNA binding domains, while the p110 isoform has only the $Z\beta$ domain.

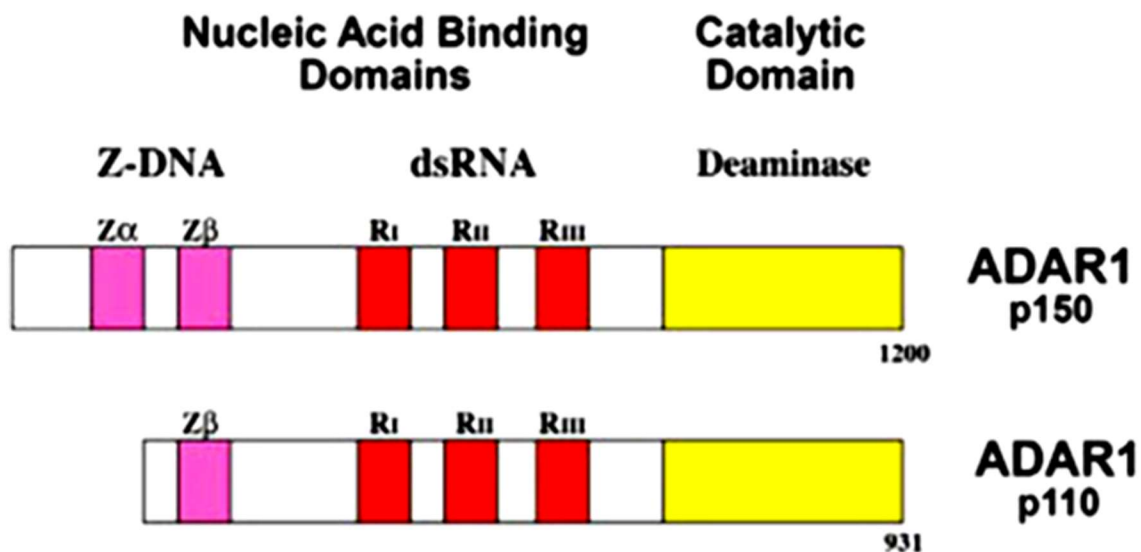


Figure 2: A representation of domain organization of ADAR1 proteins. There are three copies of the dsRNA binding domain (red, R_I , R_{II} and R_{III}) in the nucleic acid-binding domain. The N-terminal region of ADAR1 p150 possesses two copies of a Z-DNA binding domain ($Z\alpha$ and $Z\beta$, pink), while p110 possesses only one copy ($Z\alpha$). Both isoforms possess a deaminase catalytic domain (yellow) in the C terminus.

1.1.2 ADAR2

Adenosine deaminases acting on RNA 2 (ADAR2) like ADAR1, is catalytically active and is found in many tissues but is most abundantly expressed in the brain (35). Also similar to ADAR1, alternative splicing occurs in ADAR2, yielding diverse transcripts including alternative forms of exons 2, 5, 9, and 10, which indicates different isoforms of ADAR2 protein possibly with different deaminase activities (36,37). ADAR2 localizes predominantly in the nucleus (38,39) and shares the same characteristics with ADAR1 in terms of substrate editing and specificity (40). It possesses a catalytic domain at the C-terminus and a nucleic acid binding domain which consists of R_I and R_{II} dsRNA binding domains. A molecule of inositol hexakisphosphate and one zinc ion is contained in its catalytic domain. The inositol hexakisphosphate is considered a co-factor for the catalytic activity of ADAR2 (41). Although ADAR1 is implicated in most mammalian cell A-to-I editing, highly selective editing events in a few identified exonic coding sites have been attributed to compared to ADAR1 (4).

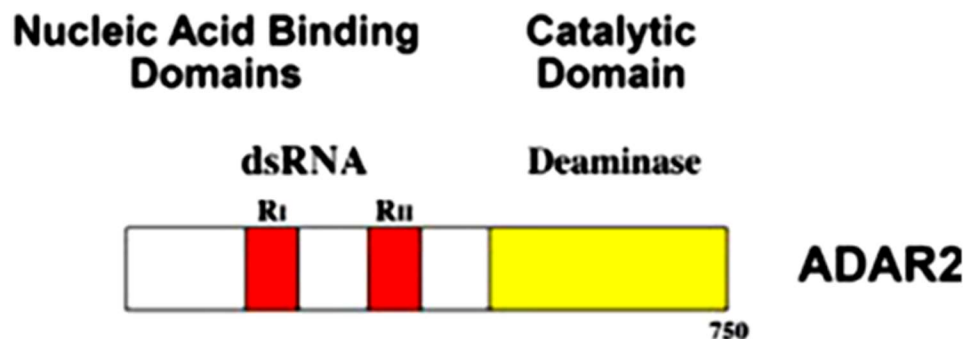


Figure 3: A representation of domain organization of ADAR2 protein. There are two copies (red, R_I and R_{II}) of the dsRNA binding domain in the nucleic acid binding domain of the N-terminal region, while the deaminase catalytic domain (yellow) is contained in the C terminus of ADAR2.

1.1.3 ADAR3

Adenosine deaminases acting on RNA 3 (ADAR3) does not exhibit any catalytic activity. Although ADAR3 has a C-terminal region similar to the deaminases domain of ADAR1 and ADAR2, it has no known deaminases catalytic activity, hence does not catalyze A-to-I editing. However, it is said

that ADAR3 regulates ADAR1 and ADAR2 activity, acting as an inhibitor of the two proteins (22,35,42,43). ADAR3 is only expressed in the brain and like ADAR1p110 and ADAR2, it is predominantly localized in the nucleus (22,35). ADAR3 possess an arginine-rich (ARG) domain, otherwise known as the R domain in its N-terminal region, that possess single-stranded RNA (ssRNA) binding activity (22) which explicitly interact with importin- α 1 and function as a nuclear localization sequence responsible for its localization in the nucleus (44).

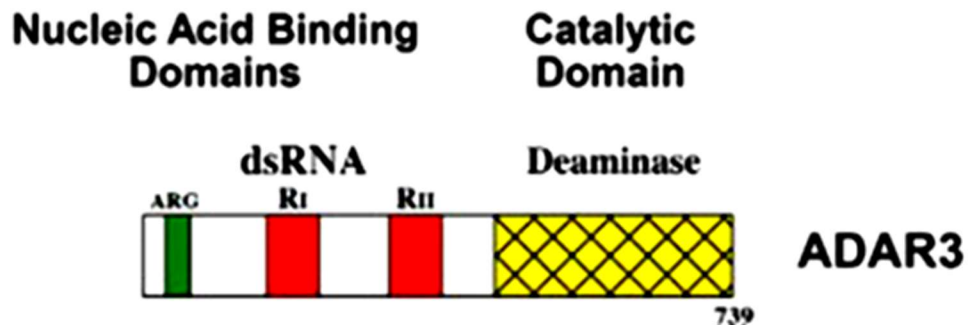


Figure 4: Domain organization of ADAR3 proteins. The N-terminal region possesses an arginine-rich (ARG) domain (green) and nucleic acid-binding domain which has two copies of the dsRNA-binding domains (red, R_I and R_{II}), while the deaminase catalytic domain (yellow) is contained in the C-terminal region.

1.2 The Role of ADAR in Viruses

During viral infection, ADAR-mediated A-to-I editing could have either an antiviral or a proviral effect. The editing activities and expression of a type of ADAR can be influenced by the editing activities and expression of another type of ADAR (19,45).

ADAR editing is classified into two main types. One is highly selective editing, where the conversion of adenosine to inosine occurs at a specific site of RNA transcripts, like the Q/R site of the glutamate receptor subunit GRIA2 (17). The other type of editing is nonselective hyper editing, where editing of multiple adenosines occurs at the same time (5). Mammalian cells mostly undergo this type of editing in the noncoding region of RNA transcripts. Both

ADAR1 and ADAR2 are involved in the two types of editing (40,46). However, details of these editing events are unknown, yet crucial in the host cell's transcriptome heterogeneity and post-transcriptional regulation (34,47,48).

Selective editing which seems to cause slight changes in a few separate codons can lead to intense alterations in target proteins. This kind of editing leads to an array of downstream neuro-physiological changes, such as neuro-developmental malfunction, decreased proliferation, as well as neuronal death (46,49). On the other hand, frequent nonselective hyper editing in edited viral genomes could have significant functional consequences. Here, the indiscriminate introduction of nucleotide changes results in missense and nonsense mutations, as well as changes in dsRNA stability that eventually disrupts the viral protein and genome functions. Interestingly, during the innate immune response, both selective and nonselective ADAR editing could have both proviral as well as antiviral outcomes (4,12,50,51). Editing by ADAR that leads to the disruption of viral protein production required for viral particle assembly could be seen as its antiviral effect and vice-versa. However, the consequence of editing could be situation specific, where the outcome is dependent on the virus and details of the host immune response. For instance, changes in the genome sequence introduced during the early infection stage through editing as an antiviral defense mechanism, could be proviral following a different direction of the immune response and given the selective context of editing (52). More understandably is the ability of ADAR1 to block the activation of IFN-stimulated protein kinase regulated by RNA (PKR), either by binding to PKR directly or by viral dsRNA editing which inhibits the activation of dsRNA immune sensors. Possibly, this is due to the antiviral role of PKR activated PKR either by stimulating apoptosis, enhancing IFN- β production, or shutting down translation (4,52,53).

1.3 ADAR in RNA Viruses

A-to-I editing attributed to ADAR proteins either during the lytic or latent stages of viral infections has so far been reported for a variety of RNA viruses. For some of the viruses, the A-to-I editing is revealed to be driven by a particular type of ADAR, which has a significant functional consequence that influences the result of the infection. For instance, for viruses replicating in the cytoplasm of the host cell, considering subcellular localization, it becomes logical to assume that the ADAR1p150 will most likely be responsible for the editing of such virus because ADAR1p150 is the only known ADAR protein predominantly in the cytoplasm. Likewise, for viruses replicating in the nucleus, either ADAR1p150, ADAR1 p110, or ADAR2 will be suspected for the viral dsRNA editing. (5,26,54).

1.3.1 ADAR in Measles Virus.

A-to-I editing by C6 deamination of adenosine has long been discovered in viruses belonging to the Paramyxoviridae family. A-to-I editing during viral infection was first reported in the Measles virus (MeV) that causes acute childhood infection and spreads via the respiratory route (55). Measles virus is an enveloped virus that belongs to the Morbillivirus genus of the family Paramyxoviridae, and possesses a nonsegmented negative-stranded ~16kb RNA genome that defines the polycistronic P/V/C gene and five monocistronic genes. The MeV P/V/C gene encodes accessory proteins C and V which regulates innate immune responses, as well as the formation of defective interfering (DI) RNAs which serve as ADAR substrates and PKR activators due to their double-stranded structure (53).

Since MeV cytoplasmic replicating virus, it is presumed that editing is performed by ADAR1p150. In the case of MeV, ADAR1 is generally proviral by counteracting PKR activation, apoptosis (56), stress granule formation (57), and type I IFN production. ADAR1p150 is known to hyper edit dsRNA-forming DI RNA in MeVs lacking the C protein (52,58). This hyper editing

activates innate immune responses (56,59). ADAR1 interferes with the recognition of DI RNA by dsRNA sensors including PKR by altering their secondary structures (52). Also, ADAR1p150 inhibits autoimmune reactions against self dsRNA by editing mRNAs with Alu-duplex structures (58,60). This suggests that both self dsRNA and viral dsRNA can stimulate PKR activation as well as MDA5 and oligoadenylate synthetase (OAS), when the entire number of dsRNA is higher than the level that can be effectively edited by ADAR1p150 (58,61,62).

1.3.2 ADAR in Influenza A Virus.

Influenza A virus (IAV) is a member of the Orthomyxoviridae virus family. They are enveloped and possess a segmented negative-stranded RNA genome made up of eight distinct ssRNAs. Tenoever⁶³ observed an A-to-G conversion in IAV RNA of infected mice having a comprehensive innate immune system. Their investigation however revealed that lack of IKKε kinase in mice leads to hyper-susceptibility to influenza virus infection, perhaps due to inadequate IFN signaling. They observed a poor expression of subsets of Interferon stimulated genes including ADAR1p150 represented by reduced A-to-I editing by ADAR in IKKε deficient mice infected with the wild-type strain of influenza virus.

