ABSTRACT

Title of Document: INTERFERENCE OF HOST INNATE IMMUNE RESPONSE BY HEPATITIS E VIRUS

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The host antiviral innate immunity mainly relies on host pattern recognition receptors (PRR) and downstream interferon (IFN) signaling. Host PRR for RNA viruses include Toll-like receptors (TLR) and Retinoic acid-inducible gene I (RIG-I) like receptors (RLR). Activation of both TLR and RLR pathways can eventually lead to the secretion of type I IFNs, which can modulate both innate and adaptive immune responses against viral pathogens, including hepatitis E virus (HEV). HEV causes acute hepatitis in humans and has been responsible for several outbreaks of hepatitis across the world. Currently, no commercial vaccine is available for the prevention of HEV infection in any country except China. HEV biology and pathogenesis as well as its responses to host innate immunity are poorly understood, though other hepatitis viruses, including the hepatitis A, B and C viruses, have been much better studied. In

this study, how HEV interferes with IFN induction and IFN-activated signaling had been examined. Results showed that the protein encoded by HEV ORF1 can inhibit type I IFN synthesis and downstream JAK/STAT signaling pathway. However, the HEV ORF3 product is able to enhance RIG-I-mediated signaling to a certain extent. These data suggest that HEV proteins interfere with the host innate immune response and may exert the diverse roles depending on the stage and/or context of infection. These studies contribute to a better understanding of HEV pathogenesis and may facilitate a strategy development for the prevention and control of HEV infection.

INTERFERENCE OF HOST INNATE IMMUNE RESPONSE BY HEPATITIS E VIRUS

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Preface

My past five years as a graduate student in the Department of Veterinary Medicine, University of Maryland, College Park had been the most exceptional part of my life. One reason is that I had incorrect conceptions and expectation about life as a Ph.D. student or a research scientist before I joined the department as I grew up in Northwest China, a remote and less developed region. Life and study as a foreign graduate student in this country for five years not only open my eyes, but also broaden my mind. I appreciate the training received here because it not only teaches me how to solve academic research problems and think logically, but also makes me be strong when facing difficulties and obstacles. No matter what kind of life will be for my next step, I am sure that I am ready for any challenge or obstacle.

Dedication

This body of work is dedicated to the memory of my grandfather Xiangqian Nan and grandmother Jinnv Cai. I wish they could see all this, and I know they would be proud for my achievement today. I also want to dedicate this work to my father Jiangzhen Nan and mother Yan Dong for raising me up. I know my achievement finally realizing your dream and expectation. You will be proud of me as well.

Lastly, but the most importantly, I want to dedicate this work to my wife Chunyan Wu and our upcoming daughter Olivia. Chunyan, reunion with you after our graduation from Northwest A&F University is the happiest time in my memory. Our love is so exciting for all of us and surprising for anyone who knows us. With your graduation with Ph.D. degree from University of Munich, you could have lived a different life by taking a job in China. However, you choose to stay with me and support me without reservation. I am highly grateful for the sacrifice you have made for me. Olivia, your will be the first member from our family who is born in the United States. I wish you grow up with a happy childhood and have the freedom to choose the lifestyle in the way you want. You will no longer suffer the hardship I experienced, just like I did not have the pain your grandfather had in the past.

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Secondly, I thank my uncle Dr. Bin He from the University of Illinois at Chicago. With his encouragement, I came to this country as a graduate student. He helped me a lot both in academic field and in personal life during the past few years here.

Thirdly, I thank my committee members Drs. Belov, Pal, Song, and Zhu for their kind help during my studies. Their kind advices and suggestions contributed to my research and study. Fourthly, I thank all the faculty members and staff I met in the Department of Veterinary Medicine. Drs. Zhu, Pal. Samal, Belov, Bossis and Shi are exemplary scientists for me. I also thank Drs. Wenxia Song, Jeffery DeStefano, Brenda Fredericksen, David Mosser and Vincent Lee for teaching me and sharing knowledge with me in the courses I took. Furthermore, I thank Drs. Suzanne Emerson in NIH, and X-J Meng at Virginia Tech University for sharing the plasmids and antibodies used in this study.

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Chapter 1: Hepatitis E Virus

Introduction

Hepatitis E virus (HEV) causes acute hepatitis in humans and has been responsible for several large outbreaks of hepatitis across the world. It has been classified as the sole member of the genus *Hepevirus*, the family *Hepeviridae* (74). Currently, there is no commercial vaccine available for prevention of this infectious disease in any country except for China. HEV is a fecal-oral transmitted viral pathogen that causes acute hepatitis with a mortality rate from 0.5 to 3% in young adults and up to 30% in pregnant women in their third trimester of gestation (139). It has been estimated that one third of total population in the world have been infected by HEV on the basis of seroprevalence. However, for a long time, HEV infection was thought to be a public health problem only for the developing countries. Hepatitis E is now frequently recognized in industrialized countries, where it was not thought to be endemic (76, 145, 185, 252, 337, 348, 430). Moreover, HEV is also a zoonotic agent and isolation of HEV from the pig, chicken, mongoose, rabbit, rat, ferret, bat, fish and deer has been reported (105, 202, 246). It is thought that transmission of HEV from animal reservoirs to humans is the major cause of sporadic cases of hepatitis E in the industrialized countries. In this chapter, the molecular virology, cell culture system, pathogenesis, epidemiology, treatment and vaccine of HEV are discussed in detail.

HEV Virology

Originally, hepatitis E was classified as enterically transmitted non-A, non-B hepatitis (ET-NANBH) due to similar clinical findings to hepatitis A and B in patients,

but the prospective causative agent was unknown (321). Although earlier research implied an RNA virus as the potential pathogen for the ET-NANBH, direct evidence came from the Reyes group. By using infectious bile to construct a cDNA library, they identified a portion of a highly conserved RNA-dependent RNA-polymerase (RdRp) motif, which is present in all RNA viruses (320). They designated this new virus as the hepatitis E virus, which was responsible for the outbreak of ET-NANBH.

The complete sequence of the HEV genome came out one year later (393). Computer based analysis of the sequencing data indicated it has a 7.2 kb single-stranded positive-sense RNA genome, which is capped and poly-adenylated. It contains three partially overlapped open reading frames (ORFs) with an order of sequences encoding non-structural proteins, followed by sequences encoding structural proteins (393, 401) [Fig.1.1]. HEV ORF1 encodes a polyprotein including non-structural proteins of HEV. ORF2 encodes the capsid protein, which is the major structural protein of the HEV virion. ORF3 encodes a small multifunctional protein with a molecular weight of 13 kDa (vp13). There are also short untranslated regions (UTRs) in both the 5′- and 3′- end of the genome.

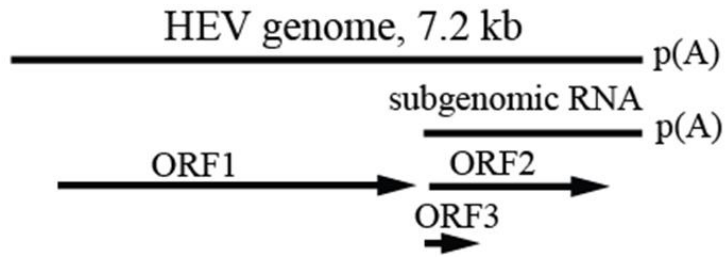

Fig. 1.1 Schematic illustration of HEV genome, sub-genomic RNA, ORFs

The ORF1 of HEV can be translated directly from its genomic RNA, while ORF2 and ORF3 can be translated only from sub-genomic RNAs (99). In an earlier report, three RNA species were detected in the liver tissue of experimentally infected macaques, with sizes of 7.2, 3.7 and 2 kb (393). The 3.7 and 2 kb RNA species were considered to be sub-genomic RNAs for ORF2 and ORF3 translation, respectively. However, a later study in Huh7 cells only identified one capped 2.2 kb sub-genomic RNA, which was considered to be a bicistronic mRNA for translation of both ORF2 and ORF3 (99). Transcription of this sub-genomic RNA initiates at nucleotide position 5122 in the Sar55 strain, which is located downstream of the first two methionine codons of the earlier presumed ORF3. The same conclusion was drawn from another *in vitro* assay of type III HEV infection in RLC/PRF/5 cells (135).

HEV was initially classified as a member of the *Caliciviridae* family. However, sequence comparison of the HEV ORF1 with corresponding ORFs from other viruses indicated no homology with Caliciviruses, or other picorna-like viruses. On the other hand, there is limited but significant homology with the alphavirus-like superfamily of RNA viruses, specifically the rubella virus (30). Consequently, HEV was reclassified as the only member of the genus *Hepevirus* (30, 74).

Genotypes of Hepatitis E viruses

HEV strains are highly diverse and heterogenic. Classification of HEV strains is under transition due to the different criteria used (372). Early classification was based on partial genomic sequences, i.e. strains with nucleotide variations differing by >20% in ORF2 should be considered as different genotypes (431). However, a comprehensive

analysis of the complete genome and sub-genome regions has classified HEV into at least four major genotypes with several subtypes in each (222). HEV genotype I with the prototype Sar55 strain is the most conserved among the four genotypes and contains five subtypes. The genotype II sequences are limited and can be divided into two subtypes. Genotype III and IV strains are highly diverse and there are ten and seven subtypes, respectively (222). Both HEV genotypes I and II are restricted to humans with no animal reservoirs, whereas genotypes III and IV are zoonotic with an expanded host range (7, 245). However, as new HEV isolates from rabbit, rats, ferret, bats and wild boar have been reported, there is concern as to whether these new isolates should be listed as a new genotype or subtype (64, 90, 144, 309, 386, 456).

There have also been HEV-like viruses isolated from avian species called avian HEV (105, 132). Avian HEV shares less than 50% nucleotide identity with mammalian HEV and has been proposed as a new genus within the family *Hepeviridae* (International Committee for the Taxonomy of Viruses; Ninth Report) (132, 414). However, the common antigen epitopes in the capsid protein are shared among mammalian HEV and avian HEV (104). A more recent study further classified HEV strains into six genotypes with two additional genotypes from wild boar (372). Moreover, although it is very rare, intra- and inter genotypic recombination of HEV has been reported and is believed to contribute to the diversity of this virus (423).

For the traditional four genotypes of HEV, there are differences in their geographic distributions. Genotype I HEV mainly includes strains from Asia and Africa including the prototype Sar55, while genotype II contains a Mexican strain and variants

from Africa. However, genotypes III, including human and swine HEV, are mainly found in industrialized countries, while genotype IV is particularly found in China (304).

Viral Proteins of HEV

ORF1 polyprotein

ORF1 of HEV is the largest ORF in the HEV genome with 5082 bp in length according to the prototype Sar55 strain (75, 401). It starts at the 5' end of the genome after a 25 bp non-coding region and can be translated directly from the HEV genome. ORF1 encodes a 1693 amino acid (aa) polyprotein, which is necessary for viral replication and protein processing. Bioinformatic analysis of the ORF1 sequence has revealed that it is highly related to the group of Rubi-like viruses including Rubivirus, Betatetravirus, Benyvirus, Omegatetravirus, *Sclerotinia sclerotiorum* debilitation-associated virus and cutthroat trout virus (26, 178, 214). The analysis also indicated eight putative functional domains according to the homology of already known functional domains from other viruses (179) [Fig.1.2]. These functional domains can be listed as follows from the protein's N terminus: methyltransferase domain (Met), Y domain (Y), papain-like cysteine protease (PCP), hypervariable region (HVR), proline-rich region (Pro), X domain, helicase domain (Hel) and RNA-dependent RNA polymerase domain (RdRp). In recent publications, the proline-rich region has been incorporated into the hypervariable region.

The current data are controversial about whether the HEV ORF1 product functions as a single polyprotein or needs to be further processed into smaller units by viral or cellular proteases (17, 289, 329, 357, 381). An earlier study by the Frey group using a vaccinia-derived expression system demonstrated that the ORF1 polyprotein can be cleaved by the PCP within it (329). However, more than ten years later, a publication from the same group indicated a lack of processing of the ORF1 polyprotein in HEK293T

cells, which contradicts their previous observation (381). In another report by Perttila et al., although *in vitro* translation of full-length pORF1 yielded smaller quantities of two fragments, these fragments were not observed in pulse–chase studies in human cells, and their production was not dependent on the predicted protease domain in pORF1(289). Furthermore, in *E. coli* and a cell-free system based on HepG2 cells, ORF1 was expressed as a 186 kDa protein without further processing (17).

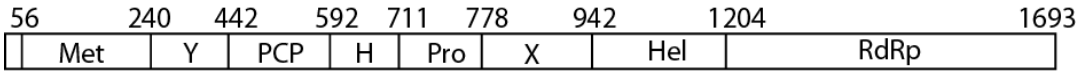

Fig. 1.2 Schematic illustration of ORF1 product.

Met: Methyltransferase domain; Y: Y domain; PCP: papain-like cysteine protease; HPX: hypervariable region, proline-rich domain and X-domain; Hel: helicase; RdRp: RNA-dependent RNA polymerase. The numbers above the boxes indicate numbers of amino acids of ORF1 polyprotein.

However, other studies demonstrated contrasting results. In one study, the ORF1 product expressed in insect cells by a baculovirus system could be processed into smaller fragments correlated with proposed functional domains, and this type of processing could be inhibited by E-64d, a cell-permeable cysteine protease inhibitor (357). In a more detailed study, transfection of HepG2 cells with *in vitro* transcribed RNA from HEV cDNA produced cleaved products with sizes of 35, 38 and 36 kDa for the Met, Hel and RdRp domains, respectively (279). Another study focusing on the analysis of the ORF1 functional domains also observed proteolytic processing of the HEV ORF1 fragment in insect cells (227). A recent publication reported that the refolded PCP domain expressed in *E. coli* is able to process ORF1 protein *in vitro* (277). Moreover, based on an HEV-Sar55 replicon system in S10-3 cells (a subclone of Huh7 cells with improved HEV replication) (99), it has been demonstrated that the putative "catalytic" aa residues in the

ORF1 protease domain are indispensable during HEV replication, as well as the putative X-domain "protease-substrate" residues (282). Overexpression of ORF1 from HEV Sar55 strain in S10-3 cells also resulted in cleaved products that were barely visible (282).

Thus, although there is no solid data to support a conclusion on the processing of the HEV ORF1 polyprotein, majority of the data available so far are in favor of the polyprotein proteolysis, which indicates that HEV ORF1 can be processed into smaller units. Additionally, some studies have analyzed the functions of the putative domains.

Met domain

The Met domain is the first functional domain of the ORF1 polyprotein. As evidence has indicated that the HEV genome is capped and that the capping is crucial for its infectivity, a viral-specific methyltransferase is expected (75, 450). Based on bioinformatic analysis, aa residues 60-240 could be the putative methyltransferase (179). The HEV Met domain shows high similarity to that of Tricornaviruses, which belong to the alpha-like supergroup of RNA viruses (414). Members of this virus superfamily code a unique methyltransferase with four conserved motifs. There are an invariant His residue, an AspXXArg signature and an invariant Tyr residue in motifs I, II, and IV of methyltransferase, respectively (333). Expression of HEV ORF1 cDNA (aa residue 1-979) in insect cells yields a 110 kDa protein (P110), along with a 80 kDa protein which is believed as the proteolytically processed product from P110 (227). *In vitro* assays have confirmed that P110 possesses guanine-7-methyltransferase as well as guanylyl transferase activity (227).

Y domain

The second functional domain following methyltransferase is the Y domain. It starts from aa residue 216 and ends at aa 442, and is highly similar to that of the rubella virus (179). Currently, no information is available for the function of this Y domain in either HEV or the rubella virus.

PCP domain

Downstream of the Y domain, there is a putative papain-like cysteine protease (PCP) domain. Just as the Y domain, the PCP domain demonstrates moderate similarity with the protease domain in the rubella virus (179). In the rubella virus, the PCP domain is responsible for the proteolytic processing of its non-structural protein (NSP) (232). Mutation of the catalytic residue within the PCP (Cys1151) abolishes its protease activity and results in inhibition of the NSP processing. It is also involved in *trans* and *cis* cleavage of the rubella virus NSP (208). However, regarding the function of the HEV PCP domain, the current data are incomplete and controversial.

In the vaccinia-mediated ORF1 expression system, mutation of the putative catalytic core (Cys483) of HEV PCP had no effect on proteolytic processing of the ORF1 product (329). Another putative catalytic site of His590 in PCP is not conserved among different HEV strains. Later studies showed controversial data for the processing of the HEV ORF1 product (17, 329, 357, 381), which leads to the speculation as to whether HEV PCP is a real cysteine protease. Recently, Parvez demonstrated that the mutation of six cystine residues (C457, C459, C471, C472, C481, C483) and three histidine residues (H443, H497, H590) in the PCP domain completely abolished HEV RNA replication in a Sar55-based replicon system in S10-3 cells. Notably, of these essential Cys and His

residues, C483 and H590 had been previously predicted as putative catalytic residues in the PCP domain (282). Furthermore, an Indian group demonstrated that the PCP domain expressed in the *E. coli* C43 strain (a strain resistant to toxic protein expression) possesses protease activity (277). The recombinant protein was shown to cleave both *in vitro* translated HEV ORF1 and ORF2 products. Protease inhibitor assays further indicated the HEV PCP domain is a chymotrypsin-like protease (277). This latter evidence indicates that HEV PCP could be a real protease responsible for HEV ORF1 polyprotein processing.

In recent years, the connection between ubiquitination and innate immunity signaling has been demonstrated (216, 231, 448), and the antiviral function of some ubiquitin-like molecules, such as ISG15 and SUMO, has been described (216). Evidence from other studies indicate that viral coded cysteine proteases possess deubiquitinase activity to inhibit host innate immunity, such as arterivirus papain-like protease 2 (415) and PCP from porcine reproductive and respiratory syndrome virus (199, 380). As a result, similar research was performed on the HEV PCP domain, which suggests that HEV PCP could act as an antagonist to ISG15 function to inhibit host innate immunity when expressed together with Met as the Met-PCP protein (157).

HVR domain

Between the PCP domain and the X domain, there are a hypervariable region (HVR) and a proline-rich region (Pro). Those two regions were first named together as the hypervariable region due to the extreme divergence between nt2011 to nt2325 (corresponding to aa 662 to 766) when comparing the HEV prototype Sar55 with two other strains (401). In a later study, aa 712 to 778 in this region was designated the

proline-rich region, which could be found in rubella virus as well. It was also considered to serve as a hinge between the X domain and its upstream domains because multiple prolines in a protein or peptide may result in an unstable tertiary structure (63, 67, 179, 400). The length and sequence of HVR and Pro is highly variable among different HEV strains and usually need to be excluded for phylogenetic analysis (302, 372). Currently, there is some confusion regarding the designation of those two regions. Some of the recent publications have named the aa 712 to 778 region as the hypervariable domain, which originally referred to proline-rich region and left out the immediately upstream domain (aa 592 to 711) (302, 303), while others still name the aa 712 to 778 domain as the proline-rich region (305). Current research mainly focuses on the Pro domain and pays less attention to the upstream HVR domain. As a result, the function of HVR is unknown. However, data gained from the rubella virus shows that deleting part of the HVR domain along with part of the Pro domain results in a mutant unable to replicate (410).

Pro domain

The Pro domain is considered as an intrinsically disordered region (IDR) with flexibility for insertion and deletion (305, 306). Data from its counterpart in the rubella virus indicates that this region is not required for viral replication (410). As expected, deletion and mutation of this region in HEV indicates that it is not required for viral replication and infectivity, but it plays a role in replication efficiency *in vitro* (302, 303). It was also demonstrated that the Pro domain is interchangeable between genotypes with genotype-specific differences and is affected by viral replication (303). More interestingly, a remarkable cell-culture adapted HEV strain Kernow-C1, which was

derived from an HIV patient with chronic HEV infection, demonstrated an insertion of a 174 bp gene fragment of human ribosomal protein S17 in the Pro region (369). This recombinant virus was adapted in cell culture and was permissible for cells from different species. It was speculated that insertion of the S17 fragment into the viral genome occurred in the host but was selected in cultured cells. This speculation needs further verification as direct detection of the inserted fragment from the host sample was not successful. Experimental insertion of the S17 fragment into the Pro domain of the prototype Sar55 strain also generated a viable chimeric virus and further demonstrated that this region could tolerate sustainable changes (369).

Although the Pro domain was considered a highly diverse region that is intrinsically disordered regions (IDR), some motifs are found in the region. Based on computer analysis and predication with comparison of other IDRs, Purdy et al. identified several linear motifs (LMS), including two protease cleavage sites, three ligand binding sites and two kinase phosphorylation sites across all four genotypes and the HEV strain isolated from a Japanese wild boar (306). The putative protein-protein interactions of the Pro domain were proposed in the same report as well, but need experimental verification. Nevertheless, this report provides some useful insight into disorder-to-order state transitions about the Pro domain. In a recent study, alignment of the Pro domain from different genotypes indicated the sequence is more conserved in genotypes I and II rather than genotypes III and IV (305). Adaption of a wider host range for genotypes III and IV was a possible reason. The authors also assessed the diversity of the Pro domain due to the higher rate of substitutions at the first and second codon positions, leading to a shift in translation to more proline, alanine, serine and threonine rather than histidine,

phenylalanine, tryptophan and tyrosine. This pattern matches the aa usage in proline-rich IDRs (305). Furthermore, it is probable that all zoonotic HEV isolates share a common ancestor for the Pro domain, and the C-terminus of this domain could tolerate more mutations than the N-terminus. HEV evolution has favored the formation of IDR structures in the Pro domain (305). Recently, the heterogeneity of the Pro and X domains is implicated in HEV persistence, which was revealed in an investigation into the association between the genetic heterogeneity of HEV quasispecies in ORF1 and the outcome of infection in solid-organ transplant patients (198).