ADAR plays diverse roles in IAV infections. Some strains of IAV are known to trigger upregulation of ADAR1 but not ADAR2. This leads to an increased A-to-I editing of both cellular (64) and viral (65) RNAs. ADAR1p150 plays a proviral role in IAV infection (56,58) by blocking RIG-like receptor signaling that triggers transcriptional induction of type 1 IFN and other genes required to establish an antiviral host response. Hence, it is required for efficient IAV replication in host cells (66). ADAR1p110 on the other hand plays an antiviral role since reduced expression of ADAR1p110 results in an increase in viral replication (52,66).

The IAV nonstructural protein 1 (NS1) also plays a role in the outcome of ADAR editing. NS1 is a dsRNA-binding protein that is an antagonist of PKR (12). It interacts with ADAR1p110 in a dsRNA-binding-independent manner by interacting with the RI and RII domain, thereby reducing the antiviral activity of p110 and of course enhancing IAV replication (52).

1.4 ADAR in Human Herpesviruses

Viruses of the *Herpesviridae* family are usually large, enveloped viruses having a linear double-stranded DNA (dsDNA) of about 125-250kb genome size. Replication of viral DNA, as well as transcription by cellular RNA polymerase II, occurs in the nucleus. The Epstein-Barr virus (EBV) and the Kaposi sarcoma-associated herpesvirus (KSHV) are the only human herpesviruses studied in the context of A-to-I editing. They are known to possess miRNAs that regulate virus-host interactions, maintain latency and mediate the reverse from latent to lytic phase during viral reactivation of several viral long noncoding RNAs (vlncRNA) (52).

1.4.1 Epstein-Barr virus

Four transcripts of EBV miRNAs (pri-miR-BHRF1, pri-miR-BART6, pri-miR-BART8, and pri-miR-BART16) are known to undergo A-to-I editing at specific sites by ADAR proteins, of which pri-miR-BART6 is more frequently edited (67). miR-BART6 targets Dicer processing and its binding to the 3' untranslated region (UTR) of the Dicer mRNA diminishes the expression of Dicer and affects mature miRNA and EBV latency. Editing by ADAR interrupts the incorporation of miR-BART6-5P into an active miRNA-induced silencing complex (miRISC) (67). ADAR proteins not only edit cellular miRNA, but also viral miRNAs. Editing by both ADAR1 and ADAR2 can affect the processing of miRNA precursor (pri-miRNA) or the targeting of mature miRNAs (68). During viral reactivation, EBV also expresses vlncRNAs like the origin of replication leftward transcript (oriPtLs) which is hyper-edited by ADAR1 and

as a result of this editing, the viral DNA replication and expression of the viral lytic gene are affected (69).

1.4.2 Kaposi Sarcoma-associated Herpesvirus

KSHV is responsible for acquired immunodeficiency syndrome-associated malignancies known as primary effusion lymphoma and Kaposi sarcoma (52). Kaposin proteins; A, B, and C are abundantly expressed by the viral K12 RNA transcript which is induced in lytic Kaposin sarcoma-associated KSHV infection and also expressed during the latent phase of the infection. The K12 transcript encodes kaposin A ORF as well as miR-K10, and possesses tumourigenic potential (70). A-to-I editing which changes glycine to serine occur at the genome position 117990 of the K12 transcript of the Kaposin A protein and ultimately altering mRNA targeting. This editing is increased during lytic KSHV infection (71). Purified ADAR1-p110 was able to selectively edit adenosine at position 117990 of the K12 RNA transcript, and the ADAR1 transcript is more abundant in the primary effusion lymphoma cells than ADAR2 (71). In KSHV, A-to-I editing seems to be involved in the control of both kaposin A and miR-K10 transcript expression. It has been observed that unedited kaposin A protein at position 117990 is involved in tumourigenicity (12).

1.4.3 Herpes Simplex Virus 1

Herpes simplex virus 1 (HSV-1) is a type of Herpes simplex virus (HSV), a member of the group of eight human herpesviruses, of the *Herpesviridae* family and *Alphaherpesvirinae* sub-family. HSVs were the first among the human herpesviruses to be discovered and are mostly investigated due to their unique biologic properties, especially their ability to cause a variety of infections, go into a life-long latency in the host cell, and be reactivated to cause lesions either at the same site of initial infection or near the site (72). HSV has two serotypes considered to be different virus species; HSV-1 and HSV-2, which are enveloped, nuclear replicating, and closely related double-stranded DNA viruses sharing 83% homology in their genome's protein-

coding regions (73). They also share many similarities in their biological functions. The HSV structure is made up of 4 elements namely; an electron-dense core containing the double-stranded DNA (dsDNA) genome enclosed as a spool in a liquid crystalline state or as a toroid, an icosadeltahedral capsid surrounding the core and made up of 162 capsomers, an unstructured tegument surrounding the capsid, and an outer envelope having spikes on its surface and consisting of a lipid bilayer with about 12 enclosed viral glycoproteins.

HSVs cause Herpes simplex disease commonly called cold sores or fever blisters which are often activated by febrile illnesses, such as a common cold. They also cause herpes gingivostomatitis, keratitis, encephalitis, and genital herpes amongst others (72). However, HSV-1 is mainly associated with facial and oral infections.

During the HSV-1 productive infection stage, a cascade of viral proteins, known as immediate-early (IE), early (E), and late (L) genes, are produced which function in viral replication and capsid formation. They stop the synthesis of protein in the host cell, degrade host mRNA, help in viral replication and regulate the viral protein expression. These proteins are expressed sequentially, from IE to L proteins. The tegument protein VP16 interacts with cellular transcription factors and initiates the transcription of the IE messenger RNA (mRNA) which encodes the infected cell protein 0 (ICP0), ICP4, ICP22, ICP27, and ICP47, expressed following the action of protein synthesis inhibitors. ICP0, 4, 22, and 27, regulate the transcription and translation of the E and L genes (74). ICP4 is known to inhibit the expression of IE genes by blocking their expression in the later stages of viral infection. When ICP4 and ICP27 accumulate in cells, the expression of E genes is initiated, primarily promoting DNA replication. E gene encodes proteins such as the ribonucleotide reductase and viral thymidine kinase, as well as DNA helicase and viral DNA polymerase directly involved in DNA replication. After DNA replication has occurred, the L genes including VP16,

structural glycoproteins required to recruit progeny viral particles, and vhs, which are integrated into the viral tegument, are produced. Once the late genes are produced, the viral capsid assembling occurs in the nucleus with an up-take of separate DNA genomes upon complete assembly assembled (75). The completely assembled capsids further associate with the tegument proteins and buds via the viral glycoprotein-containing nuclear membrane, enabling the incorporation of the virus into vesicles which then spread by syncytia formation or are discharged by exocytosis or cell lysis (72,74).

After the primary episode of infection, when the virus enters cells and starts its productive cycle, HSV-1 can enter neuronal cells where it enters into a lifelong latent state (76). The main reservoir of latently infected neurons is the trigeminal ganglia. The molecular mechanism that controls the establishment, maintenance, and reactivation from latency is poorly understood. However, miRNAs and long non-coding RNAs, the only abundantly expressed transcripts during latency, have an important role in these processes. Occasionally, under specific stimuli such as stress or long exposure to the sun, the virus can reactivate from latency and travel from neurons to epithelial cells, and cause recurrent disease. Infections with HSV-1 are not usually severe but can be life-threatening for patients with compromised or low immunity (72,76).

HSV-1 infection initiates an antiviral immune response, an inflammatory reaction such as the secretion of antiviral substances like nitric oxide and defensins as well as the production of chemokines and cytokines, in an infected cell. The type I IFNs are cytokines that are induced during the early hours of HSV-1 infection by ISGs such as the 2'-5'-OAS/RNase L system and PKR for antiviral defense (74,77), and are crucial in HSV replication and control (68,78,79). IFN acts through the induction of several ISGs (80), but the 2'-5'-OAS/RNase L system and PKR are the most important ISGs for antiviral defense. HSVs have evolved strategies to avert or repress the

innate immune response including modulation of immune signalling pathways which facilitates its gene expression, replication, and survival in the host cell. HSVs counteract IFN production, suppresses IFN signaling, and block the activation of ISGs through the expression of various viral genes, including ICP0, ICP27, ICP34.5, and host shutoffs (VHS) (74).

Since viruses generally manipulate the host's innate immune response to facilitate its replication, HSV-1 could possibly manipulate the function of ADAR being a dsRNA binding protein, to its benefit in a way that ADAR binds to the dsRNA introduced in cells by the virus and blocks recognition of innate immune sensors. The role of ADAR in HSV-1 replication is not known. Consequently, there is a need for more studies to unveil the role of ADAR proteins in DNA viruses. Hence this project, to analyze ADAR proteins during productive HSV-1 infection. This study will serve as a foundation for further investigations, especially on the latent phase of HSV-1 infection.

2.0 AIMS AND OBJECTIVES

Members of the ADAR (adenosine deaminases that act on RNA) proteins family bind double-stranded RNA, which can be their substrate for editing or a trigger of intrinsic antiviral immunity. Both functions have been shown to have an important impact on the replication of many viruses. Our group has discovered ADAR-mediated editing of herpes simplex virus 1 encoded miRNA precursors in latently infected human ganglia, but its relevance to HSV-1 infection is unknown and is yet to be discovered. In this study, we initially address the role of ADAR proteins during productive HSV-1 infection.

Our main hypothesis is that HSV-1 infection, similar to many host proteins, alters the expression of ADAR proteins and that RNA binding activity, but not editing, plays an important role in suppressing host antiviral defences.

Specific aims of the project:

- 1)** To analyse levels of ADAR proteins during productive HSV-1 infection.
- 2)** To determine the importance of ADAR1 and ADAR2 during HSV-1 infection.

Objectives:

- 1) To analyse levels of ADAR proteins during productive HSV-1 infection.
 - a. Analyse the levels of ADAR proteins during the time course of infection in different cell lines.
 - b. Determine mRNA levels of ADAR genes during HSV-1 productive infection using the transcriptome dataset.
 - c. Analyse ADAR protein levels during infection using UV-inactivated HSV-1 and inhibitors of virus DNA replication.

- 2) To determine the importance of ADAR1 and ADAR2 during HSV-1 infection
 - a. Clone ADAR1 and ADAR2 expressing plasmids.
 - b. Analyse HSV-1 replication in cells overexpressing ADAR1 and ADAR2 proteins.

3.0 MATERIALS AND METHODS

3.1 Reagents

Cell Culture Reagent

- DMEM (Dulbecco's Modified Eagles Medium) (PanBiotech) supplemented with 10% FBS (Fetal Bovine Serum), 2mM L-glutamin, and Penicillin/streptomycin.
- 1% PBS (Phosphate Buffered Saline) (PanBiotech)
- 2X Trypsin (PanBiotech, P10-024100)

Titration Reagents

- 2X Methyl cellulose (1% methyl cellulose 1mM Sodium pyruvate (Capricon), 100µg/µl Penicillin/Streptomycin (Pan-Biotech, 1X DMEM (PanBiotech), 3% FBS (PanBiotech))
- Fixative (5% methanol, 10% acetic acid in PBS)
- Giemsa stain (Carl Roth) (5% in PBS)

Cells Used

- HeLa cells, generous gift from Ivan Ahel, Sir William Dunn School of Pathology, University of Oxford.
- Human Embryonic Kidney 293T (HEK293T) cells (ATCC, CRL-3216)
- Human Foreskin Fibroblast (HFF) cells, generous gift from prof. Stipan Jonjić, University of Rijeka.

Virus Used For Cell Infection

Herpes simplex virus 1, strain KOS, generous gift from Donald M. Coen, Harvard Medical School.

Reagent Used For Interferon Induction

- IFNβ 10ng/ml (R&D Systems, #RD-8234-MB-010/CF)

- Poly I:C 10µg/ml (Invivogen, 31852-29-6)

Protease Inhibitors Used

- Phosphonoacetic acid (PAA) 300mg/ml (Sigma Aldrich, 284270-10G)
- Acycloguanosine (acyclovir), 98% - 50g/ml (Alfa Aesar, J64144)

Protein Isolation Reagents

- RIPA (radioimmunoprecipitation assay) buffer (25mM Tris-HCL pH7.6, 150mM NaCl (Sodium chloride), 1% Nonidet P-40, 1%, Sodium deoxycholate, 0.1% SDS (Sodium dedocyl sulfate)).
- Protease Inhibitor (Sigma Aldrich, #11836170001)
- 2x Sample Buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, 0.125M Tris-HCl, pH 6.8, 10% 2-mercaptoethanol)

SDS-PAGE Gel Reagent

- 10% Resolving gel: Distilled water, 40% acrylamide, Tris (1.5M, pH 8.8), 10% SDS, 10% APS (ammonium persulfate), TEMED (tetramethylethylenediamine)
- 5% Stacking gel: Distilled water (dH₂O), 40% acrylamide, Tris (1.0M, pH 6.8), 10% SDS, 10% APS, TEMED

Western Blot Buffers

- 1X Running Buffer (10X running buffer (30.0g Tris, 144g glycine, 10g SDS, 1L distilled water), distilled water)
- 1X Transfer Buffer (10X transfer buffer (30.0g Tris, 144g glycine, 1L distilled water), 96% methanol (CarlRoth), distilled water)
- 10X TBS (Tris buffered saline) (88g NaCl, 24g Tris, pH 7.6, 1L distilled water)
- Ponceau S – 0.1% (Carl Roth)

Protein Ladder

- Thermo scientific PageRuler prestained protein ladder #26616
- NEB (New English BioLabs) color prestained standard broad range

#P7719

Protein Detection Reagent

- Solution A – Amersham ECL Prime Luminal Enhancer Solution (GE Healthcare)
- Solution B – Amersham ECL Prime Peroxide Solution (GE Healthcare)
- SuperSignal West Femto Stable Peroxide Solution thermo scientific #1856191
- SuperSignal West Femto Luminol/Enhancer Solution #1856192

Membrane Blocking and Washing Reagents

- Membrane blocking: 5% milk (Carl Roth) in TBS-T (Tris buffered saline with Tween 20 detergent).
- Membrane washing: TBS-T (1:2000)

Antibody List

ANTIBODY	COMPANY	CATALOGUE NUMBER	DESCRIPTION	DILUTION
ADAR1	Santa Cruz Biotech	sc-271854	Mouse	1:1000
ADAR2	Santa Cruz Biotech	sc-73409	Mouse	1:200
ADAR3	Santa Cruz Biotech	sc-73410	Mouse	1:500
Actin (clone C4)	Millipore	2691430	Mouse	1:10 000
Gc	Abcam	ab6509	Mouse	1:2000
GFP (3F8.2)	Millipore	MAB1083	Mouse	1:1000
ICP4	Abcam	ab6514	Mouse	1:2000
ICP8	Abcam	ab20194	Mouse	1:2000
ICP27	Santa Cruz Biotech	sc-69807	Mouse	1:500
VP16(UL48)	Abcam	ab110226	Mouse	1:2000
α -mouse	Cell Signalling	7076S	Secondary antibody	1:2000

Plasmids

- Laboratory constructed plasmids (ADAR1 active, ADAR1 inactive, ADAR2 active and ADAR2 inactive) generous gift from prof. Angela Gallo, Ospedale Pediatrico Bambino Gesù, Roma, Italy.

- Commercial plasmids (ADARp110, ADAR1p150 and ADAR2) (Addgene).
- pEGFP plasmid (Addgene).

Antibiotic for Plasmid Selection

- G418 disulfate salt solution (Neomycine) 50mg/ml – (Sigma Aldrich, G8168-10ML)

Reagents for Restriction Digest

- HpaI (New English BioLabs, #R0105S)
- NdeI (Fermentas, #ER0581)
- EcoRI (New English BioLabs, #R0101L)
- HindIII (New English BioLabs, #R1014L)
- Cutsmart restriction buffer (New English BioLabs, #B7204S)
- Buffer 2.1 (New English BioLabs, #B7202S)

Reagents For Agarose Gel Electrophoresis

- 1X TAE Buffer (50X TAE buffer (242g Tris, 100ml 0.5 EDTA, 57.1ml Glacial acetic acid, 1L miliQ water), miliQ water)
- Agarose standard (Carl Roth)
- Luria Bertani broth (Carl Roth)
- GelStar Nucleic Acid Gel Stain (Lonza, #50535)
- 6X Loading Dye (New England BioLabs, #B7021S)
- 1kb Plus DNA Ladder (New England BioLabs, #N3232S)

Transfection Reagent

- Lipofectamine 3000 Transfection Reagent (Invivogen)

Reagents for Cell Preparation for Immunofluorescence Visualization

- 1X PBS (Carl Roth)
- 4% Paraformaldehyde (PFA) (Carl Roth)
- 100mM Glycine (Carl Roth) in PBS
- DAPI 1:10,000

3.2 Cell Culture

Cells were taken from the liquid nitrogen and thawed in a water bath at 37°C. Under sterile conditions, 1ml of the cell was transferred into a 15ml tube containing 6ml of cell culture medium. Cells were centrifuged at 1500rpm for 5 minutes at 4°C to pellet cells. The supernatant was discarded and the cell pellet was resuspended in 1ml of medium. Resuspended cells were then transferred to a 10cm cell culture dish containing 9ml of medium. Cells were distributed all over the dish by moving the dish left to right and up and down as in making a cross sign, or by making a figure 8 shape on a desk. After distribution, cells were stored in an incubator at 37°C and 5% CO₂.

To passage cells when they reach 70-80% confluency, the old medium was aspirated and cells were washed with 5ml of PBS under aseptic conditions. 3ml of 2X trypsin was used to detach cells adhered to the cell culture dish. After 3 minutes of incubation, cells were transferred into a 15ml tube containing 7ml of medium and centrifuged at 1500rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 1ml of medium, then transferred into a 10cm dish containing 9ml of medium. Cells were gently distributed all over the dish and stored in an incubator at 37°C and 5% CO₂.

3.3 Cell Infection with HSV-1

Wild-type and UV-inactivated HSV-1 were used to infect cells under aseptic conditions.

Cells were seeded in a multiple-well cell culture plate and incubated at 37°C and 5% CO₂ for 24 hours. The number of cells to seed depends on the cell type, size, and growth rate. MOI was calculated and the virus was taken from the -80°C refrigerator, thawed in a water bath, and used to infect cells. The total volume of the virus was pipette into the total volume of liquid

needed for the infection, usually, half the volume of medium required per well. So, for infection in a 12-well plate usually requiring 1ml of media, 500 μ l of infectious media was used to infect cells after removing the initial medium in all wells. After 1 hour post-infection, the infectious media was replaced with non-infectious media, and cells were collected over a time course by trypsinization for protein isolation and analysis. UV inactivated HSV-1 was prepared by pipetting the volume of wild-type HSV-1 needed for infection into a 6cm cell culture dish and exposing it to ultraviolet rays for 20 minutes before infection.

3.4 Protein Isolation for Western blot analysis

Cells were harvested by trypsinization for cells to detach from the cell culture plate. Detached cells were resuspended in fresh medium and collected into Eppendorf tubes. Cells were then centrifuged at 14,000rpm for 1minute 30seconds at 4°C. The supernatant was discarded and the pellet was resuspended in 50 μ l of ice-cold RIPA buffer containing protease inhibitor to lyse the cells. Lysed cells were centrifuged at 10,000rpm for 15 minutes to separate cell debris. The supernatant was transferred into a new tube and stored in -20°C freezer for future use or 2X sample (Laemmli) buffer was added immediately at a ratio of 1:1 for western blot analysis. Isolated protein was denatured by heating on the heating block at 95°C for 6 minutes and placed in ice to cool before loading on gels for the analysis.

3.5 SDS-PAGE Electrophoresis and Western blot Analysis

Isolated proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). 10% polyacrylamide gel was prepared for 2 x 1.0mm glass; for 10% resolving gel, 4.8 μ l dH₂O, 2.5 μ l acrylamide, 2.5 upper Tris, 100 μ l SDS, 100 μ l APS, 10 μ l TEMED was used. For 5ml of stacking gel, 2.5 was used. Protein samples were loaded on the gels and separated using 1X running buffer at 80V for 30 minutes and extended to

120V for about 1 hour depending on the protein sizes of interest. After separation, electrophoresis gel was transferred to a nitrocellulose membrane and ran in transfer buffer for about 1 hour 30 minutes. The membrane was stained with Ponceau S and blocked with 5% milk in TBS-T. The membrane was incubated in primary antibody overnight, washed 3 times in TBS-T (10 minutes each) then incubated in secondary anti-mouse antibody for 1 hour. The membrane was again washed 3 times in TBS-T and proteins were detected in ChemiDoc imaging system using the detection reagent. Detected bands were quantified using the ImageJ software and normalized with Actin as the loading control.

3.6 Titration of Viral Yield

200,000 Vero cells were seeded in each well of a 12-well plate and incubated at 37°C and 5% CO₂ for 24 hours to achieve cell confluency. 10µl of infectious media collected during cell infection and stored at -80°C was serially diluted to 10⁻³, 10⁻⁴, and 10⁻⁵ in 500µl of medium and used to infect cells. 1 hour post-infection, media was replaced with 2X methyl cellulose to cover the cells. Cells were incubated at 37°C and 5% CO₂ for 3 days to allow cells to form plaques. Cells were then fixed with a fixative solution for a minimum of 1 hour and stained with Giemsa stain overnight. Cells were washed and plaques counted under the microscope. The viral yield (pfu/ml) was calculated by multiplying the number of plaques by the dilution factor and by 2 since 500µl of infectious media was used to infect cells.

3.7 Plasmid DNA Isolation and Quantification

Plasmid DNA Miniprep Kit (Macherey-Nagel) Commercial Plasmid DNA Kit was used to isolate plasmids from bacterial cells according to the manufacturer's recommendation.

Briefly, a starter culture was established by inoculating E.coli bacteria transformed with ADAR plasmid, in 2ml of LB medium and incubating

overnight (12-18 hours) at 37°C in a shaking incubator. The starter culture was then used to grow a larger number of bacteria in 100ml of LB medium for another 12-12 hours. The LB culture was centrifuged for 15 minutes at 4,000 rpm to harvest cells. The supernatant was removed, and the resulting bacterial precipitate was resuspended in 8ml of buffer S1. To the suspension was added 8ml of buffer S2 and was mixed gently by inverting the tube 6-8 times after which it was incubated at room temperature for 5 minutes to lyse cells. 8ml of pre-cooled neutralization buffer S3 (4°C) was added to the suspension and mixed by inverting the tube 6-8 times to obtain a homogenous suspension containing an off-white flocculate after which it was incubated on ice for 5 minutes. The NucleoBond® AX 100 column was prepared and equilibrated by pipetting 2.5ml of buffer N2 into the column. The filtrate was discarded and a NucleoBond® folded filter paper was placed on the column and used to filter the bacterial lysate. The column was washed with 12 ml of buffer N3 and flow through was discarded. The plasmid DNA bound to the column was eluted with 5ml of buffer N5 after which isopropanol was used to precipitate the plasmid DNA. This was then centrifuged at 15000 x g for 30 minutes at 4°C. The supernatant was discarded while the DNA pellet was washed with 2ml of 70% ethanol, vortexed briefly, and centrifuged at 15000 x g for 10 minutes. Ethanol was removed after centrifugation, while DNA dried and reconstituted with TE buffer. Plasmid DNA concentration was determined using a spectrophotometer (BioDrop uLITE).

3.8 Plasmid Restriction and Agarose Gel Electrophoresis

A mixture of distilled water, 3µl of restriction buffer, 1µl of restriction enzyme, and 1µl of DNA (1µg/µl) was made in a 1.5ml Eppendorf tube in the order they were listed. The mixture was heated on the heating block at 37°C for 1 hour and separated by agarose gel electrophoresis.

For the electrophoresis, agarose gel was prepared by dissolving 1% of agarose powder in 1x TAE buffer using a microwave oven. 0.5µl of GelStar DNA dye was added to the agarose liquid and poured into the already assembled gel casting tray. Upon solidifying, the gel was transferred into the electrophoresis tank and filled with 1X TAE buffer. DNA ladder and DNA samples mixed with 6X loading buffer were loaded in each well of the gel and ran at 90V for 20-30 minutes. Results were detected using the ChemiDoc imaging system.