X Domain

The X domain is located immediately downstream of the Pro domain. In HEV, it was first identified as a domain with unknown function, which commonly flanks the PCP domain in the rubella virus, alpha virus and coronavirus (95, 179). It has now been given a new name, macro domain, due to its homology shared with non-histone domain of the histone macroH2A. It has been identified in a variety of bacterial, archaeal and eukaryotic organisms (286, 287).

Earlier studies into the human macro domain indicated that it is enriched in inactive mammalian X chromosomes, suggesting a role in gene silencing and inactivation (51). The *in vitro* assays have shown that the macro domain inhibits transcription, and this inhibition was further confirmed by binding of the macro domain to the transcription factor NF-κB (16, 288). Crystal structure analysis also identified a DNA binding motif in the macro domain, suggesting that it might interact with nucleic acids (12). A biochemical functional analysis indicated that the macro domain is involved in the downstream processing of ADP-ribose 1"-phosphate, a side product of cellular pre-tRNA

splicing (233). Furthermore, the macro domain had been found in association with proteins involved in poly(ADP-ribose) polymerization, ADP-ribosylation and ATP-dependent chromatin remodeling (6).

Information about the viral macro domain is limited. ADP-ribose 1"-phosphatase activities have been demonstrated in macro domains from three coronaviruses (233, 307, 308, 338). Crystal structure analysis and *in vitro* assays on the macro domain of the SARS virus indicate that the viral macro domain has relatively poor ADP-ribose 1"-phosphohydrolase activity, but can bind free ADP-ribose and poly(ADP-ribose) efficiently (69). In another report, the viral macro domain (Semliki Forest virus, HEV and SARS virus) along with a yeast macro domain were compared with the human macro domain. The data from this report indicated that viral macro proteins bind poly(ADP-ribose) and poly(A), but have a low affinity for monomeric ADP-ribose. This implies that viral macro domains are functionally different from homologues in the human, and the viral macro domain may participate in cellular pathways involving in RNA rather than ADP-ribose derivatives (268). Furthermore, other cell-based studies have indicated the expression of the macro domain in liver cells, leading to the inhibition of apoptosis since the macro domain is functionally related to poly(ADP-ribose) polymerase-1 (PARP-1) (12, 43), suggesting that the viral macro domain might regulate apoptosis during viral infection as well. Recently, a highly conserved “glycine-triad” (G815-G816-G817) was identified downstream of the macro domain of HEV, which is homologous to the rubella virus protease-substrate (G1299-G1300-G1301) (282). Mutagenesis assays indicated that G816V and G817V mutations in the macro domain are lethal for Sar55 replication in S10-3 cells. As mentioned above, a clinical report suggested the great quasispecies

heterogeneity in the macro domain may facilitate HEV persistence in HEV infected solid-organ transplant patients (198).

Helicase domain

The next domain downstream of the X domain is the RNA helicase domain, which is encoded by many positive-stranded RNA viruses and is essential for their replication (146). Helicases are motor proteins which are able to unwind nucleic acid strands by using energy derived from ATP hydrolysis , and helicases play an indispensable role in many positive stranded RNA viruses (146). Generally, helicases can be divided into six superfamilies (SF1-6) (371). RNA virus coded RNA helicases are mainly classified into SF1 and SF2. Helicases SF1 and SF2 contain seven signature motifs (I, Ia, II, III, IV, V and VI) that form the core of the enzyme (146). The helicase domain of HEV belongs to helicase superfamily SF1 and is proposed to possess both NTPase activity and an RNA unwinding domain (146, 179). *In vitro* experiments have demonstrated that the purified HEV helicase domain from *E. coli* has both NTPase and RNA unwinding activities. It drives the hydrolysis of rNTPs but also dNTPs with a lower efficiency, as well as unwinds RNA duplexes with 5' overhangs (159). RNA 5'-triphosphatase activity has also been observed in the HEV helicase domain, which is proposed to function along with methyltransferase for catalyzing RNA capping (160). Recently, in an *in vitro* mutagenesis study on HEV helicase demonstrated that motifs I, IV and VI are dispensable, while motifs Ia and III are crucial and unique for HEV helicase function (248).

RdRp Domain

The last domain of HEV ORF1 polyprotein is the RNA dependent RNA polymerase domain (RdRp). All positive-stranded RNA viruses code an RdRp, which is necessary for viral replication (270). The RdRp from all positive-stranded RNA viruses can be divided into three large supergroups. All RdRp domains contain approximately 300 amino acid residues, with the central and C-terminal parts showing high similarity between each other (177). RdRp from HEV belongs to supergroup III and has the highest similarity to the domains in rubella virus and beet necrotic yellow vein virus (BNYVV) (179). All eight conserved motifs can be found in HEV RdRp, including an Mg^{2+} binding sequence (GDD), which is essential for RdRp activity. The purified RdRp protein of HEV is able to bind the 3' end of HEV RNA, and two stem-loop structures at the 3' end of the poly(A) stretch are necessary for this binding (5). Expression of the RdRp domain in mammalian cells as a GFP fusion protein indicate that it localizes in endoplasmic reticulum (ER), which could be a potential replication site for HEV (315).

The capsid protein encoded by ORF2

HEV ORF2 encodes the capsid protein, which is the major component of the HEV virion. It begins from 37 nt downstream of ORF1, is 1980 nt in length and terminates 65 nt upstream of the polyadenylation tail (319). The full-length ORF2 product is predicted to have 660 aa residues with a molecular mass of 72 kDa (325). Recombinant ORF2 protein can bind to the 5′ region of HEV genome (382). The first study performed in mammalian cells showed that the ORF2 product exists as an 88 kDa protein which carries N-terminal linked glycans and a potential endoplasmic reticulum (ER)-directing signal about 15 aa from its N terminus. This 88 kDa protein can be further

processed and has the potential to form non-covalent homodimers (140). A further study from the same group demonstrated Asn-310 in ORF2 to be the major site for glycosylation (445). A mutagenesis assay indicated that the N-terminal signal peptide is required for its cell surface expression via ER transition, but glycosylation of the capsid protein is not required (445).

Since glycosylation of the capsid protein in non-enveloped viruses is not common, it is not clear whether these modifications have biological significance for HEV infection. Lately, in a more detailed study, all three putative glycosylation sites (aa137, aa310, aa562) were mutated in an infections clone to verify their function. It was found that that any mutation in the glycosylation site prevented the formation of viral particles but without an effect on virus replication in cells; the first two glycosylation sites prevented virion assembly, while the third site was related to virion particle formation and RNA encapsulation (100).

On the other hand, data acquired in insect cells provided a different conclusion regarding ORF2 expression and processing. When expressed in insect cells by the baculovirus expression system, ORF2 can be expressed as an insoluble full length protein of about 72 kDa, as well as a soluble form of 56.5 kDa which is a processed product of the intact p72 kDa form (237). Another group showed that when expressed in SF9 cells, a 62 kDa product was generated from p72 kDa, which lacks the first 111 aa residues of the putative ORF2 product (454). Further study performed in two different insect cell lines (SF9 and Tn5) for ORF2 expression showed a soluble form of pORF2 with a molecular mass of 50 kDa which lacks the first 111 aa of the N-terminal and the last 52 aa of the C-terminus but retains the ability to form virus-like particles (VLP) (203, 204). VLP

assembly is thought to involve dimer formation, and the C-terminus of the recombinant ORF2 protein is believed to be responsible for homo-oligomerization (200, 405, 432). A 3.5-Å resolution crystal structure obtained from HEV VLP indicated that the truncated HEV capsid protein has three definite domains designated as S (shell, aa 129-319), M (middle, aa 320-455) and P (protruding, aa 456-606) (437). This VLP particle is composed of 60 subunits of the truncated capsid protein, forming icosahedral 2-, 3-, and 5-fold axes (437). Mutational analyses indicated that the protruding domain is involved in binding to the susceptible cells and contains neutralization epitopes (437). Moreover, in a more detailed study focusing on the spatial configuration of VLP, ORF2 could be modified to be a delivery system for foreign epitopes (433).

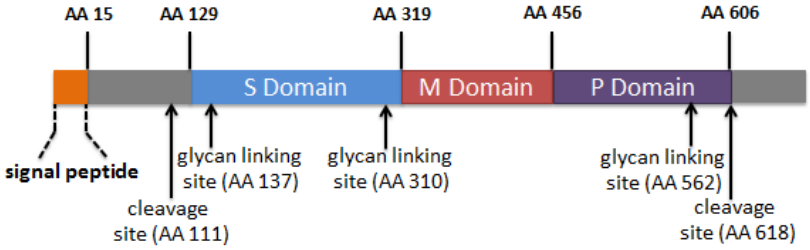

Fig. 1.3 Schematic illustration of ORF2 product.

AA: amino acid; S domain: Shell domain; M domain: Middle domain; P domain: Protruding Domain

The ORF2 product expressed in insect cells is reactive with anti-HEV antibodies (402). Another study indicated that anti-HEV serum collected from humans is reactive with a synthesized peptide (aa 414-433) from ORF2 (58). Genetic analysis of ORF2 showed over 85% homology among the four major HEV genotypes in mammalian hosts (257). Amino acid alignment indicates that divergences are mainly in the first 111 aa of

the N terminus, which is not a component of the virion (257). A study manipulating a phage display system for overlapping peptides and truncated ORF2 proteins mapped the major neutralizing domain to residues 458 to 607, which matches the location of the P domain (244, 354, 459). These data provide valuable information for vaccine development. The ORF2 truncated protein generated by baculovirus or bacterial systems was tested in clinical trials (367, 460). However, a recent study that evaluated cross-protection against heterologous HEV indicated that pigs vaccinated with truncated capsid proteins derived from swine, rat and chicken HEV only generated partial protection against a genotype III mammalian HEV (341).

Additionally, as a structural protein, the HEV capsid protein was found to interact with many cellular proteins and play a role in cell signaling. In one study, HEV ORF2 was found to activate the pro-apoptotic gene CHOP (143). The same study also demonstrated that ORF2 increases the expression of Hsp72, Hsp70B and Hsp40, and interacts with Hsp72, which may inhibit apoptosis. Moreover, it was demonstrated that ORF2 interacts with β-TRCP, a component of the ubiquitination complex that inhibits IκBα ubiquitination-mediated NF-κB activation (383). However, these data are all based on the overexpression of ORF2 in mammalian cells, which needs to be further verified in whole virus infection.

ORF3-encoded protein

The ORF3 is the smallest among the three ORFs of HEV and overlaps with ORF2 by approximately 300 nt in a different reading frame. However, it does not overlap with ORF1 (99). The overlapping region (nt 5147 to 5477) had been found to be the most conserved region between the prototype Sar55 and BUR121 (401). An early study

proposed that ORF3 codes for a protein with 123 aa and comes from a different subgenomic RNA other than that encoding ORF2 (393). However, a later study based on an HEV replicon suggested that ORF3 is translated from a bicistronic subgenomic RNA at the third AUG at nt5131 for Sar55 and expressed as a protein with 114 aa and molecular size of 13 kDa (hereinafter called vp13), which is 9 aa shorter than the predicted version (99). This observation has been confirmed by another study (134).

Sequence analysis has indicated that vp13 is unique and has no homology with any other proteins. It contains two hydrophobic domains in its N-terminal half and two proline-rich domains in its C-terminal half (121, 154). A phosphorylation site (Ser71) has been identified in the first proline-rich domain and can be phosphorylated by MAP kinase (446). Furthermore, two PSAP motifs have been identified in vp13, with the first PSAP motif located at aa 86-89 and the second located at aa 95-98 (262).

Although the full functions of HEV vp13 have not been defined yet, some studies have suggested that vp13 plays multiple roles during HEV infection. Earlier studies focusing on vp13 antigenicity and epitope mapping demonstrated that the last 32 aa of vp13 contain an immunodominant region, and a synthesized peptide from that region is reactive with anti-HEV serum from a recovered patient (58, 359). However, another study which tried to map the T cell epitopes for ORF2 and ORF3 indicated that no T cell proliferation was observed when cells were stimulated with peptides from vp13 (4). In mutagenesis studies, although vp13 is dispensable for viral replication in cell culture (72), it is indispensable for HEV infectivity *in vivo*, implying an important role for vp13 (134).

A yeast two-hybrid system was employed to screen for the interaction partners for vp13. The screen indicated that vp13 can bind to inactive mitogen-activated protein

kinase (MAPK) phosphatase and lead to activation of the MAPK (156), suggesting that vp13 can modulate host gene expression since MAPK is related to cell signaling and gene expression. Chandra et al. found that vp13 inhibits the nuclear translocation of STAT3 and down-regulates STAT3-mediated gene expression, such as acute-phase response proteins (41). The vp13 could also increase the expression of glycolytic pathway enzymes by increasing the phosphorylation and transactivation activity of p300/CBP by stabilizing HIF-1 transcription factor (253). Furthermore, microarray analysis for Huh7 cells with vp13 expression suggested that liver-specific genes can be modulated, as vp13 is able to modulate the phosphorylation of hepatocyte nuclear factor 4 (39).

The vp13 can also up-regulate mitochondrial voltage-dependent anion channel genes, which can protect cells from mitochondrial depolarization and death (254). This result implies that vp13 is able to inhibit the mitochondrial apoptosis pathway. The pro-survival role of vp13 was also demonstrated in another study showing vp13 delays the trafficking and degradation of the activated hepatocyte growth factor receptor to prolong endomembrane growth factor signaling (40). Additional interacting molecules have been identified for vp13 by yeast two-hybrid screens, including α-1-microglobulin, bikunin, and bikunin precursor protein (AMBP), fibrinogen β chain and hemopexin (312, 313, 408, 409).

Besides yeast two-hybrid screens, overexpression of the vp13-coding plasmid in mammalian cells was also employed to elucidate the function of vp13. Studies have indicated that vp13 can associate with the cytoskeleton fraction when expressed in cells, and deletion of the N-terminal hydrophobic domain of vp13 abolishes this association (446). In a more detailed study, GFP-tagged vp13 was found to interact with

microtubules to form a filamentous pattern in cells; this pattern could be altered by a microtubule-destabilizing drug (154). Furthermore, the expression of vp13 leads to an elevation in acetylated α-tubulin, indicating increased microtubule stability (154). Truncation analysis indicated that both the hydrophobic domains in the N-terminus of vp13 are required for its association with the microtubules. Moreover, salt extraction studies have suggested that the vp13-microtubule interaction is electrostatic and dynein is needed for the interaction. An earlier study showed that vp13 cannot be co-precipitated with tubulin by anti-tubulin antibody (446). These results suggest that vp13 may associate with microtubules through interactions with other proteins. This microtubule-like distribution of vp13 suggests that it may play a role in promoting virus egress, as the pUL37 protein of herpes virus can interact with dystonin, an important cytoskeleton cross-linker involved in microtubule-based transport, in order to promote capsid transport on microtubules during egress (283).

More interestingly, another study using a monoclonal antibody against vp13 to capture HEV particles showed that vp13 can associate with virions and support virus release (387). This was later confirmed by a cell culture-adapted genotype III HEV strain with vp13 deletion, indicating vp13 is required for virion release (435). Studies in Caco2 and Huh7 cells for the prototype Sar55, genotype I HEV, showed that the intact PSAP motif in vp13 is required for virion release (73, 262). For avian HEV, the PSAP motif in vp13 has also been found to play a role in virus release (170). It has been revealed that the PSAP motif in vp13 is required for the formation of membrane-associated HEV particles with the vp13 protein itself associated with lipids. This process is mediated by the cellular Tsg101 protein (262, 263). Although these results all suggest that vp13 is

associated with virion during egress, anti-vp13 antibodies are only able to capture HEV from the serum, not fecal samples from patients (387). A possible explanation for this is that viral particles could lose lipid-associated vp13 after passing through the gut (387). The role of vp13 in virus release may be one of its functions during HEV replication, and it may explain why vp13 is indispensable during *in vivo* infection.

On the other hand, as a small phosphorylated protein, vp13 can be phosphorylated at Ser71 by MAPK when expressed in COS1 and Huh7 cells (446). A later study indicated that the Ser71 phosphorylation site is required for the ORF2/ORF3 interaction as ORF3 can interact with ORF2 in a yeast two-hybrid screen, especially for non-glycosylated ORF2 (407). This finding also supports a role for vp13 in HEV structural assembly. However, a mutagenesis study indicated that HEV lacking the phosphorylation site in vp13 was able to replicate its genome in cultured cells, and infection of rhesus monkeys with wild type HEV and the phosphorylation site mutant virus induced the same viremia and seroconversion (98). These data suggest that phosphorylation of vp13 is not necessary for genome replication or for the production of infectious virions. Moreover, in addition to phosphorylation and interaction with ORF2, vp13 could form a homodimer and the 43 aa domain located in the vp13 C-terminal is responsible for its self-association (406).

Recently, our study indicated that vp13 is able to enhance host interferon induction via increasing RIG-I expression and activation (264). We found that vp13 extends RIG-I half-life and interacts with N-terminal portion of RIG-I to enhance its activation by polyIC. Especially interesting, there is a strain difference in the

enhancement of RIG-I mediated IFN induction by vp13, implicating that vp13 may contribute to HEV virulence and pathogenesis.

In summary, as the smallest among the three ORFs of HEV, ORF3 encodes vp13 that plays an indispensable role in infectivity in experimentally infected animal models. However, it appears not required for HEV replication in culture cells. Our current knowledge indicates that vp13 is a multifunctional protein. It interacts with many cellular proteins, modulates host gene expression and is required for virion release.

Replication cycle of Hepatitis E Virus

Due to the lack of an effective *in vitro* cell culture system for HEV, the replication cycle of HEV is largely unknown. The capsid protein is believed to bind to a cellular receptor to initiate viral entry, and there is evidence for the binding of recombinant capsid protein to the cell surface during HEV entry (110). It is also believed that HEV enters liver cells through clathrin-mediated endocytosis (155). However, the exact cellular receptor for HEV is still unidentified. One study based on a viral overlay protein binding assay (VOPBA) suggested that a protein with molecular weight about 55 kDa could be the candidate for HEV entry, but mass spectrometry revealed that this virus binding band contained 31 different proteins (452). Another study suggested that aa 458-607 located in the C-terminal region of the HEV capsid protein may be the putative receptor binding site of HEV virions (110).

After virus entry into permissive cells, HEV genomic RNA is uncoated by unknown mechanisms. In one study utilizing truncated capsid protein HEV239, an HSP90-specific inhibitor (geldanamycin) blocked the intracellular transport of the HEV239 capsid protein without affecting the entry of it (457). This suggests that Hsp90

may play a role in the intracellular transport of HEV particles. After that, the HEV genome will start ORF1 translation, and its genome replication is believed to rely on the necessary enzymes encoded by ORF1. Then, with the generation of sub-genomic RNA for ORF2 and ORF3 translation, there will be virion packing and egress based on our current understanding of the functions of the ORF2 and ORF3 products.

Cell culture and propagation of Hepatitis E virus

Since the discovery of HEV, many efforts had been made to develop an *in vitro* cell culture system for it. However, as of now, the cell culture system HEV is limited and ineffective. An early study tried to use primary hepatocytes from macaques supplied with serum-free medium for HEV propagation; however, virus replication was limited and the detection of virions in the medium relied on PCR (394). Almost at the same time, a group from Japan reported that HEV isolate 87A was able to replicate in A549 cells with cytopathic effect (CPE); however, PCR was used to detect viral RNA in the cell culture medium (133). Lately, a group from China also claimed that the A549 cell line could be used effectively for passaging two Chinese HEV isolates, but these findings were not confirmed for other HEV strains or in other labs (427).

On the other hand, as the commonly employed method for single-stranded, positive-sense RNA virus, transfection of capped RNA from an HEV cDNA infectious clone via *in vitro* transcription to PLC/PRF/5 and Huh7 cells demonstrates limited replication of HEV (71). Although cell lysates from RNA transfected cells is infectious in rhesus monkeys, cell to cell spread of the virus is not observed (71). S10-3 cells, a subclone of Huh7 hepatoma cells, demonstrates improved replication efficiency of HEV

for the genotype I HEV Sar55 strain but this assay still relies on the transfection of HEV RNA (99, 369).

A Japanese group reported that a genotype III isolate from acute hepatitis patient could propagate in PLC/PRF/5 (hepatocellular carcinoma) and A549 (lung cancer) cells (272). After A549 cells were seeded in a six-well plate, they were inoculated with HEV at 1.0×10^4 and 1.0×10^5 RNA copies per well, and HEV RNA reached the highest titer of 10^7 copies/ml 50 days post inoculation. However, PLC/PRF/5 cells could only support as efficient growth as A549 with a higher MOI (with 1.0×10^5 viral RNA copies per well). Moreover, in this HEV cell culture system, HEV infected cells need to be maintained at 35.5°C and cultured with a mixed cell culture medium (50% Dulbecco's Modified Eagle Medium and 50% Medium 199) supplemented with 2% (v/v) fetal bovine serum and 30 mM $MgCl_2$.