3.9 Transfection of Cells with ADAR Plasmids

Both HeLa and HEK293T cells were transfected with ADAR plasmids. Cells were seeded to be 70-90% confluent by the next day. For the transfection, a master mix of the transfection reagents was made by diluting lipofectamine in Opti-MEM in a tube and DNA in Opti-MEM before adding P3000 reagent in another tube. The solution in both tubes was properly mixed before the diluted DNA solution was transferred into the tube containing diluted lipofectamine in a ratio of 1:1. The mixture was incubated at room temperature and used to transfect cells in drops. Cells were incubated for about 24 hours before visualization using an immunofluorescent microscope or analysis. Medium in each well was changed at least 1hour before transfection.

For the selection of transfected (green) cells, cells were treated with G418 neomycin antibiotic 24 hours post-transfection.

3.10 Overexpression of ADAR in Cells

Cells were transfected following the procedure already described and infected with wild-type HSV-1 24 hours post-transfection. The transfected and infected cells were harvested 24 hours post-infection by trypsinization for protein isolation and analysis.

3.11 Immunofluorescence Visualization

Cells were grown on a glass slide in each well of a 12-well cell culture dish for 24 hours and transfected with plasmids. 24 hours post-transfection, the medium was removed and cells were washed in PBS. Cells were incubated in 400 μ l of PFA at room temperature for 20 minutes and washed 3 times with PBS (5 minutes for each wash). Cells were incubated in 1ml of glycine (in PBS) for 5 minutes and washed 3 times with PBS (5 minutes for each wash). Cells were then incubated in DAPI for 1 minute in the dark and again washed 3 times with PBS (5 minutes for each wash). Afterward, the glass slide was placed on a coverslip with a mounting medium and visualized using the fluorescent microscope.

4.0 RESULTS

The expression of proteins of the ADAR family, particularly ADAR1, is upregulated during different virus infections. We have recently discovered the editing of virus-encoded miRNAs in latently infected neurons (Cokarić et al. REF), which led us to hypothesize that ADAR proteins might have a role also in productive HSV-1 infection. In our preliminary analysis of the HSV-1 transcriptome (Zubković et al., not published) of productively infected cells, we discovered that ADAR1 transcripts are upregulated during HSV-1 productive infection (fig. 5 and 6).

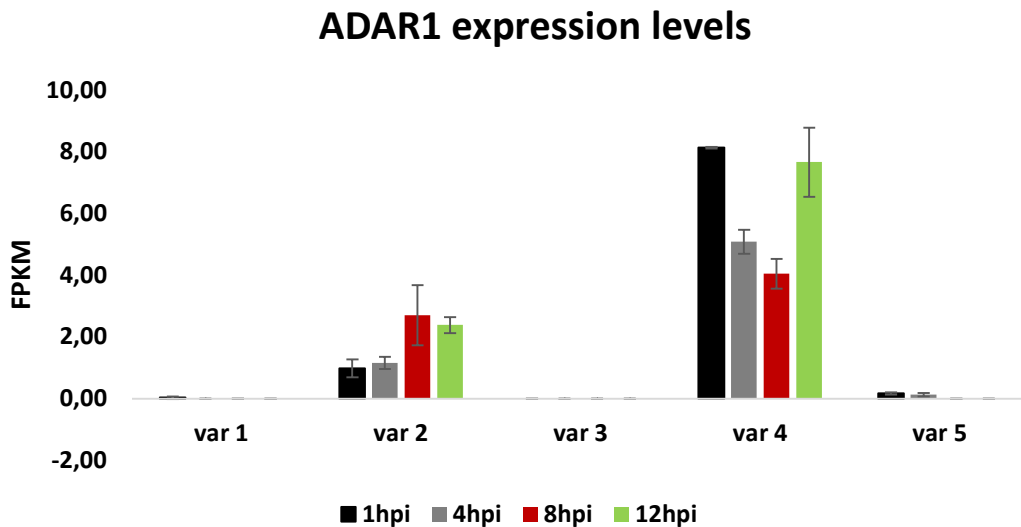


Figure 5. ADAR1 is upregulated during HSV-1 infection in certain variants. HEK293T cells were infected with HSV-1 using MOI of 5 and harvested at 1, 4, 8, and 12 hpi. RNA was isolated from cells and analysed using RNA-Seq approach.

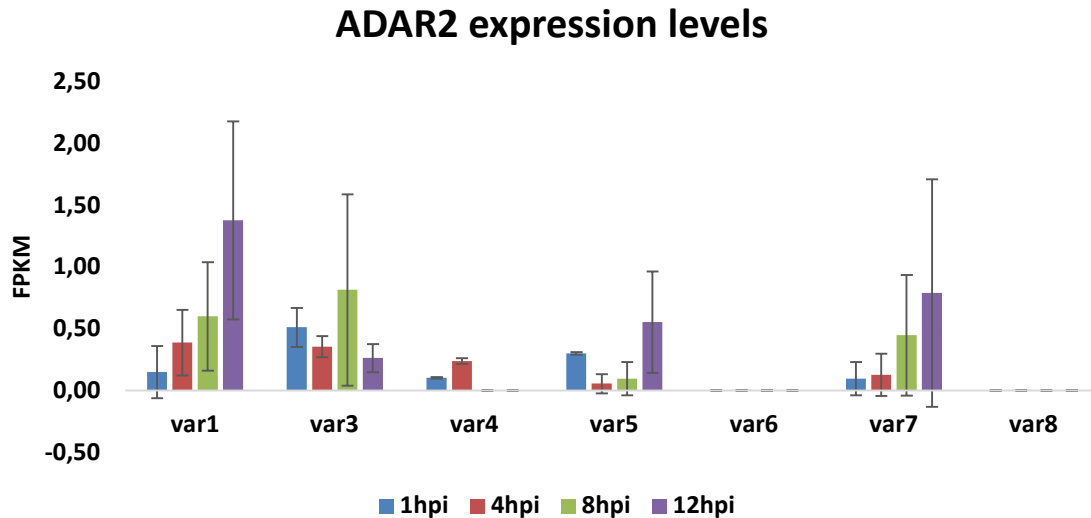


Figure 6 ADAR2 is upregulated during HSV-1 infection in certain variants. HEK293T cells were infected with HSV-1 using MOI of 5 and harvested at 1, 4, 8, and 12 hpi. RNA was isolated from cells and analysed using RNA-Seq approach.

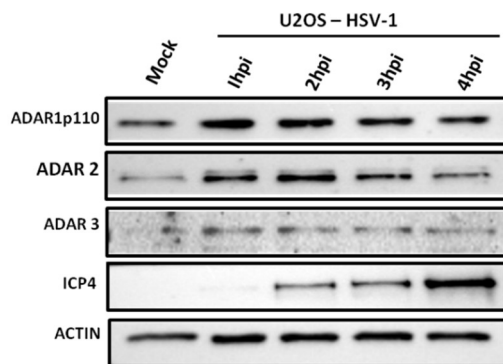
These results indicate that levels of ADAR proteins might also be upregulated and might have a functional role in efficient HSV-1 infection. The upregulation of ADAR proteins could be due to the cell's response to viral infection or viral replication strategy. In other words, it indicates that ADARs might have antiviral or/and proviral activity.

4.1 Expression of ADAR protein is upregulated during HSV-1 infection.

To investigate the dynamics of members of the ADAR protein family during the time course of HSV-1 infection, we infected the human bone osteosarcoma epithelial U2OS cells, the cell line permissive to virus infection and frequently used in laboratories, with HSV-1 at an MOI of 5. Samples for the analysis were collected at different times post-infection and the level of ADAR protein expression in whole cell extract was analyzed by Western blotting (WB). We observed a slight increase in ADAR1p110 expression as early as 1hour post-infection (hpi) (fig. 5), followed by a continuous decrease during the experiment. Levels of IFN inducible p150 form were below the detection limit of our assay. A similar induction pattern was also observed for

ADAR2. On the other hand, ADAR3 protein was barely detectible in our assays, and we did not detect a reproducible increase of this protein, and thus was omitted from further analyses. We were very intrigued with the induction of ADAR1 and ADAR2 and to investigate whether the observed phenomenon is cell-specific, we infected additional cell lines including HeLa, Human embryonic kidney (HEK) 293T, and primary human foreskin fibroblasts (HFFs). The result of the investigation in HFFs is shown and described later. Similar to U2OS cells, we observed induction of ADAR1 p110 protein and ADAR2 in all cells tested (fig. 6, 7, and 13). Interestingly, the ADAR1 p150 form was detected both in HeLa and HEK293 cells, and the results indicate a slight increase during the time course of infection.

A.



B.

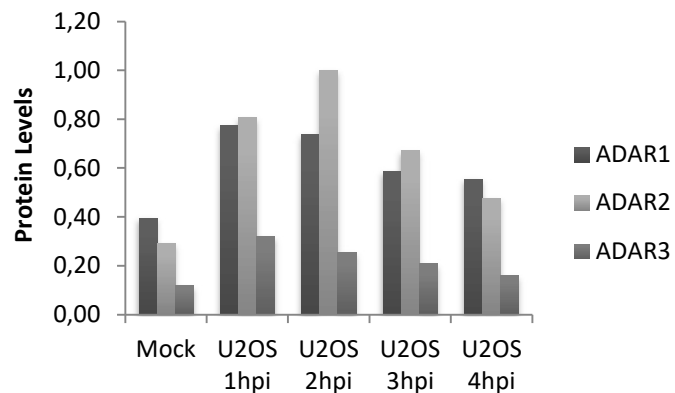


Figure 7. ADAR proteins in U2OS cells are upregulated upon HSV-1 infection. U2OS cells were infected with HSV-1 and cells were harvested at 1, 2, 3, and 4hpi. (A) Cells were lysed and isolated protein was analysed by Western blot with antibodies against ADAR1, ADAR2, ADAR3, ICP4, and β -Actin. (B) The result of the Western blot was quantified using imageJ software and normalized with β -Actin.

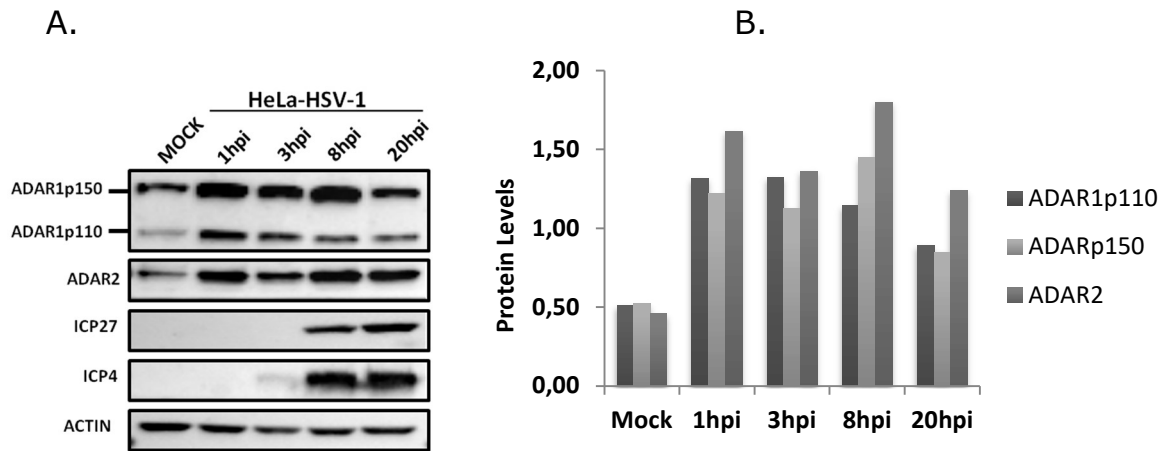


Figure 8. ADAR proteins in HeLa cells are upregulated upon HSV-1 infection. HeLa cells were infected with HSV-1 and cells were harvested at 1, 3, 8, and 20hpi. (A) Cells were lysed and isolated protein was analysed by Western blot with antibodies against ADAR1, ADAR2, ICP27, ICP4, and β -Actin. (B) The result of the Western blot was quantified using imageJ software and normalized with β -Actin.

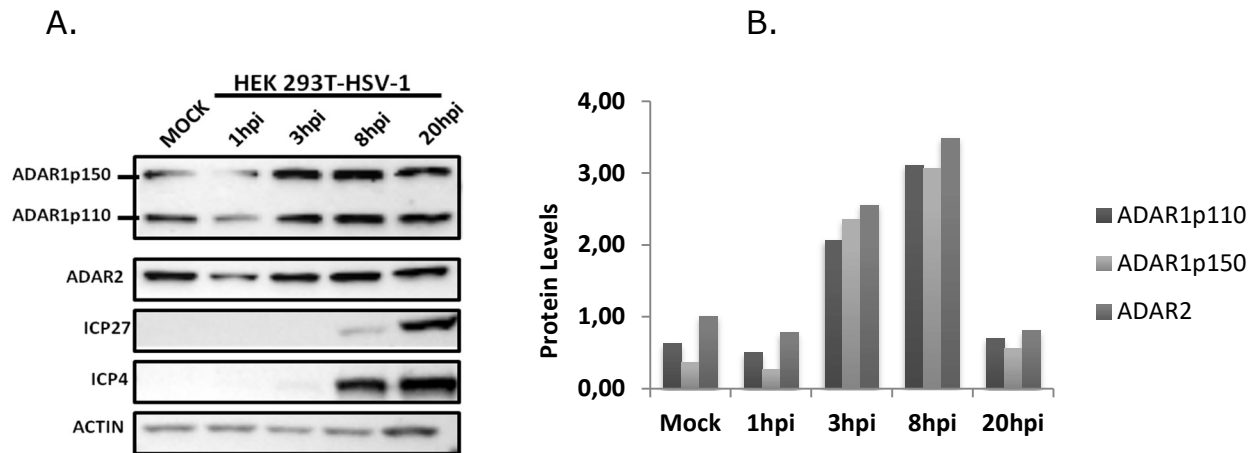


Figure 9. ADAR proteins in HEK 293T cells are upregulated during HSV-1 infection. HEK 293T cells were infected with HSV-1 and cells were harvested at 1, 3, 8, and 20hpi. (A) Cells were lysed and isolated protein was analysed by Western blot with antibodies against ADAR1, ADAR2, ICP27, ICP4, and β -Actin. (B) The result of the Western blot was quantified using imageJ software and normalized with β -Actin.

To confirm the observed upregulation of ADAR proteins in cells, transcriptome analysis was carried out by Andreja Zubkovic. Cells were infected with HSV-1 over a time course. Infected cells were harvested at 1, 4, 8, and 12 hpi. RNA was isolated and analysed using the RNA-Seq approach. The result showed an upregulation of certain variants of ADAR1 and ADAR2.

4.2 Viral and gene expression is required for ADAR1 upregulation.

ADAR, particularly ADAR1 is involved in the cell's innate immune response, although this can be hijacked by viruses to aid its DNA replication in host

cells (Toth et al., 2009; Pfaller et al., 2012; Okonsi and Samuel 2013). To investigate if virus gene expression or/and virus DNA replication, or infection per se is sufficient to trigger signaling for ADAR1 upregulation, we infected U2OS cells with wild-type HSV-1 and HSV-1 inactivated by exposure to a high dose of ultraviolet light (20 Joules for 20 minutes) at an MOI of 5. Of note, UV inactivated virus retains the ability to infect cells but is severely impaired for gene expression. In addition, cells infected with wild-type HSV-1 were either treated or not treated with acyclovir (ACV), a nucleoside analogue inhibitor of viral DNA replication, to investigate if viral DNA replication is required for ADAR1 depletion late in infection. Mock infected (i.e. non infected) cells were harvested 1 hour post-infection, while infected cells were harvested at 1, 3, 5, and 24hpi. Harvested cells were lysed and the protein extracted was analyzed by WB.

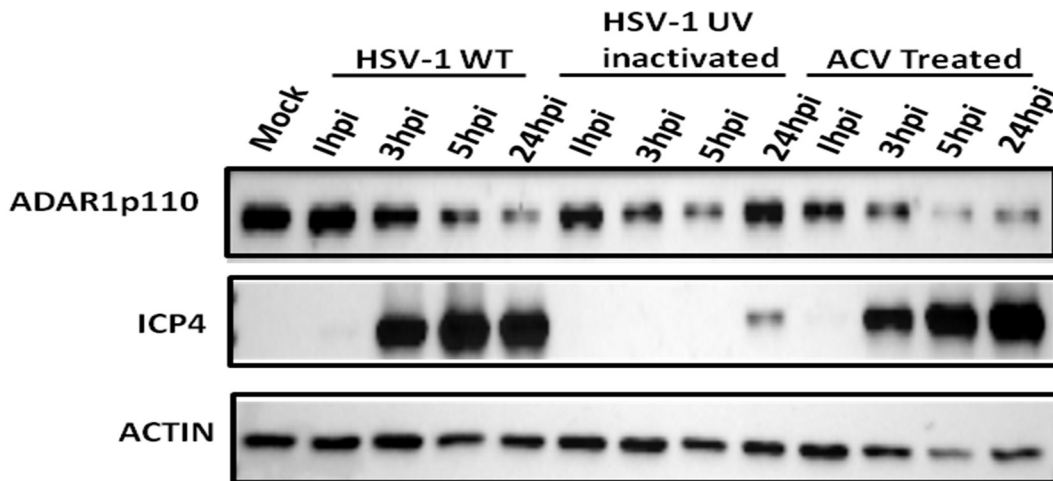


Figure 10. Upregulation of ADAR during HSV-1 infection is the virus replication strategy. U2OS cells were infected with wild-type HSV-1 and UV-inactivated HSV-1. Wild-type HSV-1 infected cells were either treated with acyclovir or not treated. Cells were harvested at 1, 3, 5, and 24hpi. (A) Cells were lysed and isolated protein was analysed by Western blot with antibodies against ADAR1, ICP4, and β -Actin. (B) The result of the Western blot was quantified using imageJ software and normalized with β -Actin.

We observed only a minor increase in ADAR1p110 in cells infected with wild type but not with UV inactivated virus or ACV treated, indicating that virus function might be required. On the other hand, we detected a drop in the

levels of protein expression observed across the time course in both wild-type (ACV treated and untreated) and UV inactivated HSV-1 infected cells when compared to the expression in mock cells, which is consistent with our previous experiments. Interestingly, there was a decrease in ADAR1 expression in UV inactivated virus infection. This result indicates that viral gene expression is not required for the depletion of ADAR1 protein. However, in this experiment we have detected a limited variety of viral proteins as only ICP4 protein, an immediate early virus protein, was detected even in ACV treated cells, indicating that some virus expressional activity has remained. To further investigate the cell's response to UV-inactivated virus, we infected another cell type (HeLa) with UV-inactivated HSV-1 (fig. 11). An increase in ADAR1p110 was observed up to 6hpi. The result seen in cells infected with UV inactivated HSV-1 did not show the same phenotype as in U2OS cells, suggesting cell-specific responses to infection.

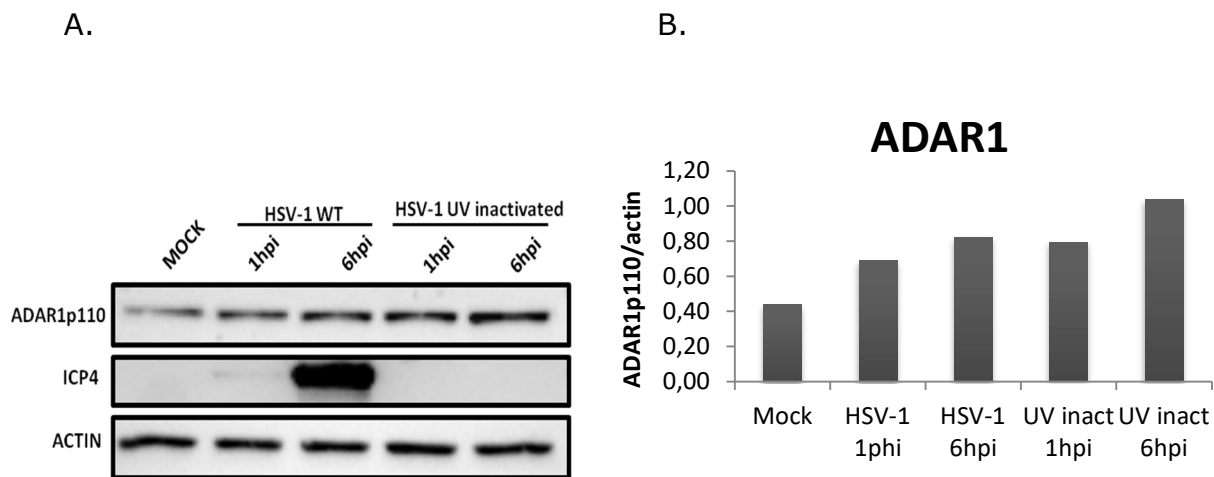
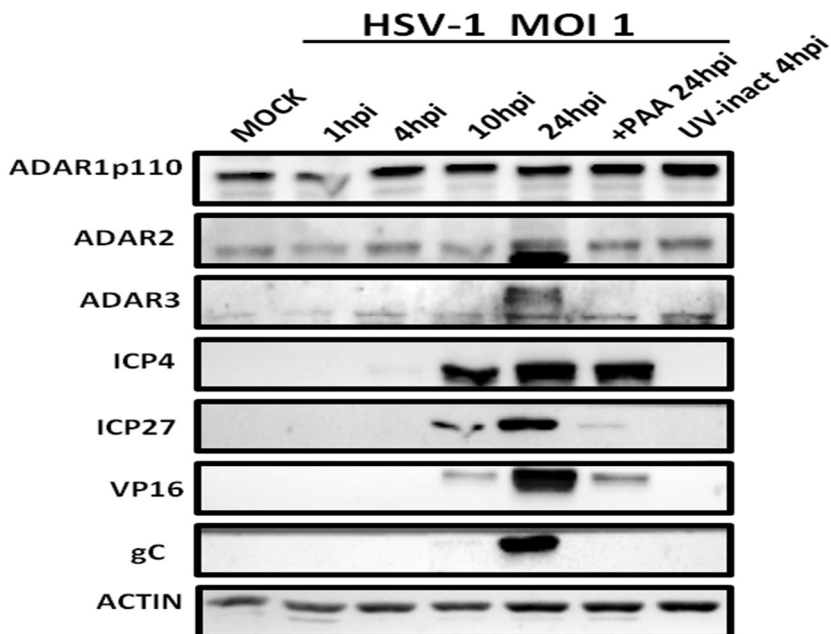


Figure 11. Upregulation of ADAR observed during HSV-1 infection is due to cells' innate immune response to viral infection. HeLa cells were infected with wild-type HSV-1 and UV-inactivated HSV-1. Cells were harvested at 1 and 6hpi. (A) Cells were lysed and isolated protein was analysed by Western blot with antibodies against ADAR1, ICP4, and β -Actin. (B) The result of the Western blot was quantified using imageJ software and normalized with β -Actin.

4.3 Upregulation of ADAR is MOI dependent.

To further confirm cell specificity, the requirement, and MOI dependency of ADAR upregulation, an all-in-one experiment was conducted. HFF cells were infected with wild-type HSV-1 and UV inactivated HSV-1 using MOI of 1 and 10. Wild-type HSV-1 infected cells were pre-treated 30 minutes before infection with Phosphonoacetic acid (PAA), an inhibitor of Viral DNA replication specifically known for its antiviral activity against herpes simplex viruses and other members of the herpes virus family. Mock cells were harvested at 1hpi, while HSV-1 wild-type infected cells were harvested at 1, 3, 5, and 24hpi. Cells infected with UV-inactivated HSV-1 were harvested 4hpi, while cells treated with PAA were harvested 24hpi. Harvested cells were lysed and the protein extracted was analysed by WB. Results of the WB were quantified using imageJ software and normalized against Actin which was used as the sample loading control.

A.