Recently, a genotype III HEV strain, Kernow, with an insertion of the human S17 gene was adapted for cell culture in HepG2/C3A cells (369). The authors speculated that this S17 insertion might occur in the HEV patient. The recombinant virus was a minor species in the host but selectively adapted to cells after six passages. In a seven-day incubation period, the replication of this HEV isolate was 7.5-fold higher in HepG2/C3A human hepatoma cells than in Huh7.5, PLC/PRF/5, A549, Caco-2 intestinal cells or rhesus kidney cells, suggesting that HepG2/C3A cells were the most permissive. Moreover, this HEV isolate was also able to infect a variety of non-primate cells, including cow, mouse, chicken, cat, dog and rabbit cells, but with less efficiency.

In summary, the current cell culture system for HEV has limitations. Until now, no cell culture system has been established for genotype I HEV except for the RNA

transfection-based system. On the other hand, although several groups have demonstrated that genotype III or IV HEV strains can be adapted to cell culture and is able to re-infect new cells, it needs a long incubation time compared to other RNA viruses. Moreover, the cell culture adapted Kernow virus may have a different phenotype compared with the wild type virus as virus-host recombination occurred in this cell culture adapted virus.

Transmission and epidemiology of HEV

The hepatitis E virus is a fecal-oral transmitted viral pathogen. The most common source of infection is contaminated drinking water in developing countries. It has been estimated that one third of the world total population have been infected by HEV on the basis of seroprevalence. However, for a long time, hepatitis E was thought to be a public health problem only for developing countries. Hepatitis E is now recognized frequently in industrialized countries where it was not thought to be endemic before (76, 145, 185, 252, 337, 348, 430).

World Health Organization (WHO) estimated that there are 14 million symptomatic cases of HEV infection with 300,000 deaths and 5200 stillborns annually in the world (429). According to WHO, hepatitis E is highly endemic in East and South Asia, where annual hepatitis E symptomatic cases are estimated at 6.5 million, with 160,000 deaths and 2,700 stillborn babies. The number of deaths indicates the more than 50% of global hepatitis E deaths occur in this region. In East Asia, large outbreaks of hepatitis E have only been described in China. Hepatitis E accounts for 20%-50% of acute hepatitis cases in this region. The seroprevalence of anti-HEV antibodies in the region varies from 10% to 50%, indicating that hepatitis E is hyperendemic in this region. In South Asia, outbreaks of hepatitis E have been reported in most countries in this region,

but variable in size. Hepatitis E virus accounts for 20-60% of sporadic acute hepatitis and fulminant liver failure in this region. Specifically, the rates of fulminant liver failure are usually higher in pregnant patients. A recent paper reported that HEV infection causes 49% acute viral hepatitis and 75% fulminant hepatic failure in pregnant women (183). However, the seroprevalence rates of prior exposure to HEV are relatively low, being 10%-40% in most studies.

In developed countries, such as North America, Western Europe and Japan, no outbreaks have been reported. These areas are considered as low or non-endemic for HEV. However, there have been sporadic cases of hepatitis E reported. Transmission of HEV from animal reservoirs to humans is the major cause of those sporadic cases. A series of cases of HEV infection in people who ate undercooked deer meat 6 to 7 weeks before the onset of disease have been reported (202, 395, 440). HEV RNA recovered from the leftover deer meat was found to be identical in sequence to the HEV RNA recovered from the patients (385). Recently, the consumption of shellfish was considered a risk factor in a documented case (175). Thus, foodborne infection could occur from the consumption of uncooked/undercooked products from infected animals. Moreover, blood transfusion-mediated HEV transmission had been also been reported. IgM and IgG against HEV have been detected in recipients of blood transfusions (426).

HEV genotype I is responsible for most endemic and epidemic cases of hepatitis E in Asia, and genotype II is prevalent in Central America and Africa (304). There is no known animal reservoir for HEV genotypes I and II (426), which may explain why HEV infections in developed countries are mainly caused by genotype III and genotype IV, as these two genotypes are zoonotic.

Pathogenesis, clinical signs and diagnosis of HEV

Hepatitis E virus infection causes acute hepatitis with a mortality rate from 0.5 to 3% in young adults (139). However, according to WHO, acute hepatitis and fulminant liver failure caused by HEV account for 20%-60% of the total cases in South Asia. Remarkably, death resulting from HEV-related fulminant liver failure has drastically increased to 30% in pregnant women in their third trimester of gestation (139). Generally, HEV has an incubation period of 3 to 8 weeks (34). The initial symptoms of acute hepatitis E are unspecific and flu-like, such as myalgia, arthralgia and weakness. After this short prodromal phase, a period of symptoms such as vomiting, jaundice, itching, uncolored stools, darkened urine and jaundice could last for days to several weeks accompanied by increased levels of liver transaminases, bilirubin, alkaline phosphatase and γ-glutamyltransferase (123, 426). Current case reports indicate that most cases are self-limited and do not result in chronic hepatitis (123). Recently, an investigation was conducted by Kumar et al. on pregnancy outcomes in hepatitis E (183). In their report, higher HEV viral loads were observed in pregnant women with acute viral hepatitis and fulminant hepatic failure, as well as higher levels of TNF-α, IL-6, IFN-γ and TGF-β1, which suggests that high cytokine levels are responsible for severe liver injury in HEV infection (183).

Chronic infection of HEV has been identified almost exclusively among immunocompromised persons, including organ transplant recipients, patients receiving cancer chemotherapy and human immunodeficiency virus (HIV)-infected persons (123). However, although such cases are rare, chronic HEV infection may occur in adults without apparent immunodeficiency, and the source of infection is often unknown. In

organ transplant recipients, the chronic course leads to persistent increases in levels of alanine aminotransferase, significant histological activity and fibrosis in some cases (426). On the other hand, although there have been reports showing that HIV infected individuals have higher positive results in anti-HEV antibody tests than individuals without HIV infection, but chronic hepatitis E was not described in HIV-infected patients before 2009 (426).

Besides hepatitis, extrahepatic manifestations have been documented as well. Neurological disorders, such as polyradiculopathy, Guillain–Barré syndrome, bilateral brachial neuritis, encephalitis and proximal myopathy have been reported in patients with acute and chronic HEV infections (150). Furthermore, a recent report provided evidence that extrahepatic replication of hepatitis E virus in the placenta of infected mothers and replication of HEV in the placenta may be associated with fetal mortality (35).

Recently, there was a report focusing on the association between the outcome of HEV infection in solid-organ transplant patients and the genetic heterogeneity of HEV quasispecies in ORF1. This report demonstrated that both sequence entropy and genetic distance during the hepatitis E acute phase were higher in patients whose infection became chronic than in those who cleared the virus (198).

Although diagnostic tests for HEV are commercially available, none of them have been formally approved in the United States by the Food and Drug Administration (FDA) (123). Current tests mainly target anti-HEV antibodies, including IgG and IgM. However, several assays are based on antigens expressed by a single HEV genotype, especially genotype III, and might be limited for the detection of all HEV genotypes. Indeed, there are variations in sensitivity, specificity and agreement in the results of these assays,

which may account for the discrepancies among positive rates of anti-HEV antibodies in various populations (65, 118, 234). On the other hand, HEV RNA can also be detected in blood and stool for several weeks after acute HEV infection. However, current RNA tests are still experimental since they have not been standardized yet (426). Furthermore, serologic and virological tests are also available for free from the Centers for Disease Control and Prevention.

Treatment and Prevention of HEV

HEV infection causes a self-limited disease and almost all infected individuals are able to clear the HEV infection spontaneously. Although the mortality in young adults is 0.5%-3%, the deaths caused by fulminant liver failure can increase to 30% in pregnant women during their third trimester of gestation in South Asia (139). Therefore, antiviral therapy may be needed for some acute cases. Although no specific treatment has been approved for HEV, off-label application of ribavirin for HEV has demonstrated promising results in both acute and chronic hepatitis E patients (91, 152, 230). For immunosuppressed patients, a reduction of immunosuppression has shown efficacy in the treatment of chronic HEV infection (426). Moreover, application of pegylated interferon in combination with ribavirin has been reported as well as a treatment for chronic HEV infection (426).

Current prevention for HEV in developing countries relies on sanitary measures, such as providing clean water. In developed countries, prevention is more complicated due to the existence of several possible transmission routes which are not fully understood (149).

Since *in vitro* culturing of HEV is limited and ineffective, HEV vaccine development mainly focuses on the expression of the capsid protein as a subunit vaccine. The capsid protein shares over 85% homology among the four major HEV genotypes in mammalian hosts (257). Currently, the capsid protein expressed by insect cells via baculovirus or bacterial vectors has been tested in clinical trials. The first candidate was a 56 kDa protein encoded by ORF2 and expressed in insect cells. In a phase 2 trial in Nepal, the vaccine was well-tolerated and highly immunogenic, with 95% efficacy for protection against hepatitis E (367). The second vaccine, HEV 239, was a 26 kDa truncated protein (aa 358-606) of ORF2 product expressed in *E. coli* (201). This vaccine was well tolerated with an efficacy of 100% protection after three doses in a tested population of China, which included both men and women aged 16–65 years (460). The HEV 239 vaccine was approved in China in 2012. Whether it will be endorsed in other countries or how effective it is against all genotypes of HEV remains unknown.

Summary

More than 20 years have passed since the discovery and complete genome sequencing of the hepatitis E virus. Our understanding of HEV is still limited, though ongoing research continues to reveal more and more information about this virus. Currently, we know that HEV is not only a public health concern in developing countries as previously thought, but also a health concern with a more complicated background in developed countries. More and more animal reservoirs have been revealed and we now understand that HEV is a zoonotic and foodborne disease. On the one hand, although approved in China, the vaccine is still unavailable to most of the world despite the fact that serum surveillance surveys indicate a high prevalence rate of HEV throughout the

world. On the other hand, virus specific treatment for HEV is not available yet. Although the off-label using of pegylated IFNs and anti-viral drugs for general purposes have demonstrated efficacy against HEV, safety is still a concern as no validation has yet been conducted for these treatments. Furthermore, due to the absence of a suitable animal model and an effective cell culture system, many details about this virus, such as its pathogenesis, strain differences, genotype differences, molecular mechanisms and vaccine efficiency for cross protection are still missing.

In this study, we attempted to determine the mechanism that HEV interferes with host interferon induction and signaling, and to identify the viral proteins responsible for this interference. As interferons play key roles in host innate immunity against viral infection and in the development of adaptive immunity, understanding the mechanism of how HEV evades host innate immunity will provide valuable information for studies on HEV pathogenesis and strain differences and provide insight into potential strategies to combat HEV.

Chapter 2: Interferons and Induction

Introduction

Host immunity can be divided to two broad categories: innate and adaptive. Vertebrates have developed strong innate immunity against viruses, and the key player in this system is interferons. Since the discovery of interferon in the 1950s, numerous studies have been conducted on this protein family. By the technology of molecular biology, many genes coding for the interferons have been cloned and sequenced, as well as expressed in *E. coli*. With the advantage of purified interferons, interferon-based therapy had been developed and applied to treat cancer and viral infection. In this chapter, interferons, their classification, function and induction are reviewed in detail.

Interferons and Their Classification

Interferons are a large family of proteins which are genetically and functionally related (193). The discovery of these mediators occurred in the 1950s, when researchers observed that virus-infected cells could be made resistant to secondary viral infection under certain circumstances. Similarly, it was also observed that treatment of cells with inactivated virus made cells resistant to infection by the same but live virus. Later, the term “interferon (IFN)” was created to describe a special substance, possibly produced by cells, which could interfere with virus replication. However, due to technical limitations, it took more than two decades until interferons could be purified for further analysis (290, 334). The antigenic differences between IFNs generated by human fibroblasts and leukocytes led to the idea that IFNs constitute a protein family (108). Currently, the functions of interferons are well-known, including antiviral activity, antiproliferative

activity, stimulation of T cell cytotoxic activity and modulation of the immune response (290). Since their approval, purified interferons have also been used for the treatment of a variety of cancers and viral diseases (290).

Interferons are divided into three different types: type I, type II and type III. In humans, type I interferons, generally called IFNs, are the largest IFN family, which includes IFN-α, IFN-β, IFN-ε, IFN-κ and IFN-ω (292, 412). They belong to the class II family of α-helical cytokines, which includes type II IFN-γ, the newly identified type III IFNs, IL-10 and several IL-10 homologs (62, 181, 291). Type I IFNs are encoded by individual genes except IFN-α, which has 13 subtypes (79). IFN-δ, IFN-τ and IFN-ζ (or limitin) have also been identified as type I IFNs in swine, ruminants and mice, respectively (79). Almost all cell types are capable of producing IFN-α/β; however, plasmacytoid dendritic cells (pDC) are considered as the major source for IFN-α production during the course of an infection (217, 370). All type I IFNs binds a ubiquitously expressed heterodimeric receptor complex, which includes the IFN-α receptor 1 (IFNAR1) and IFN-α receptor2 (IFNAR2) as subunits. Data suggest that IFNAR2 serves for ligand binding, but both subunits are required to activate downstream signaling (397). However, the various subtypes of type I IFNs display different binding affinities for the receptor complex and thus lead to various outcomes with respect to their antiviral, antiproliferative and immunomodulatory activity (138, 148, 256).

The type II interferons include only IFN-γ, which functions as a homodimer (79). unlike type I IFNs, IFN-γ production is restricted to activated T cells, natural killer cells and macrophages (413). Signaling for IFN-γ is transduced via the IFN-γ receptor complex (IFNGR). IFN-γ homodimer intimately interacts with two IFNGR1 subunits,

with further binding of two IFNGR2 subunits, which results in receptor activation (420). IFNGR is ubiquitously expressed, which means nearly all cell types are capable of responding to IFN-γ (413). IFN-γ plays a major role in establishing cellular immunity; however, it is also able to induces expression of a group of genes that respond to type I IFN treatment (56, 197).

The type III interferons are a newly discovered family of interferons which comprise IFN-λ1, IFN-λ2, IFN-λ3 (also known as interleukin (IL)-29, IL-28A, and IL-28B, respectively) (79). They were initially named "interferon-like cytokines" because IFN-λ is functionally similar to type I IFNs but has a distinct gene sequence and chromosomal location (397). Besides, IFN-λ signals through a heterodimeric receptor composed of IFRL1 (or IL-28R) and IL-10R2, which are different from type I IFN receptors (79, 291, 292). However, IFN-λ activates the same pathway and similar gene expression as type I IFNs. Recently, IFN-λ4 was described as the fourth member of the type III IFNs (301).

After the discovery of IFNs and their antiviral activity, viral RNA was proposed to be the potential inducer. Many early studies focused on the identification of possible nucleic acid inducers and numerous synthetic and biological RNAs were tested for this purpose. Unlike ssRNA, DNA or RNA:DNA hybrids, the dsRNA was found to be a potent trigger of the interferon response (28). Specifically, dsRNA from bacteria, reovirus, vaccinia virus and synthetic polyinosinic:polycytidylic acid (polyIC) were shown to be potent activators of IFN production (28). However, only recently have we begun to understand how the host detects virus infection and initiates interferon synthesis.

The discovery of Toll and the Toll-dorsal pathway in *Drosophila* led us to understand that there are conserved signaling pathways across the plants and animals as a common defense against pathogens (29). Identification of the first Toll homologue in humans finally led us to gain the concept of Toll-like receptors (TLRs) (241). The TLRs were subsequently shown to distinguish unique molecules present in a variety of pathogens from bacteria, fungi to viruses (239). Now, we understand that pathogens are recognized by a group of pattern-recognition receptors (PRRs). Besides membrane-associated TLRs, there are another group of PRRs called RIG-I-like receptors (RLR), which comprise the cytoplasmic sensors of viral nucleic acids (25). Taken together, while the host IFN response plays a key role during the early stage of viral infection, host PRRs for RNA viruses include the RLR and TLR pathways. Both RLR and TLR3 can recognize double-stranded RNA (dsRNA) of the viral genome or replication intermediate of RNA viruses. Activation of RLR and TLR3 signaling leads to the activation of interferon regulated factor-3 (IRF3), IRF7 and NF-κB. Those transcription factors translocate to the nucleus and result in the induction of type I IFNs and expression of inflammatory cytokines. In the following section, Toll-like receptors, RIG-I-like receptors and their corresponding signaling pathways are discussed further.

Toll-like Receptor Pathway

Discovery of Toll in *Drosophila*

Toll is a gene from *Drosophila* and was identified during a study for genes essential for the establishment of the dorsal-ventral axis in *Drosophila* during embryo development (269). Toll contains an extracellular domain which consists of leucine-rich repeats (LRRs), flanked by cysteine-rich regions (68). LRRs are found on a wide variety

of proteins involved in some form of protein-protein interaction, such as receptors and adhesion molecules (388). The cytoplasmic domain of Toll shows high similarity to the cytoplasmic domain of the mammalian interleukin 1 type I receptor (IL-1RI) (89, 353). In 1994, the ligand of Toll in *Drosophila*, a novel secreted protein encoded by the *spätzle* gene, was identified and confirmed to be necessary to establish the dorsal-ventral pattern of the *Drosophila* embryo (258). However, a later study demonstrated an additional, non-developmental role played by Toll that the activation of Toll could result in an immune response in *Drosophila* cell lines. This kind of immune response is mediated by transcript factors from the *Rel* family, i.e. Dorsal-related immunity factor (Dif) and Dorsal, both of which are NF-κB like transcript factors in *Drosophila* (331). Conclusive evidence from *in vivo* experiments demonstrated that Toll mediates signaling pathways and the extracellular Toll ligand, *spätzle*, controls the expression of the antifungal peptide gene drosomycin in *Drosophila* adults (192). Mutations in the Toll signaling pathway dramatically reduce the survival of *Drosophila* after fungal infection (192). These discoveries led to the formation of a conserved signaling pathway of the immune response: the *Drosophila* Toll-Dorsal pathway, which is considered the homologue of the interleukin-1 receptor (IL-1R)-NF-κB pathway in mammals (29).

Discovery of Toll-like receptors in mammalian hosts

After revealing the role played by Toll in the immunity of *Drosophila*, researchers started to look for the analogous genes in humans that are involved in this evolutionally conserved immune signaling pathway. However, the idea for the existence of certain cellular receptors which could sense pathogens and deliver signals to cells was generated even earlier, in 1989 (141). In 1997, the first human homologue of *Drosophila* Toll, later

known as Toll-like receptor 4 (TLR4), was cloned and confirmed to induce the activation of NF-κB and the expression of NF-κB activated inflammatory cytokines (241). However, since little knowledge about the existence of the natural ligand or the human homologue of the *spätzle* gene was available for the first discovered Toll homologue in humans, the strategy employed to confirm its function relied on artificial modification of this Toll homologues. Based on the previous data for generating the constitutively active mutant of *Drosophila* Toll, researchers generated a similar mutated construct and fused it to a CD4 ectodomain as a marker for verifying the expression of the hybrid protein. When this CD4–Toll fusion protein was overexpressed in a macrophage-derived cell line, it constitutively activated NF-κB and resulted in the upregulation of inflammatory cytokines as well as costimulatory molecules, as originally predicted (241). Later, a total five Toll homologues from human were identified, and designated as TLR1 to 5 (326).

The term TLR was initially assigned to describe a Toll related gene that contributes to hedgehog function in the adult eye of *Drosophila* in 1994 (44). Using of this term occurred earlier than the discovery of Toll as an immune response related receptor. However, since the discovery of Toll homologues in mammalian hosts, TLR have been assigned to describe a new receptor family more closely related to *Drosophila* Toll homologs rather than to vertebrate IL-1Rs (326). Currently, a total of 13 TLRs have been identified in humans and mice (TLR1-13) (33, 48, 49, 187, 391, 449). TLR1 to TLR10 are expressed in humans, while TLR11 to TLR13 are found exclusively in the mouse. There is indeed a TLR10 homologue identified in the mouse; however, a retroviral insertion disrupted the *TLR10* gene and rendered it non-functional (107). Furthermore, increasing evidence shows that other mammals may express TLRs that are

not found in humans. Some TLRs, such as TLR14, TLR5S, TLR20 and TLR21 have been identified in Takifugu pufferfish and catfish, and may be found in non-mammalian species with unique properties distinct from those in mammals (23, 278, 323). Of the human TLRs, TLR3, TLR7, TLR8 and TLR9 are membrane proteins localized in the endosome while the remaining TLRs are mainly locate on the cell surface (167).

TLR ligands

After the discovery of TLRs in humans, a key question remained unanswered: Do human TLRs recognize natural ligands from pathogens or are there human homologues of the *spätzle* gene that serve as ligands for TLRs (240)? Data from HEK293 cells indicated that TLR2 mediates LPS-induced signaling when co-expressed with CD14 (438). The conclusive answer came unexpectedly from a study in a mutated TLR4 gene in the LPS-unresponsive C3H/HeJ mouse strain (297). Before the identification of TLR in mammals, researchers were unable to identify the gene responsible for the LPS mediated response in different mouse strains with different phonotypes in their response to LPS stimulation, and they termed this proposed gene *LPS* (18, 250, 330, 379). In 1998, a mutation in the TIR domain of TLR4 was finally identified that led to defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice, which confirmed that LPS is the ligand for TLR4 (297). Later, TLR4 was confirmed to be the product of the proposed *LPS* gene (127). Furthermore, other studies indicated that another molecule, MD-2, which non-covalently binds to the extracellular domain of TLR4, serves as the real "receptor" for LPS, whereas TLR4 serves as the signal-transducing component of the larger "TLR4 signaling complex" (366). Since then, more TLR ligands have been identified.