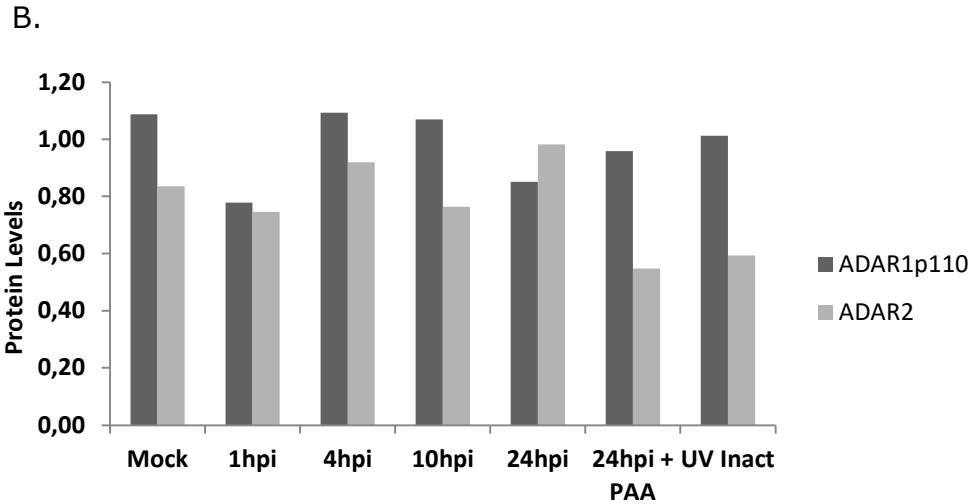
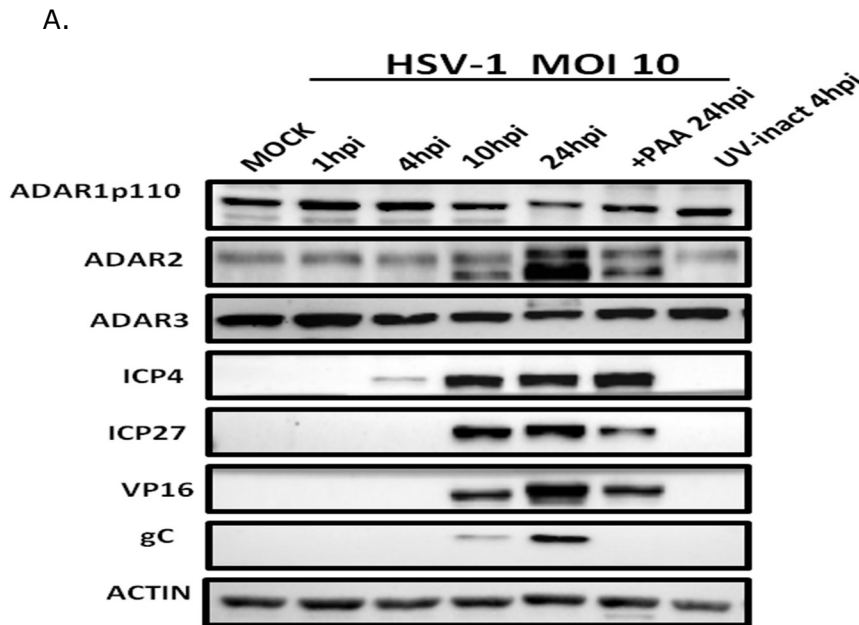


Figure 12. Upregulation of ADAR during HSV-1 infection is MOI dependent and is due to cells' innate immune response to viral infection. HFF cells were infected with wild-type HSV-1 and UV-inactivated HSV-1 using an MOI of 1. UV inactivated HSV-1 infected cells were harvested at 4hpi, while wild-type infected cells treated with PAA were harvested at 24hpi (A) Cells were lysed, and isolated protein was analysed by Western blot with antibodies against ADAR1, ADAR2, ADAR3, ICP4, ICP27, VP16, gC and β -Actin. (B) The result of the Western blot was quantified using imageJ software and normalized with β -Actin.



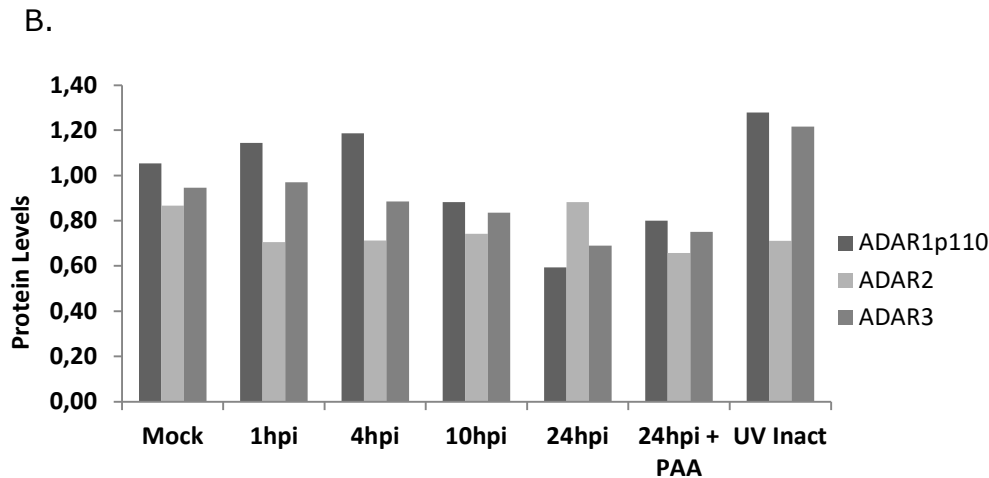


Figure 13. Upregulation of ADAR during HSV-1 infection is due to cells' innate immune response to viral infection. HFF cells were infected with wild-type HSV-1 and UV-inactivated HSV-1 using an MOI of 10. UV inactivated HSV-1 infected cells were harvested at 4hpi, while wild-type infected cells treated with PAA were harvested at 24hpi. (A) Cells were lysed and isolated protein was analysed by Western blot with antibodies against ADAR1, ADAR2, ADAR3, ICP4, ICP27, VP16, gC, and β -Actin. (B) The result of the Western blot was quantified using imageJ software and normalized with β -Actin.

From the results, we observed that the level of ADAR1p110 expression was slightly upregulated in cells infected with HSV-1 using MOI of 10, but not in cells infected using MOI of 1. This indicates that upregulation of ADAR is MOI dependent and that for ADAR to be upregulated, MOI greater than 1 is required. Upon inhibition of virus DNA replication using PAA, we have observed an expression of ADAR1p110 slightly lower than the expression in mock cells and HSV-1 wild-type infected cells. This suggests that viral DNA replication is not required for the depletion of ADAR late in infection. Interestingly, ADAR1p110 expression in cells infected with UV-inactivated HSV-1 was slightly decreased during infection with MOI of 1 but was upregulated during infection with MOI of 10, indicating MOI dependency and that virus function is not required for ADAR1p110 expression in infected cells. On the other hand, the pattern of ADAR2 and ADAR3 was similar to the expression pattern on ADAR1, although the increase of ADAR2 was somewhat delayed.

4.4 Functional analysis of ADAR protein

Our results show an upregulation of the ADAR family members, which might indicate that increased levels are beneficial for virus infection, or on other hand might represent an aspect of innate antiviral defense. Thus we hypothesize that overexpression of ADAR proteins might aid virus replication. To test this hypothesis, we obtained plasmids with cloned ADAR genes including pADAR1, pADAR1(-)(enzymatically inactive enzyme carrying point mutation WHICH ONE?), pADAR2, pADAR(-) (all generous gifts from Angela Gallo, Ospedale Pediatrico Bambino Gesù, Rome, Italy), pADAR1-150, pADAR1-110 (plasmids expression ADAR1 p150 and p110 forms, respectively), pADAR2 (all purchased from Addgene). All cloned ADAR genes are fused to a sequence encoding the green fluorescent protein (GFP), and thus we used pEGFP (Addgene) as a negative control.

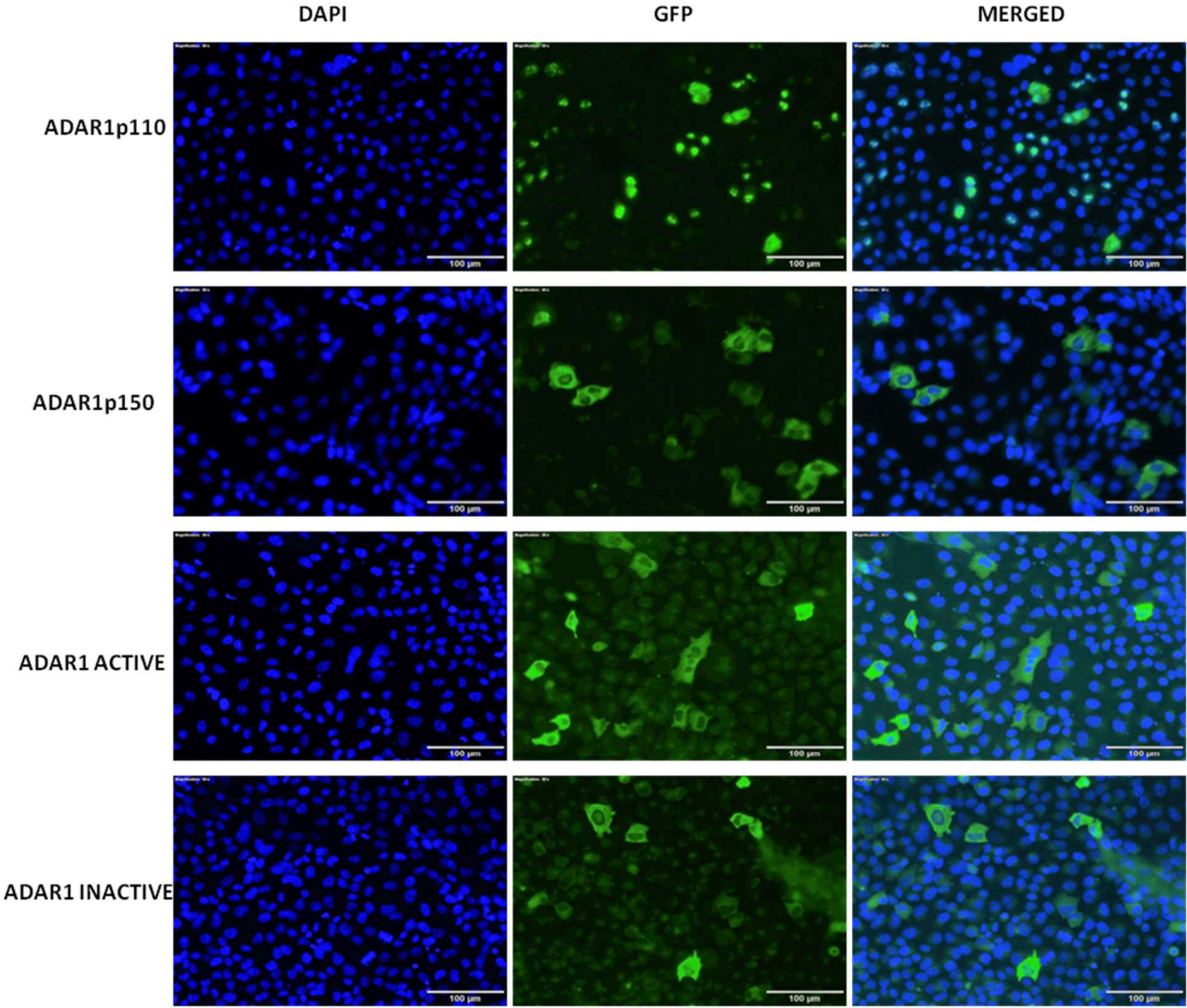
4.4.1 ADAR1 and ADAR2 localized to expected sub-cellular compartments.

Before performing functional assays, we sought to verify the specific localization of each of our ADAR proteins expressed from the plasmids in transfected cells. We also wanted to verify the effect of mutation on the deaminase domain of the ADAR inactive proteins on their subcellular localization. We know that ADAR1 isoforms are separately localized, ADAR1p110 being localized predominantly in the nucleus, while ADAR1p150 is localized both in the nucleus and cytoplasm due to its translocation ability, but is mainly expressed in the cytoplasm. ADAR2 like ADAR1p110 is also localized in the nucleus.