TLR1 in association with TLR2 as a heterodimer recognizes peptidoglycan and lipoproteins (77, 142, 356). The dsRNA is recognized by TLR3 (11). Bacterial flagellin is recognized by TLR5 (109). TLR6 recognizes lipopeptide cooperatively with TLR2 (77). TLR7 mainly recognizes single-stranded RNA, a common feature of most RNA viral genomes. Some artificially synthesized compounds, such as imiquimod, can be recognized by TLR7 as well (114). TLR7 and TLR8 are functionally related, as TLR8 recognizes viral RNA and self-RNA within snRNP autoantibody complexes (83). Unmethylated CpG DNA derived from bacteria is recognized by TLR9 (115). Currently, no specific ligand had been identified for TLR10. It has been shown that TLR10 plays a role in mediating bacterial peptidoglycan-induced trophoblast apoptosis, which implies that TLR10 may recognize a bacteria-derived component (261).

In summary, within the TLR family, TLR3, 7, 8, and 9 represent a subfamily that is localized in intracellular compartments, such as endosomes and the endoplasmic reticulum (ER), where they recognize viral nucleic acid species (167). There is cell-specific expression of those four TLRs. Innate immune cells express the endosomal TLR 7, 8 and 9, which sense GU-rich RNA and CpG-containing DNA, while TLR3 is expressed in broad cell types, such as endothelial cells, fibroblasts and astrocytes (349). On the other hand, the other subfamily includes TLR1, 2, 4, 5, and 6, which are localized on the cell surface and mainly recognize bacterial cell wall components (167).

Recently, the connection between autoimmune disease and TLRs activated by endogenous ligands was revealed (116). There is increasing evidence indicating that some endogenous ligands can activate TLR7 and TLR8 and result in autoimmune disease in mouse models (101). TLR8 and 9 are believed to be involved in the development of

some autoimmune diseases such as rheumatoid arthritis. Endogenous DNA fragments generated during cell death, either apoptosis or necrosis, are believed to serve as the endogenous ligand for these TLRs (116, 295). A full list of TLRs ligands is listed in Table 2.1 below.

Table 2.1 Toll like receptors and their ligands

TLRs	Adaptor(s)	Ligand
TLR1	MyD88	Peptidoglycan/ lipoproteins
TLR2	MyD88	Peptidoglycan/ lipoproteins
TLR3	TRIF	dsRNA
TLR4	MyD88/TRIF/TRAM/TICAM	LPS
TLR5	MyD88	Bacterial flagellin
TLR6	MyD88	lipopeptide
TLR7	MyD88	ssRNA
TLR8	MyD88	ssRNA
TLR9	MyD88	CpG DNA
TLR10	MyD88	unknown
TLR11	MyD88	Unknown
TLR12	MyD88	Unknown
TLR13	MyD88	Unknown

TLR signaling

TLRs are believed to function as dimers. Schematic illustration of the TLR signaling is shown in Fig 2.1. After recognition of their ligands, TLRs activate the same signaling components as those used in IL-1 receptor (IL-1R) signaling due to a conserved Toll-IL-1R (TIR) domain in intracellular regions (259). As a result, TLRs recruit a set of adaptor proteins with TIR domains by homophilic interaction of their TIR domains. Those adaptor molecules include myeloid differentiation primary response gene 88 (MyD88), TIR-containing adaptor protein/MyD88-adaptor-like (TIRAP/MAL), TIR-containing adaptor inducing interferon-β/TIR-domain-containing adaptor molecule 1 (TRIF/TICAM1) and TIR-domain-containing adaptor molecule/TRIF-related adaptor molecule 2 (TICAM2/TRAM) (166). Adaptor molecules used by TLRs are varied for different TLRs. TLR4 uses all four adaptors while TLR3 uses only TRIF (166).

The most well-known adaptor molecule for TLRs is MyD88, which is utilized by all TLRs except TLR3. MyD88 is composed of a death domain as well as a TIR domain. Upon TLR activation, a homophilic interaction occurs between the death domain of MyD88 and members of the IRAK (IL-1 receptor-associated kinase) family, including IRAK1 and IRAK4 (10, 428). IRAK4 is initially activated, which in turn phosphorylates and activates IRAK1. Then, phosphorylated IRAK4 and IRAK1 dissociate from MyD88 and interact with TRAF6, which is a RING-domain E3 ubiquitin ligase. TRAF6 promotes the Lys63-linked polyubiquitination of itself and NEMO (42). Ubiquitinated NEMO and TRAF6 subsequently recruit a protein kinase complex involving transforming growth factor-β activated kinase1(TAK1) and TAK1 binding proteins (TABs) (3). TAK1 and the TABs (TAB1 and TAB2) then activate two distinct pathways involving the IKK complex

and MAPK. TAK1 promotes downstream activation of the IκB kinases (IKK) IKKα and IKKβ. These IKKs directly phosphorylate the inhibitory IκB family, which normally sequester NF-κB in its inactive form in the cytosol. However, phosphorylation of IκB leads to its polyubiquitination and degradation, which enables the activation and nuclear translocation of NF-κB. The activation of the canonical NF-κB pathway results in the induction of inflammatory cytokines and co-stimulatory molecules (259).

A notable function of MyD88 is that signaling transduced via MyD88 due to activation of TLR7 and TLR8 in plasmacytoid dendritic cells (pDCs) results in induction of IFNs (165). TLR7 and TLR9, are selectively expressed by pDCs (also known as professional IFN-producing cells), which are a subset of DCs with a plasmacytoid morphology and unique in their capacity to rapidly secrete type I IFNs in response to viral infection (50, 217). In pDC, IRF7 is constitutively expressed and binds MyD88 to forms a signaling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (122, 168). Within this complex, IRF7 becomes phosphorylated, dissociates from the complex, and translocates into the nucleus to activate IFN induction upon activation of TLR7 and TLR8.

Besides MyD88, TRIF is another adaptor molecule that is specially utilized by TLR3 and represents another important pathway for TLR signaling (166). Upon activation of TLR3 by dsRNA, TRIF interacts directly with the TIR domain of TLR3 (273). TRIF also directly binds TRAF6 via its TRAF6-binding motifs in the N-terminal region (343). TRAF6 then activates TAK1 in a manner similar to that of the MyD88-dependent pathway for NF-κB activation. On the other hand, TRIF has been confirmed to associate with both IKKε and TBK1 via its N-terminal region, which shares binding with

TRAF6; the TANK family protein NAK-associated protein 1 (NAP1) might facilitate the interaction of both kinases with TRIF (342, 343). TBK1 and IKKε possess essential roles in the induction of type I IFNs through phosphorylation and activation IRF3 and IRF7 (365). Upon activation, IRF3 forms a homodimer, which translocates to the nucleus and binds to its target sequences, such as the promoter region of the IFN-β gene. Thus, there is a TLR3-TRIF-IKKε (TBK1)-IRF3 signaling axis which finally leads to IFN production.

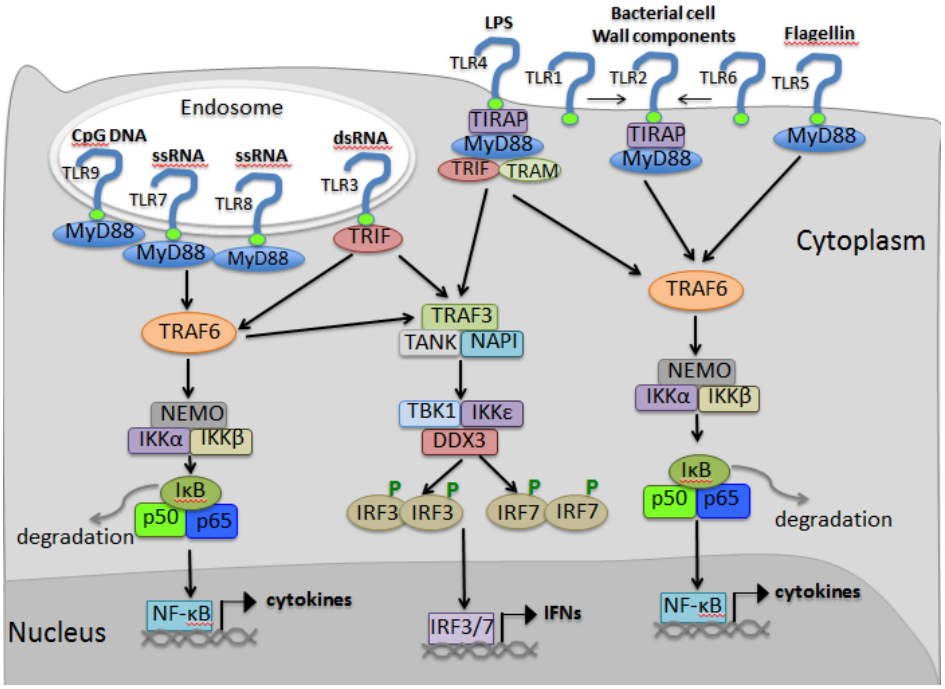

Fig. 2.1 Schematic illustration of TLR mediated signaling

TIRAP and TRAM are another two adaptors that are less investigated, but still play important roles in TLR signaling. Their functions are related to TLR4-mediated signaling. TLR4 is the only TLR that uses all four adaptors. With assistance from TRAM, TLR4 can function via TRIF as well to activate TLR3-TRIF-IKKε (TBK1)-IRF3 signaling axis. TRAM, also known as TICAM-2, acts as a bridging adaptor between TLR4 and TRIF (275). TIRAP has a crucial role in the MyD88-dependent signaling pathway shared by TLR2 and TLR4. Just like TRAM, TIRAP serves as a "bridge" for MyD88 and receptors and thus activates NF-κB (124, 147). Taken together, as the consequence of activation, all TLRs commonly promote chemokine and pro-inflammatory cytokine expression via NF-κB. However, the hallmark of endosomal TLR3, 7, 8 and 9, which sense nucleic acids, is the induction of type-I IFNs (167). A schematic illustration of TLR mediated signaling is shown in Fig. 2. 1.

RIG-I like Receptor Pathway

Discovery of the RIG-I like receptor

Retinoic acid-inducible gene I, or RIG-I (also known as DDX58), is a member of the so-called DExD/H box RNA helicase family. DExD/H box RNA helicase and its related DEAD box helicase, named according to one of the conserved protein motifs, belong to helicase superfamily 2 (94, 212). This is the largest helicase group and, in fact, a subset of it includes proteins from *E. coli*, eukaryotes, even from DNA and RNA virus (94, 212). RIG-I-like receptors (RLRs) currently include three members: RIG-I , melanoma differentiation-associated gene 5 (MDA5, also known as Helicard or IFIH1) and Laboratory of Genetics and Physiology 2 (LGP2) (236). Unlike membrane-associated TLRs that detect pathogen-associated molecular patterns (PAMPs) derived

from viruses, bacteria and fungi, RLRs are cytoplasmic proteins that are specific for detecting RNA derived from viruses in the cytosol (349).

RIG-I was originally identified as a gene induced by retinoic acid during the differentiation of an acute promyelocytic leukemia cell line (136, 453). In 1997, a Chinese research group from the Shanghai Institute of Hematology, which focused on employing all-trans retinoic acid (ATRA) as a treatment for acute promyelocytic leukemia, cloned a novel set of ATRA-inducible genes when using retinoic acid to induce the differentiation of an acute promyelocytic leukemia cell line. This group named these genes "RIG-A, RIG-B, RIG-C," and so on. They deposited the sequence of the genes into GenBank, including the sequence for RIG-I (215, 236). Later, another study identified a RIG-I homologue in swine, and named it RNA helicase induced by virus (RHIV-1). This gene was upregulated when porcine alveolar macrophages were infected with porcine reproductive and respiratory syndrome virus (PRRSV) (453). Another group from Japan also reported that stimulation of endothelial cells with lipopolysaccharide (LPS) led to the up-regulation of RIG-I (136). Since then, more reports have indicated that RIG-I can be up-regulated in cells infected with a virus or cells treated with IFN-γ (52, 255). Taken together, the earlier reports connected RIG-I with innate immunity and IFNs.

The first report to describe the function of RIG-I came from Yoneyama et al., who found that the helicase domain of RIG-I is responsible for dsRNA recognition and the caspase activation and recruitment domain (CARD) for transmitting the signal downstream, leading to the activation of NF-κB and IRF3 and then IFN induction (442). After revealing the function of RIG-I, the same group started searching mammalian

databases for RIG-I related genes, leading to the discovery of two other members of the RLR family, MDA5 and LGP2 (441).

MDA5 is another member of the RLR family. Similar to RIG-I, MDA5 was identified in a differentiation induction subtraction hybridization (DISH) screen which was designed to define genes regulated by the induction of terminal differentiation in human HO-1 melanoma cells (130, 131). The genes identified by the DISH screen were named melanoma differentiation-associated (MDA) genes. MDA5 was one of these novel genes that was induced by IFN-β in human melanoma cells and had melanoma growth-suppressive properties (153). MDA5 shares 23% and 35% identical aa with RIG-I in the CARD and helicase domains, respectively (441).

LGP2, the latest member of the RLR family discovered, was identified based on a set of studies in mammary gland development and remodeling, which are regulated by two members of the STAT (signal transducer and activator of transcription) family, STAT3 and STAT5. Since *Stat3*, *Stat5a*, and *Stat5b* are closely related in a region of mouse chromosome 11, this led the research team to hypothesize that additional genes involved in mammary gland development might harbor in this locus (53, 251). Finally, two novel genes, *Lgp1* and *Lgp2*, were identified and LGP2 was found as a cytoplasmic protein harboring a DExH/D-box helicase domain (53). Unlike RIG-I and MDA5, which contain both the CARD and helicase domains, LGP2 contains only the helicase domain with 31% and 41% identical aa residues to the helicase domains of RIG-I and MDA5, respectively (441).

Ligands of RLRs

RLRs present in the cytosol of all cell types induce type I IFNs and cytokines upon activation (349). As RNA helicases, RLRs mainly recognize RNA molecules derived from viruses, and in some cases, RNA from bacteria (349). However, ligand preference varies among different RLRs. Specially, even though the details of the RLRs ligands have been revealed, there are still many questions remaining. Currently, RIG-I is better defined than MDA5 and LGP2, and considerable efforts have been made to identify its ligands.

Initially, polyIC was used as an artificial ligand for RIG-I to activate IFN production, consistent with a previous concept of dsRNA as a physiological viral trigger for IFN induction due to the simple explanation that RNA viruses are bound to make mistakes during replication and are therefore likely to expose at least some dsRNA molecules in the cell (28). However, this assumption is questionable. Studies employing dsRNA-specific antibodies have shown that positive-stranded RNA and DNA viruses make dsRNA during replication; however, negative-strand RNA viruses do not appear to produce detectible amounts of dsRNA, such as influenza A virus (425). On the other hand, an important addition to this field was the discovery that a 5′ triphosphate (5′ppp) group on an RNA molecule serves as a activator of RIG-I in addition to dsRNA (125, 294). This discovery gives a potential explanation to the earlier observation that 5' triphosphate bearing siRNA generated from *in vitro* transcription is able to induce interferon induction, while removing the 5' triphosphate from artificially synthesized siRNA maintains full efficacy but no longer induces IFN (172).

On the contrary, later studies challenged the earlier observation that the 5' triphosphate alone is sufficient for RIG-I activation and indicated the requirement for a double-stranded component in addition to the triphosphate (28). Specifically, synthetic 5′ppp ssRNA was not capable of inducing IFNs when introduced into cells, while the same RNA molecule generated by *in vitro* transcription served as a competent activator of the IFN response since the RNA mixture from the T7 transcript contained a significant portion of dsRNA molecules (28). Further characterization of RIG-I activation requirements showed that dsRNA complementarity of at least 10–18 nt is required at the 5′ppp containing end in order to induce RIG-I activity. These types of RNA molecules can form a panhandle structure, and is supported by the data that ends of defective interfering (DIs) genomes from negative-stranded RNA viruses are very potent IFN inducers (378). Thus, for a negative-stranded RNA virus, the complementary 5′ and 3′ terminal sequences of its genomes bear the potential to hybridize as dsRNA panhandle structures with blunt ends and 5′ppp groups, thereby meeting the requirements of a RIG-I ligand (351).

In addition to 5' triphosphate dsRNA, another study defined the RIG-I ligand as blunt-ended dsRNA longer than 23 bp with higher activity when the RNA contains two blunt ends (350). Since the dsRNA antibody recognizes dsRNA longer than 40 bp, this ligand size requirement gives a potential explanation for why dsRNAs generated by negative-stranded RNA virus replication were not detected since they maybe too short for the antibody detection (349). Moreover, a recent study indicated that RIG-I is also involved in DNA-initiated IFN induction (349). dAdT functions as a template for

endogenous RNA polymerase III, which generates 5′triphosphorylated AU-polymers in the cytosol to stimulate RIG-I (47).

There has been less attention on the ligand of MDA5. Although polyIC indeed binds and stimulates RIG-I, it is worth noting that polyIC fails to induce IFN-α when injected intravenously into MDA5-deficient mice or transfected *in vitro* into MDA5-deficient cells (93, 162). While RIG-I is essential for interferon induction for paramyxoviruses, influenza virus and Japanese encephalitis virus, MDA5 is critical for picornavirus detection (162). This implies that MDA5 and RIG-I helicases have differential roles in the recognition of RNA viruses. Another study demonstrated that MDA5 was only stimulated by long polyIC fragments when using polyIC fragments of different sizes generated from RNase-III digestion as the activator (161). Briefly, dsRNA of 1 kb was entirely dependent on RIG-I for IFN induction, while increasing the length to 4 kb progressively led to dual MDA5 and RIG-I dependence. MDA5 can recognize dsRNA more than 7 kb long. This also shows that viral dsRNAs differentially activate RIG-I and MDA5, depending on their length as well. Furthermore, an mRNA fragment from the negative-stranded RNA parainfluenza virus 5 activated type-I IFN expression in a MDA5-dependent manner (225). Since type I IFN induction by this mRNA fragment requires the involvement of RNase L, it implies that RNase L may recognize and process viral mRNA into a MDA5-activating structure (225).

As the third member of the RLRs, LGP2 is not fully understood and the current data on its function are controversial. No specific research has been performed on ligand identification for LGP2. Unlike RIG-I and MDA5, LGP2 structurally lacks CARD

domain, suggesting a putative ligand sequestering role (349). Indeed, earlier reports suggested an immune suppressive function for LGP2 (176, 332, 339). Moreover, a mutant LGP2 with abolished RNA binding ability was still able to inhibit RIG-I mediate signaling (205). However, further studies on LGP2-deficient mice revealed an indispensable role of LGP2 for the immune response to viruses that are mainly detected by MDA5 (344, 417). In some cases, an impaired RIG-I antiviral response was observed as well (344). Another study also demonstrated that LGP2 contributed to sustained RLR signaling for IFN-β expression in myeloid cells, and that LGP2 promotes an essential pro-survival signal in response to antigen stimulation to induce CD8+ T cell expansion and effector functions against divergent RNA viruses (384). Therefore, although no finalized conclusion has yet been made, LGP2 appears to have a modulatory role in fine-tuning the innate immune response to viruses depending on the context of viral infection.

Collectively, the current data indicate that RIG-I and MDA5 recognize different types of viruses with a possible overlapping recognition by both, while LGP2 plays a role as a potential regulator. However, both RIG-I and MDA5 require the same adaptor molecule, mitochondrial antiviral signaling (MAVS) (also known as virus-induced signaling adaptor (VISA), IFN-β promoter stimulator 1 (IPS-1) and the CARD adaptor inducing IFN-β (Cardif)) to activate innate immune defense. In the next section, the downstream signaling pathway of RLRs will be discussed.