In brief, transfection was done in a 12-well cell culture plate having glass slides in them. Afterward, cells were fixed to the glass slide and stained with DAPI, a nuclear marker. Based on the GFP fluorescent signal detected and visualization by fluorescent microscopy, the individual sub-cellular

localization of the proteins as shown in figure 14 was determined. As expected, the result showed that ADAR1p110, ADAR2, ADAR2 active, and ADAR2 inactive proteins are predominantly localized in the nucleus of the cell, while ADAR1p150, ADAR1 active, and ADAR1 inactive proteins are localized in the cytoplasm. The result of the ADAR1 active and ADAR1 inactive shows that they are both ADAR1p150 isoform of ADAR1. Based on the result, we observed that mutation in the catalytic domain of the ADAR1p150 (ADAR1 inactive) and ADAR2 (ADAR2 inactive), does not affect ADAR protein localization in cells under normal physiological conditions.

A.



B.

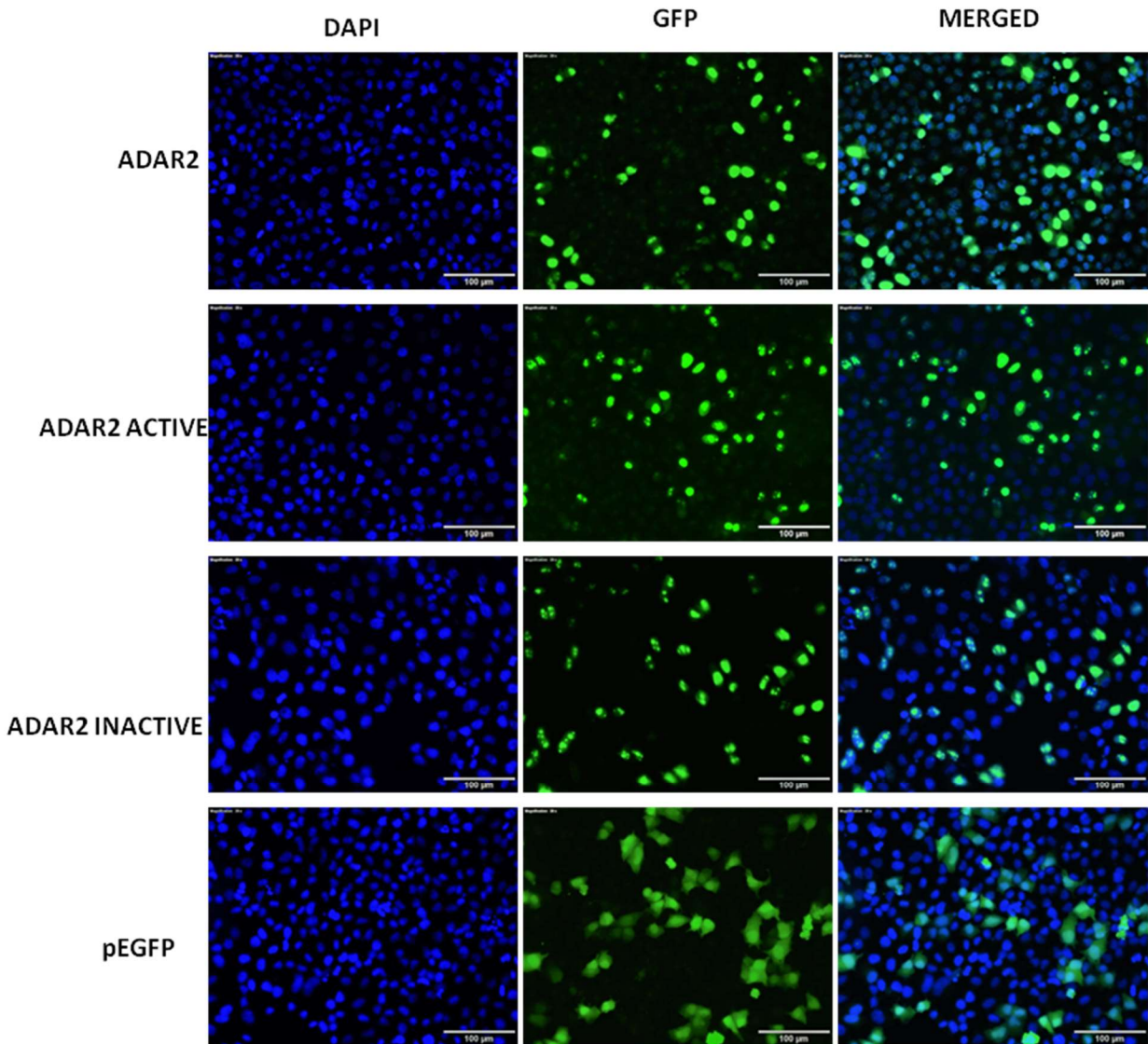


Figure 14. ADAR proteins are localizing to the nucleus and cytoplasm. (A) HeLa cells were transfected with ADAR1p110 and ADAR1p150, ADAR1 active and ADAR1 inactive plasmids fixed onto a glass slide and stained with Dapi (nuclear marker). Cells were visualized and pictures were taken with the fluorescent microscope 24 hours post-transfection. (B) HeLa cells were transfected with ADAR2 active, ADAR2 inactive and commercial ADAR2 and pEGFP plasmids, fixed onto a glass slide and stained with Dapi (nuclear marker). Cells were visualized and pictures were taken with the fluorescent microscope 24 hours post-transfection. pEGFP plasmid was used as a control.

Having determined the subcellular localization of ADAR proteins expressed from all our plasmids, it was necessary to additionally verify the plasmids before further experiments to determine the role of ADAR proteins during productive HSV-1 infection.

So, we performed a restriction digest to verify the plasmid profile. ADAR1 active, ADAR1 inactive, ADAR2 active, and ADAR2 inactive plasmids (from Angela Gallo) were restricted with a combination of HpaI and NdeI restriction enzymes, while the commercial ADAR1p110, ADAR1p150, and ADAR2 plasmids were restricted with a combination of EcoRI and HindIII restriction enzymes. 1µg of DNA was used in the reaction, using cutsmart restriction buffer for HpaI and NdeI restriction enzymes and 2.1 restriction buffer for EcoRI and HindIII restriction enzymes. 3µl of restriction buffers and 1µl of restriction enzyme (0.5µl for each enzyme) were used in the reaction. After digestion, the DNA fragments were separated in 1% agarose gel.

The results of ADAR1p110, ADAR1p150 and ADAR2 correspond to the results predicted using the Clone Manager, a DNA cloning application. In contrast, the results of ADAR1 active, ADAR1 inactive, ADAR2 active and ADAR2 inactive did not correspond to their predicted results, suggesting incorrect plasmid sequence and that there might be additional restriction sites contained in the plasmid maps. Therefore, we will proceed with further experiments using the plasmids obtained from Addgene.

ENZYMES	EXPECTED SIZES					
	ADAR1+/-	ADAR2+	ADAR2-	ADAR1p110	ADAR1p150	ADAR2
HpaI+NdeI	2149,2757, 3449	3384, 3449	3378, 3449			
EcoRI+ HindIII				336,1011, 7569	336,1896, 7569	849, 7385

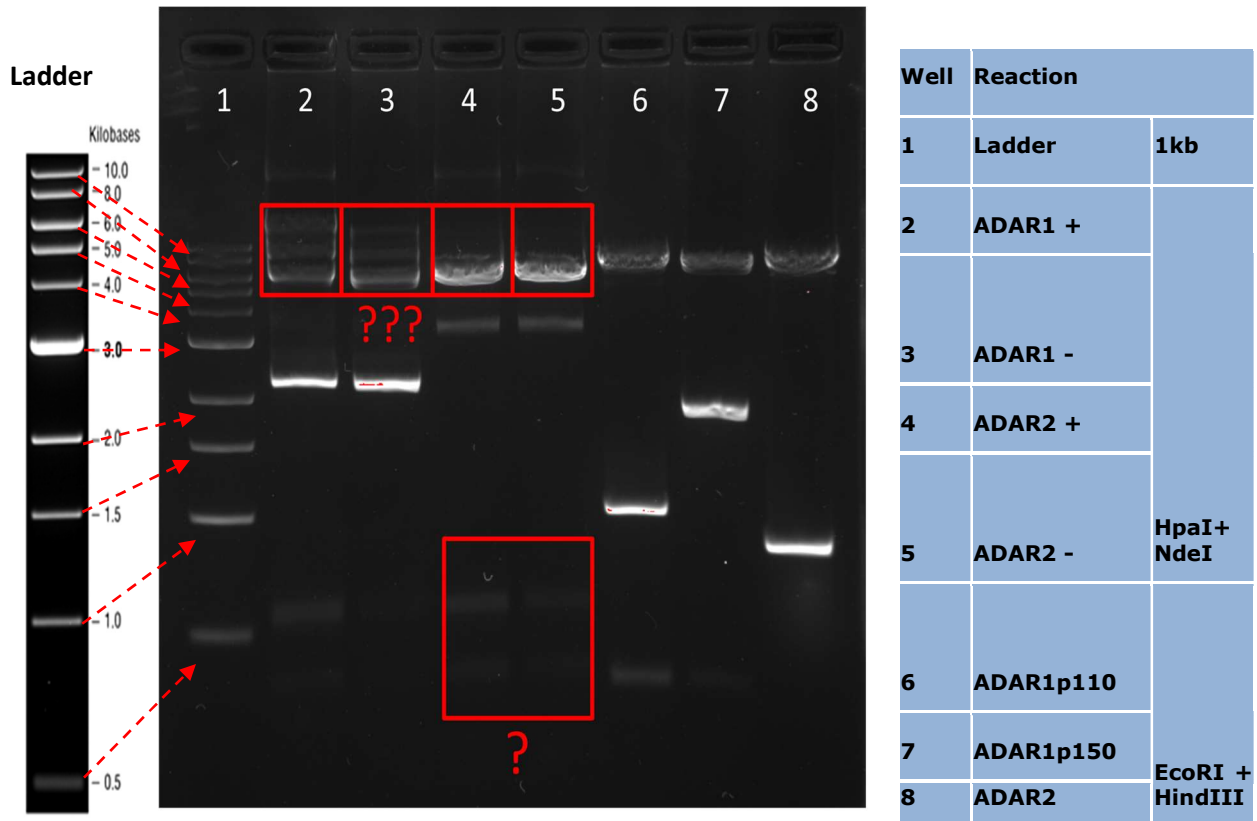


Figure 15. The commercial ADAR plasmids correspond to their predicted sizes, while the laboratory-constructed ADAR plasmids did not. ADAR1 active (ADAR1+), ADAR1 inactive (ADAR1-), ADAR2 active (ADAR2+), and ADAR2 inactive (ADAR2-) plasmids were restricted with a combination of HpaI and NdeI restriction enzymes, while ADAR1p110, ADAR1p150 and ADAR2 plasmids were restricted with a combination of EcoRI and HindIII. Additional unexpected bands which did not fall within the predicted sizes (red boxes) were gotten for all laboratory-constructed plasmids (ADAR1 and ADAR2), while exactly predicted band sizes were obtained for all the commercial plasmids (ADAR1p110, ADARp150, and ADAR2).

4.4.2 Overexpression of ADAR protein in cells does not affect viral replication.

To investigate how overexpression of ADAR1 and ADAR2 influences HSV-1 replication, HEK293T cells were transfected with GFP-infused ADAR1p110,

ADAR1p150, and ADAR2 plasmids. The GFP infusion enables visualization under the fluorescent microscope. The responsiveness of cells to the plasmids and the level of expression of the plasmids were determined by transfecting and observing cells under the fluorescent microscope 18 hours post-transfection. The transfection efficiency was low at about 20-30% which could be a result of less number of cells seeded (150,000) in a 12-well cell culture plate. 24 hours post-transfection, cells were infected with HSV-1 at an MOI of 1 and a total virus concentration of 9.2×10^7 pfu/ml. Cells were harvested at 24hpi and proteins were isolated with RIPA lysing buffer and analysed by WB with Actin as the sample loading control. The experiment was performed in duplicate for reproducibility of results. The result of the WB analysis (fig. 16a) showed that exogenous ADAR proteins were strongly expressed with the evidence of strong bands detected for ADAR1 and ADAR2. This means that overexpression was achieved in cells even at low transfection efficiency and that the introduction of exogenous proteins did not inhibit the expression of endogenous ADAR proteins. We also observed a correspondence in size between GFP and ADAR proteins, as the sizes of the detected ADAR proteins should include the size of GFP. That being the case and since the size of GFP is 28kDa, the detection of ADAR1p110 protein at around 130kDa, ADAR1p150 at around 180kDa, and ADAR2 below 130kDa was appropriate.