Signaling of RLRs

When RLRs were found to be the intracellular receptor of viral RNAs, it was less clear which molecule acts downstream of these helicases and facilitates the activation of

IRF3, IRF7 and NF-κB, which are required for IFN transcription and utilized by TLRs. Soon thereafter, MAVS was identified as the necessary adaptor for RLR signaling from different research groups with different names (MAVS, IPS-1, VISA or Cardif. hereinafter, MAVS is used) assigned to the same molecule (169, 247, 363, 434). MAVS contains an N-terminal CARD-like domain just like RLRs and a C-terminal transmembrane domain which defines its mitochondrial localization. Both domains are essential for MAVS signaling (363). MAVS also interacts with TRIF, TRAF6, IKKα, IKKβ and IKKε, thus leading to the activation of NF-κB and IRF3 (247, 434). Although the details of these interactions and activation pathways are still incomplete, available data for RLR signaling indicates a schematic picture. Briefly, in the absence of its ligand, RIG-I is in an

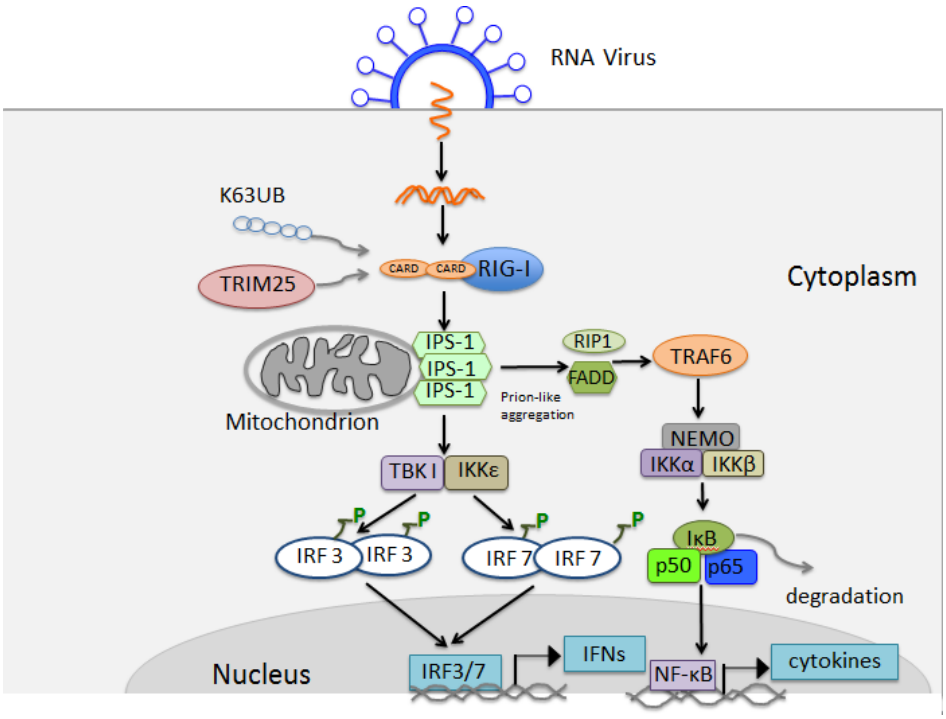

Fig. 2.2 Schematic illustration of RIG-I mediated IFN induction

inactivated form. Binding of dsRNA or 5'ppp-RNA to the basic cleft in the C-terminal domain (CTD) induces a conformational change, which causes uncovering of the CARD domain in the presence of ATP (389). TRIM25, a member of the tripartite motif (TRIM) protein family, is an ubiquitin E3 ligase reported to bind to the N-terminal CARD of RIG-I and conjugate the lysine 172 in the internal CARD with a lysine 63-linked polyubiquitin chain (86, 322). Then, the CARD domain of RIG-I interacts with the CARD domain of MAVS, which causes the conversion of MAVS on the mitochondrial membrane to a prion-like aggregate (128). MAVS then activates the cytosolic kinases IKKs and TBK1, which activate the transcription factors NF-κB and IRF3. Finally, type I interferons and cytokines are induced (238). A schematic illustration of RIG-I mediated IFN induction is shown as Fig. 2.2.

In this RLRs-MAVS-IRF3/7 axis, another study identified that receptor-interacting serine-threonine kinase 1(RIP1) and Fas-associated death domain (FADD) are involved in the TLR-independent innate immune pathway. Both RIP1and TBK1 are required for the activation of the transcription factor IRF3 (22). Moreover, lysine-63 (K63) polyubiquitin chain-mediated ubiquitination of TRAF6 and TBK1 is also required for the activation of TRAF6 and TBK1 (59, 403).

Viral antagonism of IFN induction

Antagonism via direct interaction with host molecules in the IFN induction pathway

It is well-known that viruses employ multiple strategies to evade host TLR and RLR mediated signaling. Some viral proteins can directly interact with host signaling molecules to inhibit their functions. For example, the NS1 protein of influenza A virus is

able to interact with RIG-I to block downstream signaling (249). The V protein of paramyxoviruses binds MDA5 to inhibit the downstream activation (14). Respiratory syncytial virus (RSV) NS1 protein associates with MAVS during early infection to disrupt the RIG-I and MAVS interaction as well as downstream signaling (38). The $\gamma_1$34.5 protein of HSV forms a complex with TBK1 and disrupts the interaction of TBK1 and IRF3, which prevents the induction of interferons (418). The Ebola virus protein VP35 interacts with IKKε and TBK-1 to impair the activation of IRF3 (300). Human papillomavirus 16 (HPV16) E6 oncoprotein binds to IRF3 and inhibits its transcriptional activity as well (328).

Antagonism via degradation or cleavage of host molecules in the IFN induction pathway

On the other hand, viruses can encode proteins that target host molecules to degrade or be cleaved as another way to inhibit the activation of IFN-β. For example, viral-encoded 3C protease by encephalomyocarditis virus (EMCV) induces RIG-I degradation during EMCV infection (280). MDA5 protein is degraded in poliovirus-infected cells in a proteasome and caspase-dependent manner (24). HCV protease NS3/4A cleaves MAVS to evade innate immunity (206). The 3Cpro cysteine protease of coxsackievirus B3 (CVB3) cleaves both MAVS and TRIF to escape host immunity (260). CSFV Npro induces the proteasomal degradation of IRF3 (27).

Antagonism IFN induction via inhibiting IRF3 activation or function

As a key transcription factor, IRF3 is a frequent target of viral proteins to inhibit its phosphorylation, such as HCV NS3/4A protease and PRRSV nsp1β (32, 84). Hepatitis A virus and hepatitis B virus suppress IRF3 activation and nuclear translocation as well (78, 444). Varicella-Zoster virus (VZV) immediate-early protein 62 blocks IRF3

phosphorylation (361). IRF3 can be inhibited by viral coded homologues vIRF3 from Kaposi's sarcoma-associated herpesvirus (KSHV) (137). vIRF3 have been shown to interact directly with cellular IRF3 (137). Furthermore, immediate early ICP0 protein from herpes simplex virus (HSV) can recruit activated IRF3 and CBP/p300 to ICP0 nuclear foci to block IFN-β induction (243).

Antagonism of IFN induction via virally-encoded deubiquitinases

Recently, the connection between ubiquitination and activation of the IFN induction pathway was defined, and cellular and viral deubiquitinases have been demonstrated to play a negative role in IFN induction (216, 231, 448). Cysteine proteases represent a large family of deubiquitinases (13), which implies that viral-encoded cysteine proteases can function as deubiquitinases to inhibit ubiquitination-dependent activation of host IFN signaling. Evidence from virus studies indicates that viral-encoded cysteine proteases indeed possess deubiquitinase function and inhibit host innate immunity, such as arterivirus papain-like protease 2 (415), the PCP domain of PRRSV (199, 380) and the leader proteinase of foot-and-mouth disease virus (FMDV) (422). Furthermore, as an ubiquitin homologue, small ubiquitin-related modifiers (SUMO) cause the sumoylation of IRF3 and IRF7, which has been demonstrated as a means of negative regulation of IFN induction by viruses (182).

Antagonism via viral homologues of host molecules

Viruses can encode some homologues of the host TLR and RLR signaling molecules or related molecules to interfere with host signaling, especially for some DNA viruses with a larger genome. For example, vaccinia A46R and A52R, which share amino acid sequence similarity with TIR domain, are antagonists of TLR signaling (36, 374).

KSHV encodes four homologs of IRFs, vIRF1–4, which inhibit the activity of their cellular counterparts. vIRF1, 2, and 3 have been shown to interact directly with cellular IRFs (137).

Other functions of TLRs and RLRs

TLRs and RLRs form the most important sensor families for viral infection, resulting in an innate immune response within minutes of infection to produce type I IFNs and pro-inflammatory cytokines. For TLRs, besides their important roles played in host innate immunity, TLR mediated signaling is involved in many other biological processes. TLRs have also been shown to be an important linker between innate and adaptive immunity through their presence in dendritic cells (DCs) (113). TLR activation in DCs causes an enhanced display of MHC peptide ligands for T cell recognition. TLR activation can also up-regulate co-stimulatory molecules that are important for T cell clonal expansion and the secretion of immunomodulatory cytokines to direct T cell differentiation into effectors (318). TLRs are able to regulate neutrophil migration, activation and apoptosis (335). TLR signals also regulate B-cell activation and survival (92). Due to their unique feature linking innate and adaptive immunity via DCs, considerable effort has been made to testing TLR ligands as novel vaccine adjuvants to enhance the efficacy of vaccination.

TLRs also play important roles in some diseases and are regarded as potential therapeutic targets. It was demonstrated that TLR4 plays a crucial role in the development of contact allergy to nickel (352). The application of a TLR4 antagonist in the treatment of severe sepsis has entered into clinical trial as well (398). Moreover, besides their roles in the immune system, TLRs in the intestinal epithelium affect

intestinal function (1), and TLRs are also able to modulate adult hippocampal neurogenesis (327). Activation of TLR3 is required for efficient nuclear reprogramming during the generation of induced pluripotent stem cells (iPSCs) (190).

For RLRs, other than its ability to initiate an innate response, RIG-I has been proposed to participate in a variety of intracellular events. ATRA-induced differentiation and normal myelopoiesis are accompanied by the induction of RIG-I (215, 236). Conversely, disruption of the RIG-I in mice leads to the development of a progressive myeloproliferative disorder (451). It has been suggested that RLRs also participate in cell differentiation, and RIG-I participates in TLR-stimulated phagocytosis (215). *In vivo* studies and work in clinical samples suggest that RIG-I may be involved in immune responses associated with both non-infectious and infectious diseases, such as atherosclerosis, psoriasis and rheumatoid arthritis (236).

Taken together, TLRs and RLRs play crucial roles both in immunity and non-immune events. As IFNs are the key consequence of TLR and RLR activation, IFNs induce the synthesis of proteins with antiviral activity. In the next chapter, IFN signaling via the JAK/STAT pathway will be reviewed in detail.

Chapter 3: Interferon Signaling and Function

Introduction

IFNs activate cells to establish an antiviral state that is characterized by the expression and antiviral activity of IFN-stimulated genes (ISGs) (163). More than 300 ISGs can be upregulated by IFNs (60). Those ISGs include antiviral effectors, such as ISG15 (an IFN-stimulated protein of 15 kDa), the GTPase MX1 (myxovirus resistance 1), ribonuclease L (RNaseL) and protein kinase R (PKR). The major signaling pathway results in ISG expression is JAK/STAT (Janus kinase and signal transducer and activator of transcription) pathway (346). There are other pathways involved in IFN-mediated signaling as well, such as the MAPK-p38 pathway and the phosphatidylinositol 3-kinase (PI3 kinase) pathway (296). In this chapter, the classical JAK/STAT pathway in type I IFN signaling and the effectors of IFNs for their antiviral functions are reviewed in detail.

Discovery of IFN-mediated signaling

The concept that individual cytokines or peptides bound to specific cell surface receptors cause the transcription of specific sets of genes was established 30 years after the initial definition of IFNs (196, 373). The study of IFNs started to focus on what kind of genes would be upregulated by IFNs. Early studies indeed demonstrated that IFN binding to distinct cell surface receptors leads to the synthesis of several unique polypeptides and their corresponding mRNAs (186). Due to technical limitations, less attention was paid to the function of those ISGs during that time. However, by analyzing the genomic 5'-flanking regions of the ISG54 and ISG15, an IFN-stimulated response element (ISRE) was identified (196). Using the ISRE as a probe to react with an IFN-

treated cell lysate, a cellular factor (named ISGF3) was identified as well (196). Subsequent studies revealed that ISGF3 consisted of four pre-existing proteins in a large complex with sizes of 48, 84, 91 and 113 kDa for each protein. Three of those proteins with sizes of 91, 84 and 113 kDa were suggested to come from the same protein family and are currently known as STAT1α, STAT1β and STAT2, respectively. The 48 kDa protein was later confirmed as IRF9, belonging to the interferon regulator factor (IRF) family (416). STAT1α and STAT1β have different sizes of 91 and 84 kDa due to alternative splicing of the same gene product (368). STAT1β, which lacks the 38 C-terminal residues, including the Ser727 site and most of the C-terminal transactivation domain (TAD), is believed to serve as a negative regulator (368). Since that time, more details about the STAT family have been revealed, and different signaling pathways for type I IFNs and type II IFN have been discovered. The IFN-γ specific promoter (GAS, gamma IFN-activated sequence), with a different sequence from the ISRE and another factor, only contains STAT1 bound to this promoter (GAF, gamma IFN activated factor) (57, 197). Further studies have demonstrated the involvement of a kinase in IFN-α induced ISGF3 formation and ISG expression, indicating a requirement for kinase activity in IFN signaling (316). Additionally, phosphopeptide mapping confirmed that STAT1 and STAT2 are phosphorylated at tyrosine (Y) 701 and 690, respectively, in response to IFN-α (346, 368). Collectively, this evidence finally led to the formulation of the concept of the JAK/STAT pathway. In the next part, the details of the JAK/STAT pathway will be discussed in detail.

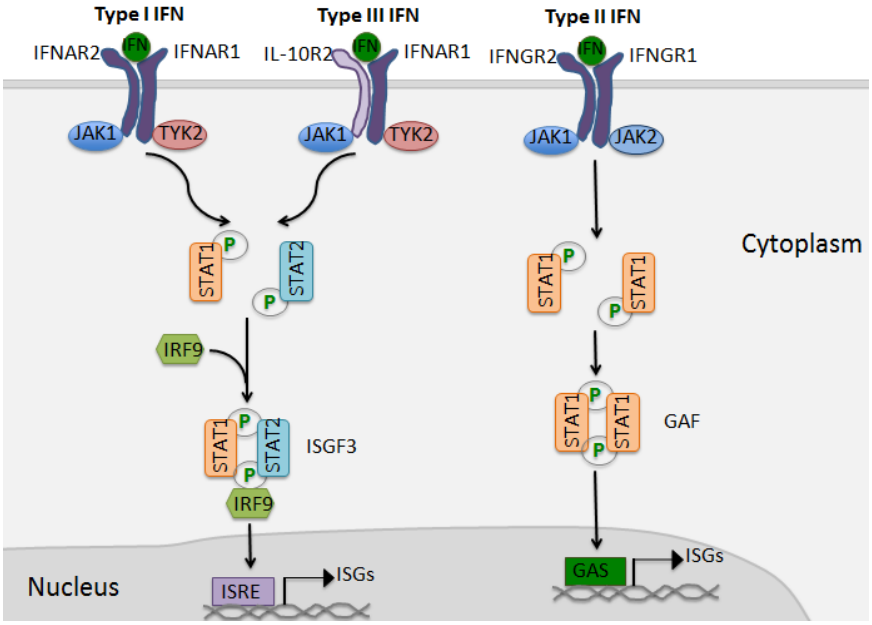

Fig. 3.1 Schematic illustration of IFN-mediated JAK/STAT activation

JAK/STAT pathway

The JAK/STAT pathway was first characterized in the type I interferon (IFN) response (54). Beyond IFN signaling, STAT proteins are critical for signal transmission of many different membrane receptors, such as cytokine and hormone receptors (195). The JAK/STAT pathway is conserved during eukaryotic evolution. In *Drosophila*, the JAK/STAT pathway is genetically well-characterized and has been shown to be important for larval hematopoiesis, sexual identity, embryo segmentation and the formation of polarity in the eye (447). JAK/STAT are the transducers of cytokine-mediated signaling in mammals after cytokines bind to specific cell-surface receptors (376).

There are four members in the JAK family: JAK1 to 3 and tyrosine kinase 2 (TYK2). They are all characterized by a C-terminal catalytic domain and a related, but enzymatically inactive, pseudo-kinase or kinase-like domain (271). They also share five additional domains of sequence similarity throughout the N-terminal region (195). All those seven domains are now called Janus homology domains (JHD) 1 to 7.

The STAT family contains seven members: STAT1, 2, 3, 4, 5A, 5B and 6. STAT1 contains two isoforms: STAT1α and STAT1β. All STATs except STAT1β share a very similar structure: an N-terminal domain (ND), a coiled-coil domain (CCD), a DNA-binding domain (DBD), a linker domain (LD), a Src homology 2 (SH2) domain, and a transactivation domain (TAD) (376). Generally, JAKs are non-covalently associated with the cytoplasmic tail of specific receptors. Upon cytokine binding, receptor dimerization or oligomerization leads to JAK apposition and autophosphorylation on tyrosine residues, releasing their intrinsic catalytic activity.

Tyrosine phosphorylation by activated JAKs of cytokine-receptor cytoplasmic domains then provides binding sites for SH2 domain of the STAT proteins. The STAT proteins are then recruited to the JAKs, whereupon they are phosphorylated on a tyrosine residue (around residue 700 of their 750–850 aa-long sequence) (195). Depending on the nature of the activating ligand, STAT/STAT interactions occur immediately through reciprocal SH2 interactions (126). All STAT proteins are able to form homodimers and heterodimers depending on the signaling pathway activated by different cytokines (195, 296).

STATs transduce signals for a large subfamily of cytokines due to the conserved receptor families to which JAKs bind. These cytokine families include the IFN family (IFN-α/β, IFN-γ; IL10, IL-19, IL-20, IL-22), the gp130 receptor family (IL-6, IL-11, OSM, LIF, CT-1, G-CSF, IL-12, IL-23, leptin, CTNF, NNT-1/BSF-3), the γC family (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) and the single chain family (Epo, GH, PRL, Tpo) (345, 347). STAT1 and STAT2 are the major players involved in type I IFN-mediated signaling. The activation of such STATs is a common response to different type I IFNs since the same receptor is utilized by those IFNs, thereby activating TYK2 and JAK1. Phosphorylated STAT1 and STAT2 form heterodimers, with the further association of IRF9 to form the ISGF3 complex. Other complexes induced by type I IFNs include STAT1-STAT1, STAT3-STAT3, STAT4-STAT4, STAT5-STAT5 and STAT6-STAT6 homodimers, as well as STAT1-STAT3, STAT1-STAT4, STAT1-STAT5, STAT2-STAT3 and STAT5-STAT6 heterodimers (296). On the other hand, type II IFN activates JAK1 and JAK2 and results in the phosphorylation of STAT1 on the tyrosine residue at Tyr701 as well, but this leads to the formation of STAT1-STAT1 homodimers as GAF,

which translocates to the nucleus and binds to GAS elements to initiate transcription (296). Karyopherin α1 (KPNA1) is the essential importin for the nuclear transport of phosphorylated STAT1 (358). However, the interaction of STAT1 and KPNA1 does not appear to be involved in the "classical nuclear localization signal (NLS)" recognition (358). Additionally, STAT1 possesses a non-classical NLS and KPNA1 binds between two STAT1 monomers, with two major binding determinants in the SH2 and DNA binding domains (266).

For the function of other STATs, STAT3 was initially identified as an IL-6 dependent transcription factor that promotes acute phase gene expression (9). It is now known to transduce signals for the entire IL-6 (IL-6, IL-11, IL-31, LIF, CNTF, CLC/CLF, NP, CT1, OSM) and IL-10 (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26) families, as well as granulocyte colony stimulating factor (G-CSF), leptin, IL-21 and IL-27 (173). Recently, STAT3 was implicated for its involvement in transformation. It has been demonstrated that STAT3 is constitutively active in some murine and human tumors, and is able to regulate the Src-dependent transformation of fibroblasts (37).

STAT4 was initially identified through screens for STAT homologues and found to share 52% identical amino acids to STAT1 (436). Unlike STAT1, neither IFN-α nor IFN-γ activate STAT4 (436). The expression of STAT4 was found to be limited to myeloid cells, NK cells, dendritic cells and T lymphocytes (436, 458). Subsequent studies determined that STAT4 is activated by IL-12, which plays a critical role in the development of the Th1 subset of T helper cells (21, 173).

STAT5A and STAT5B were found to be encoded by two linked genes, *STAT5a* and *STAT5b*, which shared 96% identity and only diverged at their carboxyl termini (173).

STAT5 was originally identified as mammary gland factor (MGF), which is the central mediator in the lactogenic hormone response in mammary epithelial cells (419). In addition to be a prolactin-induced transcription factor, STAT5 proteins are activated by the IL-3 family (IL-3, IL-5 and GM-CSF), the IL-2 family (IL-2, IL-7, TSLP, IL-9, IL-15 and IL-21), growth hormone (GH), Epo and Tpo (173). STAT5A and STAT5B display functional redundancy. Despite these structural and functional similarities, STAT5A single knockout mice are predominately defective in prolactin (RPL) dependent mammary gland development, while STAT5B single knockout mice exhibit defects similar to GH receptor deficient mice (173). STAT5 can also act as an oncogene and was found to be constitutively phosphorylated in cancer cells (267).

STAT6 was originally identified from cell extracts as the IL-4 stimulated STAT and it was soon shown to be also activated by IL-13 as well as to the common receptor chain shared by IL-4 and IL-13 (129, 210). As mediator for IL-4 and IL-13 signaling, STAT6 plays an important role in regulating acquired immunity since IL-4 is secreted by activated T and B lymphocytes, mast cells and basophils and promotes the activation of several cell types, most notably Th2 cells (173).

Functions of ISGs

The binding of type I IFNs to their receptors on the cell surface initiates the signaling cascade, which leads to the induction of more than 300 ISGs. Some of these ISGs can be upregulated by type II IFNs as well (55, 60). The functional diversity of ISGs indicates that IFNs play multiple roles in the host beyond their anti-viral or anti-tumor function. Evidence from microarray studies have also confirmed that the expression of ISGs is cell type-specific, ranging from 50-1000 genes with 200-500 genes

that are commonly expressed (355). This implies that IFNs can also have different functions in different cells. ISGs can be further classified into different categories according their functions. Depending on the category, ISGs are involved in many distinct pathways, such as amino acid and lipid metabolism, antigen processing and presentation, host cell signaling, transcriptional regulation, ubiquitination, apoptosis, host defense, immune modulation, inflammation and tumor suppression (55).

The ISGs can be divided into at least two large groups based on their major functions. The first group is proteins involved in host defense, including proteins directly possessing antiviral activity at the cellular level, as well as immunomodulatory genes including cytokines and chemokines, which recruit lymphocytes to sites of inflammation or infection (55). There are also genes in this group that promote lymphocyte adhesion to endothelial cells, such as ICAM1, SELL and CD47. Adhesion of lymphocytes to vessel walls is an important first step in the trafficking of lymphocytes to areas of infection (55).

The second large group of ISGs includes genes involved in host signaling. In this group, there are PRRs, such as TLRs and RLRs, which detect pathogens and initiate the signaling of the host innate response. Some adaptor proteins for TLRs that mediate signaling, such as MyD88, can be upregulated as well to enhance PRR signaling (189). There are also transcription factors, such as members of the IRF family, the STAT family and NF-κB, which form amplification loops, resulting in increased IFN production or ISG expression (55, 189, 336). Moreover, some proteins that are involved in other pathways can be upregulated as well, such as genes of ubiquitin activating E1 and conjugating E2, as well as IL1-β converting enzyme (189). IFNs have anti-proliferation

and tumor suppression functions. There are indeed some ISGs classified as proteins involved in apoptosis and tumor suppression (189).

Mechanisms of antiviral ISGs

Among all the ISGs, the most important ones for viral infection are those molecules possessing antiviral activity as the effectors of IFNs, such as ISG15, MX1, the 2'-5' oligoadenylate synthetase (OAS) family, RNaseL, PKR and ISG56 or the IFIT1 family. In this section, each of these ISGs will be discussed.

ISG15

ISG15 was identified soon after the discovery of ubiquitin, and was recognized as an ubiquitin homologue (219). As an ubiquitin-like molecule, ISG15 can be conjugated to protein substrates; this process is called ISGylation, and is reversible. However, ISGylation does not result in the degradation of the substrates as K48-linked ubiquitination, but instead parallels the activating effects of K63-linked ubiquitination (336). ISG15 has been reported to interfere with Ub-E2 and E3 interactions to inhibit protein ubiquitination (228), and can also prevent virus-mediated degradation of IRF3 to increase the induction of IFN-β expression (221). Besides, ISGylation has also been shown to modulate the function of enzymes. As an example, the conjugation of ISG15 to protein phosphatase 1B (PPM1B) suppresses its activity and enhances NF-κB signaling (392). Moreover, mice deficient in ISG15 show increased susceptibility to influenza A and B viruses, Sindbis virus and HSV-1 (194, 276).

MX1

In addition to components of the ISGylation pathway, IFNs induce the expression of several guanine-hydrolyzing proteins such as MX proteins. MX1 and MX2 were first identified as antiviral proteins by the observation that mutations within the MX locus on chromosome 16 confer susceptibility to orthomyxovirus infection in mice (19). Two human homologues, MXA and MXB, have been identified as well. MX proteins can target viral nucleocapsid-like structures (174).

The smooth endoplasmic reticulum localization of MXs allows them to screen exocytic events and mediate vesicle trafficking to trap essential viral components, and therefore, prevent viral replication at early time points (2). Furthermore, MX1 can associate with subunits of the influenza virus polymerase to block viral gene transcription (404).

OAS1 and RNaseL

The OAS protein family and RNaseL are another two important ISGs. The OAS family and RNase L are functionally related. The OAS proteins are unique in their capacity to synthesize 2' to 5' linked phosphodiester bonds to polymerize ATP into oligomers of adenosine (314). These unique 2',5'-oligomers specifically activate the latent form of RNaseL, which can then mediate RNA degradation (229). RNA degraded by RNaseL can then serve as the ligand for MDA5, resulting in enhanced induction of IFNs (229).

PKR

PKR is another important ISG that belongs to a small family of protein kinases responding to environmental stresses to regulate protein synthesis (324). PKR is constitutively expressed in all tissues at a basal level and is upregulated by IFNs. Under normal circumstances, PKR is maintained as an inactive monomer. This repression is released by activating ligands, such as viral RNAs, which elicit a conformational change and result in the activation of PKR. The active PKR enzyme consists of a homodimer with autophosphorylation at several key residues (336). Activation of PKR following dimerization leads to the phosphorylation of initiation factor eIF2α to halt translation.

ISG56

The ISG56/IFIT1 family is comprised of four members, ISG56/IFIT1, ISG54/IFIT2, ISG60/IFIT3 and ISG58/IFIT5, whose homologs are evolutionarily conserved (80). ISG56 expression is extraordinarily responsive to not only IFN treatment but also to certain viral and bacterial molecular patterns. ISG56 family members can inhibit translation by binding to specific subunits of eIF3, presenting a mechanism of inhibition distinct from PKR and OAS (80). Translation of the hepatitis C virus (HCV) positive-sense RNA genome is initiated by IRES-dependent ribosome recruitment, which is different from cap-dependent translation, and can be compromised in the presence of ISG56 (224, 421). For translation-unrelated inhibition, human ISG56 can directly bind the E1 helicase of human papillomavirus (HPV) to sequester the E1 helicase in the cytoplasm, separating it from the viral genome in the nucleus (396). Furthermore, ISG56 can also act as a negative feedback regulator to dampen virus-induced innate immune signaling (207). Recently, the crystal structure revealed that ISG54 binds specifically to

some RNAs, such as adenylate uridylate (AU)-rich RNAs, with or without a 5' PPP, suggesting a new mechanism underlying the antiviral activity of this ISG family (439).

Viperin

Viperin (also known as cig5 and RSAD2) is a highly conserved, 361-amino-acid protein with a molecular mass of 42 kDa (112). It was first identified as a human cytomegalovirus (HCMV)-inducible gene in fibroblasts that was renamed viperin (for virus inhibitory protein, endoplasmic reticulum (ER) associated, interferon inducible). Viperin was first shown to be antiviral against HCMV. Overexpression of viperin in human fibroblasts prior to HCMV infection was found to significantly decrease the expression of the late viral proteins such as gB, pp28 and pp651(45). Since then, viperin has been demonstrated to have antiviral activity against a broad range of viruses from both DNA and RNA viral families, such as CMV, RSV, DENV, influenza A and HIV (112). Viperin had been shown to inhibit influenza A and HIV-1 via blocking viral particle release (112). It also regulates cellular lipid metabolism during HCMV infection (362). However, the full details of the antiviral mechanisms of viperin remain unknown and need further investigation.

PML protein

PML (promyelocytic leukemia protein) protein is an ISG as well, containing both an IFN-stimulated response element and a gamma-activated site, meaning its expression is responsive to both type I IFNs and type II IFN, respectively (375). PML mediates PML-nuclear body (PML-NB) formation, generating electron-dense nuclear punctate areas within the intrachromosomal regions of the nucleus (46). The integrity of these structures has been correlated with the regulation of cell proliferation, and PML functions

as a tumor suppressor (340). In addition to these processes, PML-NBs have been implicated in multiple cellular responses, including apoptosis, DNA damage repair, the cellular stress response, transcriptional regulation, antiviral defense and post-translational modifications (411).

Viral interference of IFN-activated JAK/STAT signaling

As IFN signaling induces the expression of ISGs to inhibit virus replication, viruses employ different strategies to antagonize IFN-activated JAK/STAT signaling to facilitate their replication. Viruses can target different stages of the JAK/STAT pathway to inhibit signaling. All flaviviruses examined thus far, including the West Nile virus, Japanese encephalitis virus, Langat virus and Dengue virus (DENV), can suppress JAK/STAT signaling by inhibiting JAK phosphorylation (31, 102, 119, 211). This blocks downstream phosphorylation of STAT1 and STAT2. The hepatitis C virus proteins phosphatase 2A and NS5A disrupt STAT1 phosphorylation and suppress type I interferon signaling (184, 364). Rotavirus NSP1 inhibits interferon-mediated STAT1 phosphorylation as well (360).

Besides STAT1 phosphorylation, STAT degradation is also a common mechanism of viral IFN antagonism. The V protein of human parainfluenza virus 2 can lead to the proteolytic degradation of STAT1 and STAT2 (15, 281, 298, 299). The activity of DENV NS5 protein can also mediate STAT2 degradation (20). HCV also targets the interferon-α-activated JAK/STAT pathway by promoting proteasome-mediated degradation of STAT1 and STAT3 in immune cells and hepatocytes (377).

Nuclear translocation of STAT1 is another antagonizing target for viruses. VP24 of Ebola virus is known to antagonize interferon signaling by binding host KPNA1

proteins, thereby preventing them from transporting tyrosine-phosphorylated STAT1 to the nucleus (317). PRRSV nsp1β is able to induce ubiquitin-mediated degradation of KPNA1 to inhibit nuclear translocation of the ISGF3 complex (284, 424). The 3Cpro of FMDV contributes to the degradation of KPNA1 and thus blocks STAT1/STAT2 nuclear translocation as well (66).

Moreover, activation of the JAK/STAT pathway can be regulated by suppressors of cytokine signaling (SOCS) proteins (8), as SOCS proteins negatively regulate signaling pathways by facilitating ubiquitination and proteasome degradation of signaling molecules (8). However, viruses have also developed strategies to induce robust SOCS protein expression, which essentially "hijack" SOCS function to promote virus replication. These viruses include HIV-1, HBV, HCV, HSV1, RSV, Ebola virus, influenza A virus and coxsackievirus (8).

Summary

The IFNs are responsible for inducing expression of a large group of ISGs, which play roles in host resistance to viral infections and activate key components of the adaptive immune system, including antigen presentation, activation of T cells, B cells, and natural killer cells. IFNs activate signaling via an evolutionally conserved JAK/STAT pathway, which transduces signals from other cytokines as well. Though the induction and signaling of IFNs are well-defined and antagonism of many viruses are known, further studies are needed to address how viruses regulate IFN induction and signaling to facilitate their replication and pathogenesis *in vivo*. On the other hand, elucidation of ISG functions is also interesting as most ISGs are not well-defined, though they play important roles during the host defense. However, the combined roles of the

ISGs in antiviral responses make it difficult to tease apart the functions of individual ISGs. In the coming decade, we expect more data will be available to increase our understanding of the IFN pathway.

Chapter 4: Hepatitis E Virus Inhibits Type I Interferon Induction

ABSTRACT

HEV causes both endemic and epidemic human hepatitis by fecal-oral transmission in many parts of the world. Zoonotic transmission of HEV from animals to human has been reported. Due to the lack of an efficient cell culture system, the molecular mechanisms for HEV infection remain largely unknown. In this study, we found that HEV replication in hepatoma cells inhibited polyIC-induced IFN-β expression and that HEV ORF1 product was responsible for this inhibition. Two putative domains, X and the papain-like cysteine protease domain (PCP), from HEV ORF1 were identified as the IFN antagonists. When overexpressed in HEK293T cells, the X domain (or Macro domain) inhibited polyIC-induced phosphorylation of IRF3, which is the key transcription factor for IFN induction. The PCP domain was shown to have deubiquitinase activity for both RIG-I and TBK-1, whose ubiquitination is a key step in their activation in polyIC-induced IFN induction. Furthermore, replication of a HEV replicon containing GFP (E2-GFP) in hepatoma cells led to impaired phosphorylation of IRF3 and ubiquitination of RIG-I and TBK-1, which confirmed our observations of X and PCP inhibitory effects in HEK293T cells. Taken together, our study identified the IFN antagonists within the HEV ORF1 polyprotein and expanded our understanding of the function of several of the HEV ORF1 products as well as the mechanisms of HEV pathogenesis.

INTRODUCTION

Hepatitis E Virus is a fecal-oral transmitted viral pathogen, which causes acute hepatitis with a mortality rate at or below 3% in young adults and to up 30% in pregnant women in the third trimester (151, 171). While previously thought as a public health problem only for developing countries, hepatitis E has now been recognized frequently in industrialized countries (171). Isolation of HEV from the pig, chicken, mongoose, rabbit, rat, ferret, bat, fish and deer has been reported (105, 202, 246). Zoonotic transmission of HEV from animals to human has been documented (171), and is considered as a major transmission route for sporadic cases in the industrialized countries.

HEV contains a 7.2 kb single-stranded positive-sense RNA genome, which is capped and poly-adenylated (74). It has been classified as the sole member of the genus Hepevirus, family Hepeviridae (74, 151). There are four major genotypes and a single known serotype for HEV (105, 252). There are three ORFs in the HEV genome (393). ORF1 encodes a polyprotein that has all non-structural proteins needed for HEV replication. ORF2 encodes the capsid protein of HEV virion. ORF3 encodes a small multifunctional protein with a molecular weight of 13 kDa (vp13).

As an invader, HEV faces host innate immune responses, which are mainly induced by activation of host pattern recognition receptors. For recognition of RNA viruses, those receptors include RLRs and TLRs. Stimulation of the RLR and TLR signaling pathways leads to activation of transcription factors such as IRF3, IRF7 and NF-κB. These transcription factors mediate expression of type I IFNs and inflammatory cytokines, which not only lead to an antiviral state of the neighboring uninfected cells, but also serve as regulators to evoke adaptive immune response. Thus, viruses have

evolved many strategies to evade from host innate immune responses. Little is known about how HEV evades from host IFN induction. Microarray analysis from Hepatitis C virus (HCV) and HEV-infected chimpanzees showed that HEV evoked a lower magnitude of IFN response than HCV, indicating that HEV must employ an effective strategy to dampen host innate immune responses (443).

The objective of this study was to elucidate the mechanism of HEV interference with type I IFN induction. We found that HEV replication in S10-3 hepatoma cells inhibited IFN-β induction stimulated by polyIC, a dsRNA homologue. Further studies identified two putative domains (X and PCP) from ORF1 to be the IFN antagonists. The X domain (also known as Macro domain) inhibited polyIC-induced IRF3 phosphorylation while the PCP led to deubiquitination of both RIG-I and TBK-1. These findings were also confirmed in hepatoma cells with HEV replication. Our findings provide valuable information about function of the HEV ORF1 product and improve our understanding of HEV pathogenesis.

MATERIALS AND METHODS

Cells, transfection, viruses and chemicals. HEK293T and HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). S10-3 cells, a subclone of Huh-7 hepatoma cells (99), were maintained in DMEM-Reduced Serum (DMEM-RS) medium supplemented with 3% FBS. Transfection of HEK293T and HEK293 cells with plasmid DNA was performed by using FuGeneHD (Promega, Madison, WI), according to the instructions of the manufacturer.

Full-length RNAs of HEV and HEV-GFP were obtained by in vitro transcription from plasmids pSK-E2 and pSK-E2-GFP (71), respectively, using AmpliCap-Max T7 High Yield Message Maker Kit (Cellscript, Madison, WI). Transfection of S10-3 cells with RNA was performed using an optimized protocol with DMRIE-C reagent (Invitrogen, Grand Island, NY). Briefly, after S10-3 cells reaching 70% confluence in a 12-well plate, medium was discarded and the monolayer cells were washed twice with PBS pH7.4, followed by addition of 0.5 mL serum-free Opti-MEM to each well. For each well, 1 μg RNA was add to 50 μL Opti-MEM and mixed evenly with 4 μL DMRIE-C. After incubation under room temperature for 20 minutes, the mixture was added to the S10-3 cells. One mL DMEM-RS with 3% FBS was added to each well 5 h post transfection. The cells were cultured at 34.5^{o}C.

HEK293 cells stably expressing VenusC1-IRF3 (HEK293-IRF3) were established by transfection of the cells with VenusC1-IRF3 and selection under antibiotic G418. The surviving cells were cloned by limited dilution and cell sorting by flow cytometry.

Polyinosinic-polycytidylic acid (*polyIC*, LMW), a synthetic analog of double-stranded RNA (dsRNA) (Invivogen, San Diego, CA) was used to induce interferon production. The HEK293T and S10-3 cells were transfected with the polyIC at a concentration of 1 μg/ml and incubated for 12 h before harvested for further analysis.

Plasmids. Putative ORF1 domains were PCR amplified and cloned to vector pCAGEN (Addgene plasmid# 11160) with HA-tag at N-terminus as previously reported (235, 424). VenusC1-IRF3 was constructed in house by subcloning IRF3 from pFLAG-CMV-IRF3 (a gift from Dr. Michael Gale Jr.). Full length RIG-I cDNA from S10-3 cell RNA was cloned into KpnI site in pCMV-Flag-MAT-Tag-1 vector (Sigma). All primers used for plasmid construction in this chapter were listed in Table 4.1. All in house constructed plasmids were subjected to DNA sequencing to confirm the inserts.

HEV replicons: pSK-E2 and pSK-E2-GFP were described previously (71, 75, 97). Construction of Myc-RIG-I(N) (87), Myc-IPS1 (220), MDA5(N) (441), FLAG-TBK-1 (399), and FLAG-IKKε (293) plasmids were previously described.

Immunofluorescence assay (IFA) An immunofluorescence assay (IFA) was carried out as previously reported (154), by using chimpanzee antibody against the HEV capsid protein. Specific antibody-capsid reactions were detected by DyLight 549 goat anti-human immunoglobulin (Ig) G conjugate (Rockland Immunologicals, Gilbertsville, PA). The coverglass was mounted onto slide using SlowFade Gold antifade reagent containing 4'6'-diamidino-2-phenylinodole (DAPI) (Invitrogen) and observed using fluorescence microscopy.

Western blot analysis. Cells were lysed in the Laemmli sample buffer. The whole proteins in the lysate were analyzed by sodium dodecylsulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) and Western blot as previously described (154, 455). Antibodies against GFP (Santa Cruz Biotechnology, Santa Cruz, CA), phos-IRF3-S396 (Cell Signaling Technology, Danvers, MA), HA (Thermo Fisher Scientific, Waltham, MA), IRF3 (Santa Cruz) and tubulin (Sigma-Aldrich, St. Louis, MO) were used in the blotting. The chemiluminescence signal was recorded digitally using a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). Digital signal acquisition and densitometry analyses were conducted using the Quantity One Program, Version 4.6 (Bio-Rad).

Reverse transcription and real-time PCR (RT-qPCR). Total RNA was isolated from cells with TRIzol® Reagent (Invitrogen). RNase-free DNase was used to remove carryover DNA from the RNA isolation procedure. Reverse transcription using AMV reverse transcriptase was conducted along with oligo dT and random 15-mer. Real-time PCR with SYBR Green detection (Invitrogen) for IFN-β was done as described previously (285). Transcripts of RPL32 (ribosomal protein L32) were also detected from the same samples to serve as an internal control for normalization. Gene expression was quantified by $2^{-\Delta\Delta CT}$ method (218). Primers of IFN-β and RPL32 were described previously (265).

Ubiquitination Assay. Immunoprecipitation (IP) was conducted as previously described (284, 424) with modifications. Briefly, S10-3 and HEK293T cells were lysed with a lysis buffer (50mM Tris pH 7.4, 150 mM NaCl, 0.2 mM EDTA, 2 mM EGTA, 0.5% IGEPAL CA-630, 10% glycerol, 1 mM sodium vanadate) as described previously (424), with the supplement of a protease inhibitor cocktail (Sigma) as recommended by the manufacturer and N-Ethylmaleimide (NEM) (Sigma) at a final concentration of 50 μM.

The lysate was clarified by centrifugation at 14000 xg for 5 min at 4ºC. Antibodies against RIG-I or TBK-1 were added to the supernatant. IP with protein G agarose (KPL Inc, Gaithersburg, MD) was done following the manufacturer's instructions. The IP samples were subjected to immunoblot analysis with antibody against ubiquitin.

Reporter assay. HEK293T cells were transfected with the reporter pGL3.0-IFN-β promoter, and testing plasmids. Renilla vector pRL-TK (Promega) was also transfected for normalization. At 48 h after transfection, the cells were lysed for luciferase activity assay of Firefly and Renilla luciferases, respectively, by following manufacturer's instructions (Promega). Lysate of cells transfected with empty vector of testing plasmids was used as a control for calculation of IFN-β promoter activation level. Relative fold of luciferase activity is shown.

Statistical analysis. Differences in indicators between treatment samples, such as IFN-β mRNA level between the group in the presence of HEV replication and the control sample, were assessed by Student *t*-test. A two-tailed *P*-value of less than 0.05 was considered significant.

RESULTS

HEV replication inhibits IFN-β expression induced by polyIC transfection

We first tested whether HEV replication would interfere with interferon induction. S10-3 cells, a subclone of Huh-7 cells that is more susceptible to transfection with whole HEV genomic RNA (369), were used. HEV genomic RNA was transcribed from pSK-E2 plasmid containing whole cDNA of Sar55 (genotype I) HEV genome, as previously described (71). Full length HEV RNA generated from the pSKE2-GFP construct, in which insertion of GFP disrupts expression of ORFs 2 and 3 (71), was included for comparison. S10-3 cells were transfected with the two different HEV RNAs. HEV replication was detected by immunofluorescence assay with HEV antibody (Fig. 4.1A). The cells were transfected with polyIC 10 days post-HEV RNA transfection to stimulate IFN production. RT-qPCR was then conducted to detect IFN-β transcript. Compared with uninfected cells, S10-3 cells with HEV replication (from pSK-E2 RNA) had significantly lower level of IFN-β transcript by 3.2-fold (Fig. 4.1B), which indicates that HEV replication reduced IFN expression evoked by polyIC. Similarly, the cells transfected with RNA from pSKE2-GFP had significantly lower level of IFN-β transcript by 4.8-fold. This result suggests that ORF1 product was at least partially responsible for the inhibition as GFP insertion interrupts expression of both ORF2 and ORF3 in pSKE2-GFP.