The result of virus protein expression shown in figure 16a, suggests that overexpression of ADAR protein in cells does not affect HSV-1 replication in cells.

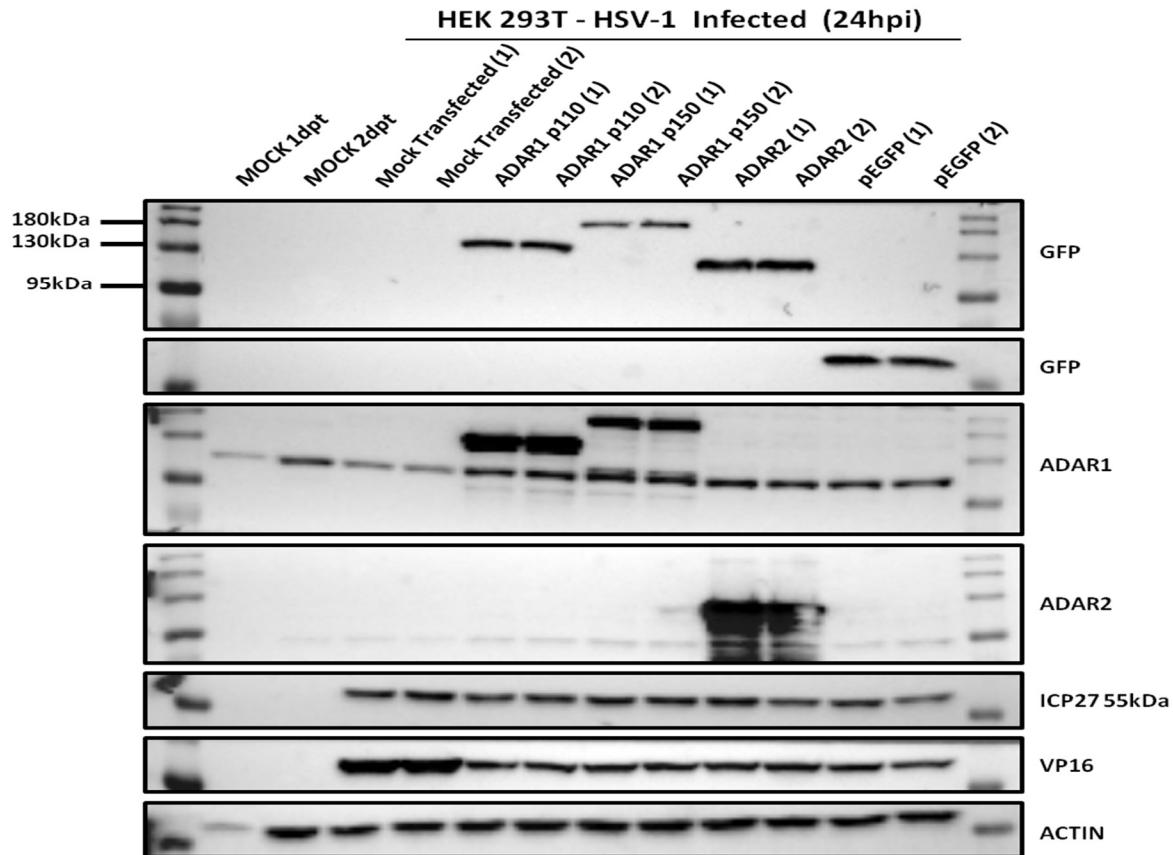


Figure 16a. Overexpression of ADAR proteins does not affect HSV-1 replication in HEK293T cells. HEK293T cells were transfected with ADAR plasmids and infected with HSV-1 24hpi. Infected cells were harvested 24hpi, and protein was isolated and analysed by WB. Viral early and late genes were detected confirming viral infection and replication in cells. pEGFP plasmid was used as a negative control, while Actin served as the loading control.

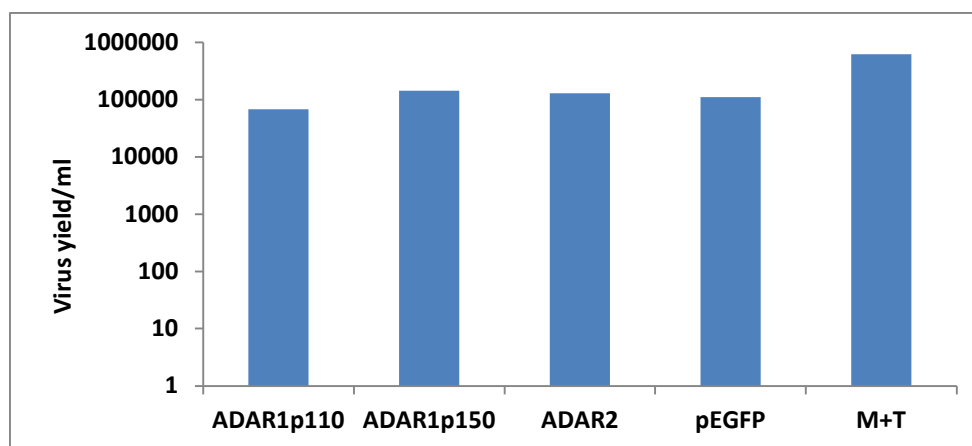


Figure 16b. Titration of viral yield corresponds to the result of Western blot analysis. Vero cells were infected using infectious media collected before harvesting HEK293T cells infected with HSV-1 after transfection. Cells were left for 3days to form plaques before they were fixed with fixative and stained with Giemsa stain in PBS. Plaques were counted and the result gotten was plotted into a graph.

The virus titration was performed to determine the virus yield and confirm the result of the Western blot analysis. This was done by collecting 100µl of infectious media before harvesting infected cells. As shown in figure 16b virus yield was more in mock transfected (No DNA introduced) and infected cells tagged as mock transfected confirming the result of viral protein expression shown in figure 16a.

5.0 DISCUSSION

ADAR proteins are endogenous proteins known for their ability to edit dsRNA. These enzymes also play an important role in immune system function. HSV-1 infection initiates an antiviral immune response in infected cells. The virus dsRNA is sensed by immune sensors like MDA-5 and RIG-1 which initiate a signalling response that drives the activation of type 1 IFN by ISGs as a key step in the antiviral innate immune response. One of the genes expressed as a result of this response is ADAR1 which has also been shown to suppress the RIG-1-like receptor (RLR) signalling pathway, by editing the virus dsRNA in a way that it cannot be recognized by immune sensors. This being in favour of the virus helps it escape antiviral response and facilitates its gene expression, replication, and survival in the host cell.

Before now, the role of ADAR (ADAR1) proteins has mostly been investigated in RNA viruses such as the measles virus (56), influenza virus (64,66), hepatitis C virus (81), etc. Although it is known that editing events by ADAR occur in DNA viruses, the role of ADAR in DNA viruses is unknown. Through in vitro experiment, we show that ADAR is upregulated during HSV-1 infection in U2OS cells (fig. 7), particularly ADAR1p110. This is in contrast to the findings by Dachmani et al., which have shown upregulation of ADAR1 in HCMV infected cells but not in HSV-1 or HSV-2. However, these authors have analysed the late time point of infection, and thus might have not observed early induction (82). We report herein that ADAR is also upregulated in HeLa, HEK293T, and HFF cells (fig. 8, 9, and 10) during HSV-1 infection, suggesting that upregulation of ADAR expression is not cell-specific as also seen during HCMV infection (82). We further show that upregulation of ADAR is MOI dependent since the same pattern of upregulation of ADAR in all cell lines was observed during infections with an MOI greater than 1 (MOIs 5 and 10) (fig. 7, 8, 9, and 13).

Whether upregulation of ADAR is a viral replication strategy or cells' innate response to viral infection remains unknown. In an attempt to solve this, we infected U2OS cells with both wild-type HSV-1 and UV-inactivated HSV-1. UV inactivated virus infects cells and triggers defence mechanisms that are not dependent on virus gene expression, but it is unable to replicate or express its genes. In our experiments, we did not observe any upregulation of ADAR1p110 in cells infected with UV-inactivated HSV-1, indicating that viral gene expression is required. On the other hand, in experiments in which we have inhibited virus DNA replication, we observed depletion in ADAR1p110 late in infection, indicating that HSV-1 gene expression is sufficient for this depletion. This strongly points to the function of viral protein ICP0, an important virus transcriptional transactivator and well-described ubiquitin ligase that directs proteins for degradation. It would be rather interesting to test whether ICP0 deficient virus induces ADAR1 depletion. HeLa cells infected with wild-type and UV-inactivated HSV-1 showed a similar phenotype. In our effort to probe the functional relevance of ADAR proteins for virus infection, we confirm the subcellular localization of ADAR1 and ADAR2 proteins expressed from plasmids. It was shown that ADAR1p110 and ADAR2 are predominantly localized in the nucleus, while IFN inducible ADAR1p150 is predominantly localized in the cytoplasm (12).

While the mechanism behind ADAR upregulation is uncertain, we see that overexpression of ADAR in cells does not affect viral replication. However, this might not be true since HEK293T and even HeLa cells are permissive to HSV-1 infection. It is unlikely that overexpression of ADAR proteins in cells will facilitate viral replication beyond the level they already attain in these cells, confirmed by the result showing virus yield (fig 16b). The use of viruses that have protracted or impaired replication such as mutants deficient for certain important genes, e.g. ICP0 deficient virus, might be important to test. However, although overexpression seemed to be achieved

in our experiments, as we see a strong expression of exogenous ADAR proteins (fig. 16a), the negative results can be also explained by low transfection efficiency during the experiment. At best, only about 30% transfection efficiency was achieved (i.e. only about 30% of cells seeded were successfully transfected), which might not be sufficient to observe less obvious phenotypes. In addition, we noticed a significant number of disintegrated cells, indicating a relatively high level of apoptosis, in transfected cells which might have an influence on viral replication. This can explain a failed attempt to generate a pure culture of transfected cells by selection with G418 antibiotic. Taken together, further research is needed to decipher the potential roles of these proteins in herpes virus infection.

CONCLUSION

The discovery of editing of virus-encoded miRNAs by ADAR protein during latent herpes simplex virus infection in neurons led to the curiosity about the role of ADAR proteins during productive HSV-1 infection. In this project, we analyzed the levels of ADAR proteins during productive herpes simplex virus 1 infection and sort to determine the importance of ADAR1 and ADAR2 during HSV-1 infection. We have shown that ADAR proteins are upregulated during HSV-1 infection. Also, we have proven that upregulation of ADAR protein expression occurs early during HSV-1 infection and that it is MOI dependent. All tested cell lines showed the same pattern of ADAR upregulation, suggesting that the observed upregulation of ADAR protein expression is not cell-specific. Depletion of ADAR proteins was observed following upregulation. Treatment of cells with PAA before infection showed that DNA replication is not required for the depletion of ADAR during infection. We have reported contrary findings in U2OS, HeLa, and HFF cells infected with UV-inactivated HSV-1 which led to the conclusion that response to HSV-1 infection is cell-specific.

Interestingly, we confirmed that the isoforms of ADAR1 are localized in different cellular compartments. ADAR1p110 is localized in the nucleus, while ADAR1p150 is localized in the cytoplasm. ADAR2 was also shown to be localized in the nucleus. We also confirmed that mutation on the deaminases domain of ADAR proteins does not affect their subcellular localization. Overexpression of GFP plasmids cloned to express ADAR proteins in cells did not show any proviral activity as we had expected.

Unfortunately, we were not able to determine the importance of ADAR1 and ADAR2 during HSV-1 infection. So, to satisfy this curiosity, further study is required. For instance, an experiment performed with high transfection efficiency and using a pure culture of cells expressing ADAR proteins by cell

sorting is needed. Also, it is important to carry out investigations using ADAR knock-out cells.

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