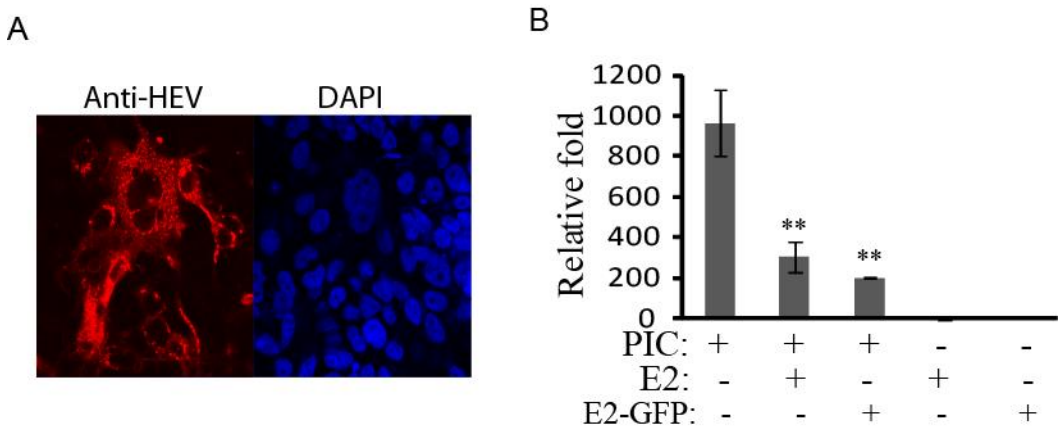

Fig. 4.1 HEV replication in S10-3 cells reduces IFN production induced by polyIC.

A. HEV replication in S10-3 cells detected by immunofluorescence assay. The cells were transfected with full length HEV genomic RNA from pSK-E2. Red fluorescence on the left panel indicates HEV ORF2 protein. Nuclear DNA shown on the right panel was counterstained using DPAI. B. Reduction of IFN-β expression in HEV-infected S-10 cells detected by real time RT-PCR. The cells were transfected with polyIC (PIC). Relative folds of IFN-β mRNA level in comparison to control cells without HEV RNA transfection are shown. Significant differences from HEV-negative group are denoted by "**", which indicate $P < 0.01$.

HEV ORF1 product inhibits IFN production

After finding that the inhibition of IFN induction was potentially due to the ORF1 product, we cloned 6 fragments of ORF1 into pCAGEN plasmid with HA tag at N terminus according to a previous analysis of the putative domains (179) (Fig. 4.2A). They were methyltransferase domain (Met); Y domain (Y); papain-like cysteine protease (PCP); a fragment covering hypervariable region, proline-rich domain and X-domain (HPX); helicase (Hel); and RNA-dependent RNA polymerase (RdRp). Protein expression of these HA-tagged fragments in HEK293T cells transfected with these plasmids was confirmed with Western blotting using HA antibody (Fig. 4.2B). All the proteins except HPX appeared to be one band. The HPX lane had two bands, indicating an excision of the product.

Next we examined their effects on IFN expression in HEK293T cells. Empty vector and PRRSV nsp1β, which is known to inhibit IFN induction (32), were included as controls. At 48 h after the transfection, the cells were transfected with polyIC to stimulate IFN induction. RT-qPCR was conducted to detect IFN-β mRNA level. Compared with the cells transfected with empty vector, PCP and HPX led to a significant reduction of IFN-β expression by 2.6 and 7.6-fold, respectively (Fig. 4. 2C). Likewise, PRRSV nsp1β led to 4.6-fold reduction. However the other fragments of ORF1 did not have an inhibitory effect on IFN-b induction, indicating that ORF1 PCP and HPX were IFN antagonists.

HEV X domain inhibits IFN induction via blocking phosphorylation of IRF3

After finding the two ORF1 products inhibit IFN induction, we selected HPX to determine its interference mechanism first. HEK293T cells were transfected with plasmids encoding the IFN-β reporter, HPX, and specific component of the RIG-I signaling pathway, specifically RIG-I(N-terminal domain), MDA5(N-terminal domain), TBK-1 or IKKε. Compared with cells transfected with empty vector, HPX expression resulted in a significant reduction of RIG-I and MDA5-induced IFN-β reporter expression by 1.8 and 2.2-fold, respectively (Fig. 4. 3A). Similarly, when the cells were transfected with TBK-1 or IKKε plasmid, HPX reduced luciferase yield significantly by 2.26 and 2.35-fold, respectively (Fig. 4. 3B). PRRSV nsp1β reduced luciferase yield of IFN-β reporter, as expected. These results indicated that HPX interfered with IFN induction via RLR pathway.

As HPX inhibits RLR pathway, we tested the activation status of IRF3 in cells expressing HPX. Since the phosphorylation level of endogenous IRF3 in HEK293T cells

after polyIC stimulation is hard to detect, we co-transfected the cells with FLAG-IRF3 and HPX plasmids. At 40 h after the transfection, the cells were transfected with

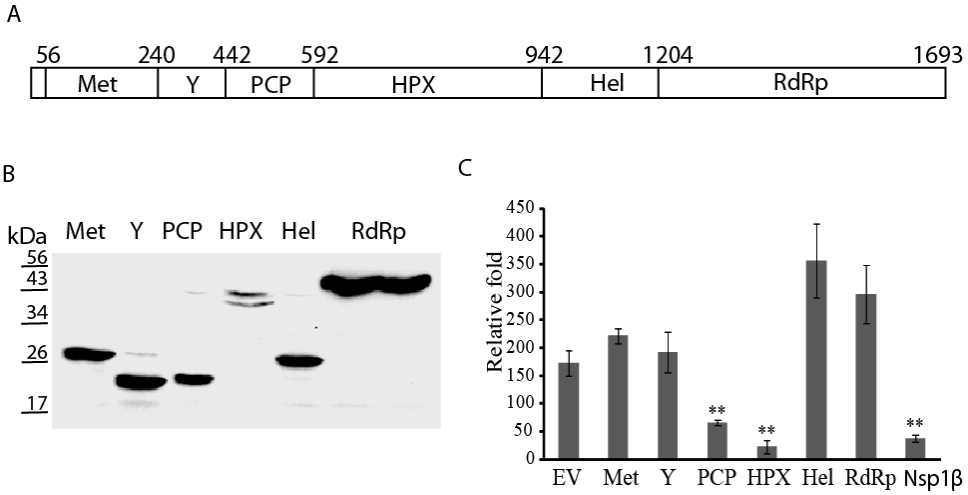

Fig. 4.2 Screening of ORF1 products for potential IFN antagonists.

A. Schematic illustration of ORF1 products. Met: Methyltransferase domain; Y: Y domain; PCP: papain-like cysteine protease; HPX: hypervariable region, proline-rich domain and X-domain; Hel: helicase; RdRp: RNA-dependent RNA polymerase. The numbers above the boxes indicate numbers of amino acids of ORF1 polyprotein. B. Cloning and expression of ORF1 fragments in HEK293T cells detected by Western blotting. The fragments were expressed as HA-tagged proteins. C. Inhibition of polyIC-induced interferon-β expression by ORF1 fragments in HEK293T cells detected by RT-qPCR. The cells were transfected with plasmids of the ORF1 fragments and then transfected with polyIC. At 12 h after polyIC treatment, the cells were harvested for RNA isolation and real time PCR to detect IFN-β transcript. PRRSV nsp1β was included as a control. Relative folds in comparison with untreated control cells are shown. EV: empty vector. Significant differences from EV control are denoted by "**", which indicate $P < 0.01$.

polyIC and harvested 8 h later for Western blotting. Compared with cells transfected with empty vector, the cells with HPX had much lower level of IRF3 phosphorylation (Fig. 4. 4A), while the levels of total IRF3 and tubulin were similar between the samples. The result indicated that HPX interfered with IRF3 activation.

The HPX regions contains 3 putative domains. To determine which domain was capable of antagonizing IRF3 activation, we cloned the three domains individually into pCAGEN expression vector, which encodes an HA tag at the N-terminus. When co-

expressed with FLAG-IRF3 in HEK293T cells, expression of the three small HPX domains was very low. However, unexpectedly, FLAG-IRF3 expression was interfered in the cells transfected with plasmids of HV and Pro domains, while endogenous IRF3

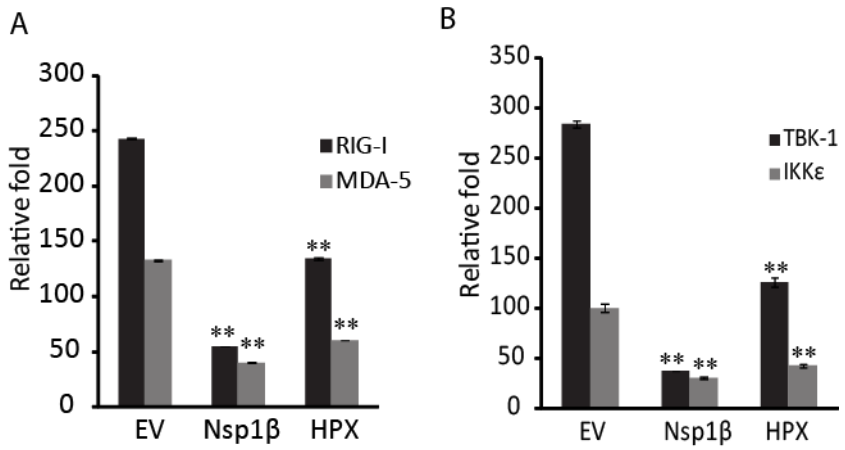

Fig. 4.3 Reporter assay showing ORF1 HPX product inhibits IFN-β induction

A. HPX product inhibits RIG-I or MDA5-activated IFN-β induction. HEK293T cells were transfected with IFN-β reporter plasmid, along with RIG-I(N) or MDA5(N), and nsp1β or HPX DNA. Firefly and renilla luciferase activities were measured at 48 h post transfection. PRRSV nsp1β was included as a positive control. EV: empty vector. Significant differences from EV control are denoted by "**", which indicate $P < 0.01$. B. HPX fragment inhibits TBK-1 or IKKε-activated IFN-β induction.

expression was not affected (data not shown). To solve the issue of plasmid interference in co-expression, we established HEK293 cells stably expressing VenusC1-IRF3, which makes it easy to observe IRF3 expression and select positive clones. Phosphorylation of VenusC1-IRF3 after polyIC stimulation in the stable cells in the presence of HV, Pro or X domain was examined by Western blotting. The X domain was identified to be the inhibitor for IRF3 phosphorylation, while HV and Pro domains had minimum effect (Fig. 4. 4B).

PCP domain deubiquitinates both RIG-I and TBK-1

During our screening for the IFN antagonists from ORF1 domains, the papain-like cysteine protease domain also inhibited polyIC-induced IFN expression. It was reported that HEV Met-PCP polyprotein had deubiquitination activity for ubiquitin, SUMO and ISG15-conjugated cellular proteins (157). We reasoned that PCP could potentially lead to deubiquitination of both RIG-I and TBK-1 as ubiquitination of these two molecules is essential for their activation (226). To test this, we co-transfected 293T cells with plasmids expressing PCP and RIG-I or TBK-1, since the levels of endogenous RIG-I and TBK-1 are too low to be detected in Western blotting. Samples were immunoprecipitated with antibodies to RIG-I or TBK-1 and the ubiquitination status assessed by Western blotting with ubiquitin antibody. Decreased ubiquitination level of both RIG-I and TBK-1 were observed in cells co-transfected with PCP cells (Fig. 4. 5A and 4. 5B), while the total RIG-I and TBK-1 levels were not affected in whole cell lysate. These results suggested that PCP may inhibit RIG-I and TBK-1 activation via its deubiquitinase activity.

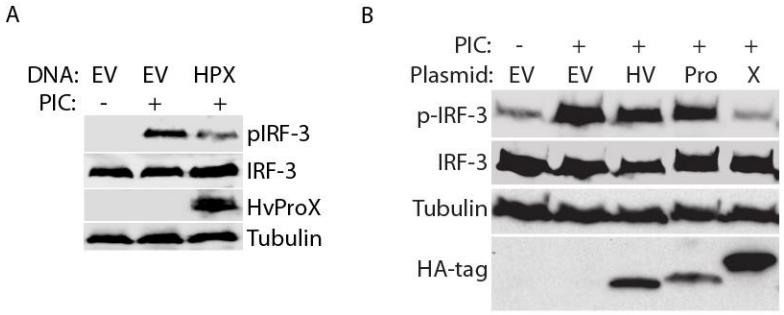

Fig. 4.4 Inhibition of IRF3 phosphorylation by X domain

A. Expression of HPX fragment inhibits IRF-3 phosphorylation. HEK293T cells were transfected with IRF-3 and empty vector (EV) or HPX plasmids, and then transfected with polyIC (PIC). At 8 h post-PIC treatment, the cells were harvested for detection of IRF-3 phosphorylation by Western blotting with antibody against phosphorylated IRF-3 (pIRF-3). Total IRF-3 and HPX expression were detected with IRF-3 and HA antibodies, respectively. B. Identification of X domain in inhibition of IRF3 phosphorylation. HEK293-VenusC1-IRF-3 stable cells were transfected with HV, Pro or X domain plasmids and Western blotting were done similarly as detailed in "A" caption.

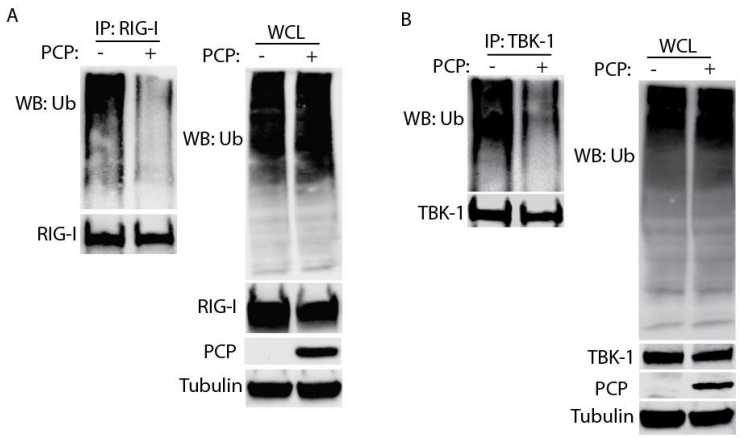

Fig. 4.5 Deubiquitination of RIG-I and TBK-1 by PCP domain

A. RIG-I deubiquitination by PCP. HEK293T cells were transfected with PCP and RIG-I plasmids, and then transfected with polyIC (PIC). At 8 h post-PIC treatment, the cells were harvested for immunoprecipitation with RIG-I antibody. Western blotting with antibodies against ubiquitin and RIG-I was conducted. Whole cell lysate (WCL) was used to detect ubiquitin, RIG-I, PCP and tubulin. B. TBK-1 deubiquitination by PCP. Experiment was done similarly as in "A" with the exception of TBK-1 plasmid used.

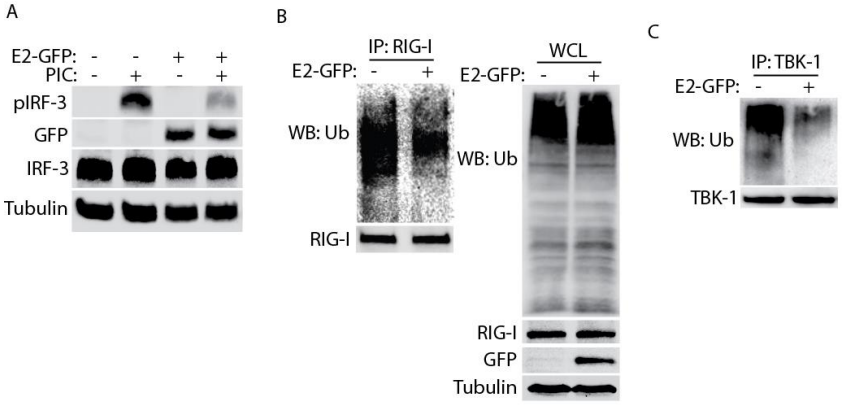

Fig. 4.6 HEV replication leads to downregulation of IRF3 phosphorylation and deubiquitination of RIG-I and TBK-1 in S10-3 cells.

A. Reduction of PIC-induced IRF3 phosphorylation in S10-3 cells transfected with HEV RNA from pSK-E2-GFP replicon. The cells were treated with PIC to induce IRF3 activation. At 8 h post-PIC treatment, the cells were harvested for detection of IRF3 phosphorylation by Western blotting with antibody against pIRF3. Total IRF-3, GFP and tubulin were detected too. B. Deubiquitination of RIG-I in S10-3 cells with HEV replication. At 8 h post-PIC treatment, the cells were harvested for immunoprecipitation with RIG-I antibody. Western blotting with antibodies against ubiquitin and RIG-I was conducted. Whole cell lysate (WCL) was used to detect ubiquitin, RIG-I, PCP and tubulin. C. Deubiquitination of TBK-1 in S10-3 cells with HEV replication. Ubiquitin level in WCL is the same as in "B".

HEV replication in hepatoma cells inhibits IRF3 phosphorylation and ubiquitination of RIG-I and TBK-1

The above results showed that overexpression of X and PCP domains from HEV ORF1 in HEK293T cells led to inhibition of IRF3 phosphorylation and RIG-I and TBK-1 ubiquitination, respectively. We reasoned that the HEV ORF1 product could perform the same functions during whole viral infection in hepatoma cells. To test this speculation, we employed HEV replicon pSK-E2-GFP. S10-3 cells that were transfected with RNA from E2-GFP and cultured for 10 days were stimulated with polyIC for 10 h. Compared to the cells without HEV RNA, the cells with E2-GFP replication had much lower polyIC-induced IRF3 phosphorylation, while the total IRF3 level had minimum change (Fig. 4. 6A). This result was consistent with the findings for X domain expression in HEK293 cells.

In the S10-3 cells transfected with E2-GFP RNA, ubiquitination levels of RIG-I and TBK-1 were also examined. IP results showed that both RIG-I and TBK-1 in S10-3 cells transfected with E2-GFP RNA had lower ubiquitination levels than the control without HEV replication (Fig. 4. 6B and 4.6C). Again the results were consistent with the observation of PCP overexpression in HEK293T cells. Endogenous RIG-I in S10-3 cells was also detectable, and no change in its expression was observed between control cells and E2-GFP RNA-transfected cells (Fig. 4. 6B). So was the total cellular ubiquitination level in the whole cell lysate. However, endogenous TBK-1 in whole cell lysate of S10-3 cells was below detection level in the Western blotting, though it was detectable after enriched by IP (Fig. 4. 6C). These results demonstrated that HEV replication inhibited

polyIC-induced IFN production by blocking phosphorylation of IRF3 and ubiquitination of RIG-I and TBK-1.

DISCUSSION

Type I IFNs, such as IFN-α and β, are critical to innate immunity against viral infection and contribute to the modulation of adaptive immunity (390). Viruses have developed a variety of strategies to subvert or evade the innate immune response. IRF3, a critical transcription factor, is frequently targeted by viruses to interfere with IFN induction. VP35 of Ebola virus inhibits IRF3 activation and this inhibition is related to the viral virulence (106). PRRSV nsp1β inhibits IRF3 activation (32). Npro of Classical swine fever virus induces proteasome-mediated degradation of IRF3 (27, 96). In this study, we demonstrated that HEV replication inhibited IFN production. Furthermore, the ORF1 product was responsible for this inhibition. Out of 8 putative domains in HEV ORF1 product, the PCP and X domains were demonstrated to inhibit RIG-I mediated signaling in different steps. PCP may mediate the deubiquitination of RIG-I and TBK-1, while the X domain may inhibit polyIC-induced IRF3 phosphorylation.

The X domain of HEV is known as a macro domain due to its homology with the C-terminal non-histone domain of histone macroH2A, and such domains have been identified in a variety of bacterial, archaeal, and eukaryotic organisms (reviewed in reference (103)). Studies of human macro domains indicate they possess a DNA binding motif, and are involved in DNA repair, chromatin remodeling and transcriptional regulation. Little information about viral macro domains is available. The coronavirus macro domains were shown to possess relative poor ADP-ribose 1"-phosphohydrolase activities, implying they are functionally different from their human homologues (69).

In our study, the HEV X domain was found to inhibit IRF3 phosphorylation, which appears to be a new function for a viral macro domain. However, we were unable to detect interaction between X domain with IRF3 or upstream kinase TBK-1 in IP experiments (data not shown). Thus, the mechanism that X domain inhibits IRF3 phosphorylation needs to be further elucidated. In addition, we also found that X domain from HEV could bind tightly with chromosomal DNA when overexpressed in HEK293T cells (data not shown). The human macro domains have a DNA binding motif and are involved in down-regulation of gene activation, suggesting that HEV X domain could potentially regulate host gene expression such as IFN-stimulated genes, which may further dampen IFN signaling. However, due to the lack of X antibody and an efficient cell culture system for HEV, we do not know whether X domain perform the function during HEV replication.

Another IFN antagonist we identified from HEV ORF1 is the PCP domain, which has moderate similarity to protease domain of Rubella virus (179). While the PCP domain encoded by Rubella virus has been shown to be responsible for the proteolytic processing of its non-structural protein (232), the role of the HEV PCP in proteolytically processing the ORF1 product into small subunits has yet to be confirmed. Recently, the connection of ubiquitination and activation of IFN induction pathway was defined (reviewed in reference (216)). Since cysteine proteases represent a large family of deubiquitinases (13), it implies that viral-encoded cysteine proteases could function as deubiquitinases to inhibit ubiquitination-dependent activation of host IFN induction pathways. Evidence from other viruses shows that viral coded cysteine proteases indeed possess deubiquitinase function and inhibit host innate immunity, such as arterivirus

papain-like protease 2 (415) and the leader proteinase of foot-and-mouth disease virus (422).

In a previous report, HEV Met-PCP polyprotein that contains the Met, Y and PCP domains was found to act as a deubiquitinase for ubiquitin, SOMO and ISG15-linked proteins in a cell-free system (158). However, the Met-PCP was unable to cleave a LXGG motif, which should be recognized by cellular or viral PCP. In this study, we demonstrate the PCP domain could act as the deubiquitinase for RIG-I and TBK-1 in HEK293T cells. In addition, our data indicate HEV replication in S10-3 cells could lead to deubiquitination of RIG-I and TBK-1. Since the PCP domain does not contain a Zn-binding finger, which is required for protease activity (117), our data suggests that the PCP deubiquitinase activity does not rely on its protease activity. Another study from the foot-and-mouth disease virus also indicated the deubiquitinase activity of viral leader protease does not rely on its proteolytic activity (422).

What is more, SARS virus processes a papain-like cysteine protease that is capable of cleaving ubiquitin and ISG15 conjugated proteins and helping virus evade from host innate immunity (213, 311). One study demonstrated that targeting the viral cysteine protease by virus-specific inhibitor blocks SARS virus replication while not affecting cellular deubiquitinases (310). It implies that deubiquitinase function of viral PCP could be a potential therapeutic target.

In conclusion, in this chapter, we identified PCP and X domains of HEV ORF1 polyprotein are the IFN antagonists and they inhibit RIG-I-mediated signaling in different steps, ubiquitination of RIG-I and TBK-1 and IRF3 phosphorylation, respectively.

Table 4.1 List of primers used in Chapter 4

Primer[a]	Sequences (5' to 3')[b]	Target gene
H1F1	GC*GAATTC*GAGGCCCATCAGTTTATCAAGG	Cloning of "Met"
H1R7	CC*CTCGAG*TTAAATCCAGGAGCGCAGGTTGG	
H1F3	GC*GAATTC*AGAACCACTAAGGTTACCGG	Cloning of "Y"
H1R6	CC*CTCGAG*TTACTGAGCGTAGAACTCCAAC	
H1F4	GC*GAATTC*TGTAGGCGCTGGCTCTCGGC	Cloning of "PCP"
H1R5	CC*CTCGAG*TTAGAGATTGTGGCGCTCTGG	
H1F5	GC*GAATTC*TCTTTTGATGCCAGTCAGAG	Cloning of "HPX"
H1R4	CC*CTCGAG*TTAGCCGGCACAGGCCCGGCCG	
H1F6	GC*GAATTC*TGTCGAGTCACCCCCGGCG	Cloning of "Hel"
H1R3	GC*GATATC*TTAACCAGCAAGGAAAAAGTTATTA	
H1F7	GC*GAATTC*GGCGAAATTGGCCACCAGCG	Cloning of "RdRp"
H1R2	GC*GATATC*TCATTCCACCCGACACAGAAT	
H1F10	CG*CTCGAG*CTTTTGATGCCAGTCAGAG	Cloning of "HV"
H1R16	GC*GAATTC*TAAACAGCATCAACCTCCGAC	
H1F9	GC*GAATTC*CCTAGTCCAGCCCAGCCCG	Cloning of "Pro"
H1R18	GC*GAATTC*TAGCGATGCCGGGCCGTCTGG	
H1F8	GC*GAATTC*CCGGATGGCTCTAAGGTG	Cloning of "X"
H1R13	CC*CTCGAG*TGCTGTCCGCGCAACATCC	
IRF3-F2	CG*GAATTC*GGGAACCCCAAAGCCACGGATC	Cloning of IRF3
IRF3-R2	CC*GGTACC*TCAGCTCTCCCCAGGGCCCTG	
RIG-I-F1	TTA*GGTACC*ATGACCACCGAGCAGCGAC	Cloning of RIG-I
RIG-I-F2	GGA*GGTACC*TCATTTGGACATTTCTGCTG	

a. F: forward primer, R: reverse primer. The "H1" before a primer name indicates the primer is based on sequences of HEV ORF1.

b. The italicized letters indicate restriction enzyme cleavage sites for cloning.

Chapter 5: Inhibition of Interferon-Activated Signaling by Hepatitis E Virus

ABSTRACT

Since the initial identification of HEV, lots of effort have been made to study on HEV virology and epidemiology. However, the molecular mechanisms for HEV infection remain largely unknown. As an invader, HEV faces host innate immune responses, which are mainly mediated by type I IFNs and inflammatory cytokines. Our earlier study indicates that HEV inhibits host IFN synthesis. Yet it was not known whether HEV is able to inhibit downstream IFN-activated JAK/STAT signaling pathway. In this study, we demonstrated that HEV replication in hepatoma cells significantly inhibits type I IFN signaling and downstream gene expression and that ORF1 product of HEV is responsible for this inhibition. Further analysis of the domains of ORF1 product indicates that methyltransferase (Met) and ProX domains are able to inhibit IFN-mediated signaling. The expression of Met domain appeared to have no effect on IFN-induced STAT1 phosphorylation. Immunofluoresce assay shows that majority of Met is located in the nucleus and the rest as speckles in the cytoplasm. Further analysis will be conducted to elucidate the mechanisms of Met and ProX domains in the inhibition of IFN-activated JAK/STAT signaling.

INTRODUCTION

HEV has been classified as the sole member of the genus *Hepevirus* (74). There are at least four major genotypes and a single known serotype for HEV strains (105, 252). There are three ORFs in the HEV genome (393). ORF1 encodes a non-structural polyprotein that is required for HEV replication. ORF2 encodes the capsid protein of HEV virion. ORF3 encodes a small multifunctional protein with a molecular weight of 13 kDa (vp13).

As an intruder for the host cell, HEV faces innate immune responses that are mainly induced by activation of host pattern recognition receptors. Activation of pattern recognition receptors results in production of type I IFNs and proinflammatory cytokines. Type I IFNs, such as IFN-α and β, activate cells via JAK/STAT pathway and are critical to innate immunity against viruses (390). Therefore, viruses evolve different strategies to evade from IFN-mediated innate immune responses. Hepatitis C virus phosphatase 2A and NS5A disrupts STAT1 phosphorylation and suppresses type I IFN signaling (184, 364). VP24 of Ebola virus antagonizes IFN signaling by binding host KPNA1 protein, thereby preventing them from transporting the tyrosine-phosphorylated STAT1 to the nucleus (317). The nps1β of PRRSV is able to induce ubiquitin-mediated degradation of KPNA1 to inhibit nuclear translocation of ISGF3 complex (284, 424). Dong et al also demonstrated that a type III HEV is able to inhibit IFN-α mediated signaling in A549 cells via blocking STAT1 phosphorylation by vp13(61). However, in our recent publication (also Chapter 6 of this thesis) which demonstrated vp13 from type I HEV can enhance IFN induction (264).We did not observe any inhibition of JAK/STAT pathway by vp13, because IFN bioassay in vp13-stable HeLa cells indicated an intact IFN

signaling. It is possible that there is strain or genotype difference for vp13 interaction with JAK/STAT pathway. Furthermore, it is unknown that what role the other HEV proteins play in JAK/STAT1 signaling pathway.

In this chapter, we found that HEV replication significantly reduced expression of ISGs after IFN treatment of hepatoma cells and that HEV ORF1 product is responsible for this inhibition. Further analysis indicates that methyltransferase domain and a fragment encoding the hypervariable (HV), proline rich (Pro) and X domains from ORF1 are able to inhibit IFN signaling. Our findings provide valuable information about function of the HEV ORF1 product and improve our understanding of HEV pathogenesis.

MATERIALS AND METHODS

Cells, transfection, viruses and chemicals. HEK293T cells, HEK293 cells and S10-3 cell were maintained in the same way as described in Chapter 4. Transfections of HEK293T, HEK293, and HEK293-ISRE cells with plasmid DNA were conducted in the same way as described in the Chapter 4. HEV RNA transfection of S10-3 cells was described in the Chapter 4.

HEK293 cells stably expressing pGL3.0-ISRE were established by transfection of the cells with pGL3.0-ISRE and selection under antibiotic G418, towards which the resistance gene was provided by VenusC1 empty vector. The surviving cells were cloned by limiting dilution and cell sorting by flow cytometry. Quantification of firefly luciferase activity was described in the Chapter 4.

Plasmids. Cloning of putative ORF1 domains to vector pCAGEN (Addgene plasmid# 11160) with HA-tag at N-terminus was described in the Chapter 4. *In vitro* RNA transcription of HEV replicon pSK-E2 and pSK-E2-GFP was described in the Chapter 4.

Western blot analysis. Cells were lysed in the Laemmli sample buffer for SDS-PAGE and Western blot as described in the Chapter 4. Antibodies against STAT1-Y701 (Santa Cruz Biotechnology), STAT2 (Santa Cruz Biotechnology), ISG56 (Thermo Fisher Scientific, Rockford, IL) and tubulin (Sigma-Aldrich, St. Louis, MO) were used in the blotting. The chemiluminescence signal was recorded digitally using a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). Digital signal acquisition and densitometry analyses were conducted using the Quantity One Program (Bio-Rad).

RESULTS

HEV replication inhibits ISGs expression induced by IFN-α

We first transfected the S10-3 heptoma cells with full-length HEV RNAs. Ten days after the tansfection, replication of HEV was detected by IFA using chimpanzee anti-HEV serum. HEV-GFP replicon was also included in the transfection, and GFP-positive cells were observed under flouresence microcsopy. To investigate whether HEV replication can inhibit IFN-mediated JAK/STAT activation, we treated S10-3 cells with human IFN-α2 at the concerntration of 500 U/mL. At 24 hours after the treatment, the cells was lyzed for SDS-PAGE and Western blot assay. STAT2 and ISG56 are two ISGs that can be upregulated by IFN treatment in a variety of cell lines such as MARC-145 and VERO cells (265, 284). In this experiment, compared with normal cells treated with the IFN, cells with HEV replication had significant lower levels of STAT2 and ISG56 proteins (Fig.5.1) , indicating an inhibition of the JAK/STAT signaling. Notablely, in the cells with HEV-GFP replicon transfection, expression of STAT2 and ISG56 were also inhibited as well. As insetion of GFP in the HEV replicon disrupts expression of HEV ORFs 2 and 3 (71), this result indictaes that the ORF1 product was the major player for the inhibition of IFN signaling in the hepatoma cells.

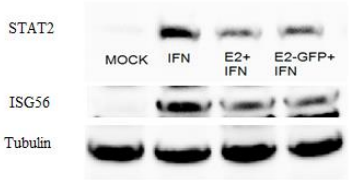

Fig. 5.1. Inhibition of ISG expression by HEV replication in S10-3 cells.

IFN-α2 treatment was conducted to stimulate JAK/STAT pathway in normal S10-3 cells, and cells with HEV replication. HEV-GFP replicon was included for comparison. At 12 hours after the IFN treatment, STAT2 and ISG56 levels in the S-10 cells were detected by Western blot.

Met domain and ProX domain of HEV ORF1 inhibit IFN signaling

After finding that the inhibition of IFN signaling was potentially due to the ORF1 product, we used plasmids constructed in the Chapter 4 that express 6 fragments of ORF1 polyprotein with HA tag for screening for the IFN antagonist. Our earlier experience with co-transfection of several plasmids suggests that some viral plasmids interfere with expression of the other plasmids. The interference leads to difficulty in normalization of the transfection efficiency in such a reporter assay. We also observed that endogenous expression of proteins is rarely affected by the transfection. To overcome such shortcoming of the co-transfection of multiple plasmids in the screening, we create a HEK293 cell line with stable expression of an ISRE reporter containing firefly luciferase driven by the ISRE promoter. The cells also express Veuns protein driven by a CMV promoter for easy selection during the cell cloning and later use. IFN treatment of the stable cells can lead to increased luciferase activity while fluorescence reading from the Venus protein can be used as an internal control for normalization. We transfected the HEK293 ISRE-stable cells with each of the plasmids and treated the cells with IFN at 500 U/mL 48h after the plasmid transfection. Based on luciferase activity, the Met domain and ProX domain demonstrated the strongest inhibition of IFN-stimulation of the ISRE promoter (Fig. 5.2). This result indicates the Met domain and ProX fragment of ORF1 are able to play the role as IFN antagonist in genotype I HEV.

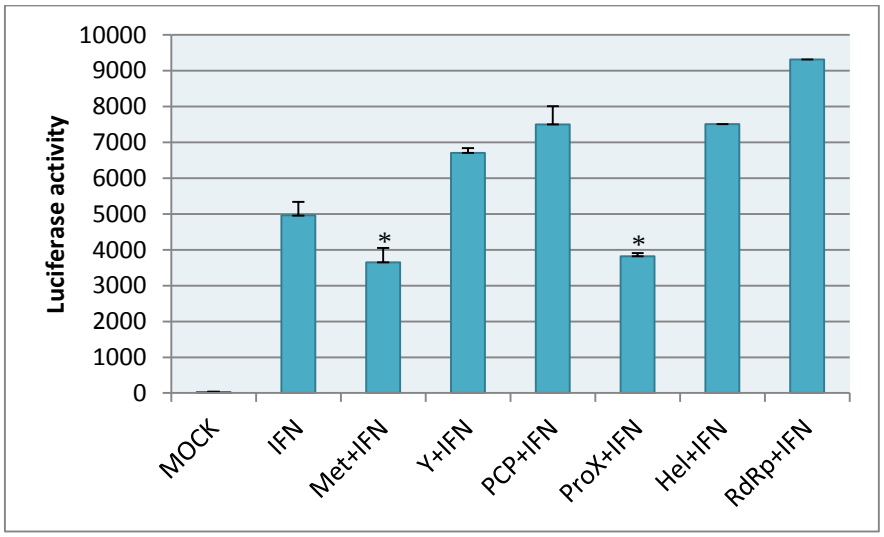

Fig. 5.2. Screening of ORF1 products for potential JAK/STAT antagonists.

HEK293 ISRE-stable cells were transfected with the plasmids of the ORF1 fragments and then treated with IFN-α2 at the concentration of 500 U/mL. At 24 h after the IFN treatment, the cells were harvested for luciferase activity assay. Significant differences from EV control (IFN) are denoted by "*", which indicates $P < 0.01$.

Met domain inhibits IFN signaling without affecting STAT1 phosphorylation

To further confirm our finding that HEV Met domain inhibits IFN-activated signaling, we test whether Met domain inhibit the endogenous ISG expression after IFN treatment. Just as expected, when Met domain was expressed in HEK293T cells, IFN-induced expression of ISG56 was dampened in comparison with normal cells (Fig.5.3A). This result is consistent with the ISRE reporter assay.

Activation of ISG expression needs ISGF3 complex binding with ISRE promoter in the nucleus. STAT1 phosphorylation is the key step for the ISGF3 complex formation. So we tested whether STAT1 phosphorylation was affected by the Met domain. HEK293T cells were transfected with Met plasmid and treated with IFN-α 48 h after the transfection. Western blotting revealed that Met domain had minimum effect on STAT1 phosphorylation (Fig.5.3B).

In addition, we conducted IFA to observe the sub-cellular localization of the Met domain by confocal microscopy. We noticed that majority of the Met protein was located in the nucleus, while the rest exists in cytoplasm as speckles (Fig.5.3C). Whether the nuclear or cytoplasmic localization of the Met domain relates to its inhibition of JAK/STAT pathway needs further investigation. A plausible speculation is that the nuclear Met might inhibit ISGF3 interaction with ISRE promoters because the Met domain potentially causes methylation of target nucleic acid sequences.

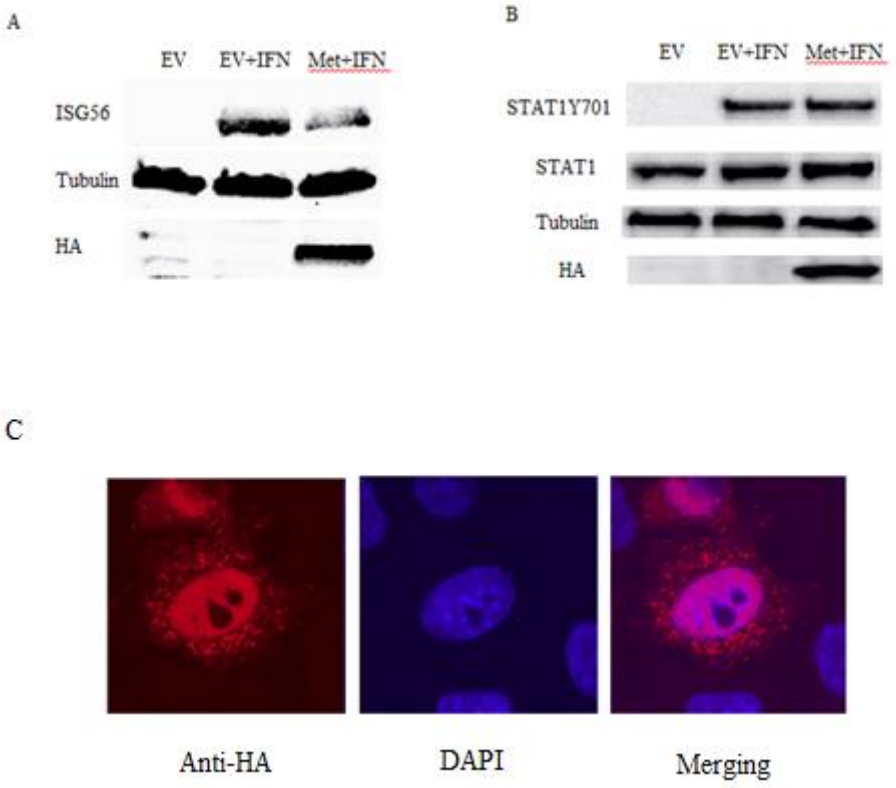

Fig. 5.3. Met domain inhibits ISG56 expression without affecting STAT1 phosphorylation.

A: Met domain inhibits ISG56 expression. HEK293T cells were transfected with pCAGEN empty vector (EV) or pCAGEN-HA-Met for 48 hours, then treated with IFN-α2 at 500 U/mL for 24h. Western blotting with ISG56, tubulin and HA was conducted. B: HEK293T cells were transfected with EV or Met domain for 48 hours, then treated with IFN-α2 at 500 U/mL for 1h. C: Met domain is mainly located in the cell nucleus. HeLa cells were transfected with HA-Met plasmid and, 24 h later, fixed and stained with anti-HA antibody for confocal microscopy. Nuclear DNA was counter stained with DAPI.

DISCUSSION

In the Chapter 4, HEV replication in hepatoma cells was found to inhibit the polyIC induction of type I IFNs. However, as IFNs need to activate the expression of ISGs in the cells via JAK/STAT pathway, many viruses develop varieties of strategies to block the pathway to dampen the host defense. In a previous study based in genotype III HEV, vp13, the product of HEV ORF3, was shown to bind with STAT1 and inhibit STAT1 phosphorylation, thus block IFN-α mediated signaling (61). However, our data indicate that the HEV-mediated inhibition of IFN signaling was mainly due to the ORF1 product as pSK-E2-GFP replicon had similar inhibition to the full length pSK-E2 replicon. The ORF 2 and 3 are truncated in the pSK-E2-GFP. This difference may be due to genotypic difference as our study used a genotype I HEV strain.

In our screening for IFN antagonist from ORF1 product, the Met domain was identified as a potential inhibitor in HEK 293 ISRE reporter cell line. Overexpression of Met domain in HEK 293T cells resulted in the inhibition of IFN-stimulated ISG56 expression. However, our data also showed that the Met domain does not inhibit the phosphorylation of STAT1, which is the key step for the JAK/STAT pathway. Thus, the inhibition step must be somewhere downstream of STAT1 phosphorylation: either STAT1 nuclear translocation or its interaction with ISRE promoters.

Confocal microscopy showed that majority HA-tagged Met domain has a nuclear localization. As the Met domain was proposed to cooperate with HEV helicase domain for catalyzing capping of HEV genomic RNA in the cytoplasm (160), it is intriguing that majority of Met-domain is located in the nucleus. It is possibly the Met in cytoplasm as speckles performs the proposed function. Furthermore, as no nuclear location sequence

(NLS) has been identified from Met domain, the mechanism for Met-domain's nuclear transportation is not known. It is possible that the Met domain possesses a non-classical NLS and competes with STAT1 for the KPNA1 for nuclear transportation since phosphorylated STAT1 possesses a non-classical NSL to bind to KPNA1 as well.

In addition to the Met domain, the ProX domain is able to inhibit the IFN-induced ISRE activation as well in HEK293-ISRE reporter cells. As ProX fragment codes for HV, Pro and X domain, further research is needed to identify the exact domain that is responsible for the JAK/STAT inhibition. Among these three domains, it seems that the X domain could be the potential inhibitor as it possesses a DNA binding domain and was confirmed to play a role in gene silencing and inactivation. On the other hand, our results also indicate that X domain is able to bind tightly with chromosome in HEK293T cell (data not shown). It is possible that this DNA binding of X domain plays some role to inhibit ISG expression.

Chapter 6: Enhancement of Interferon Induction by HEV ORF3 Product

ABSTRACT

The HEV ORF3 encodes a 13-kDa multifunctional protein (vp13), which is essential for HEV to establish an infection in animals. The exact role of vp13 in HEV infection remains unclear. In this chapter, vp13 was found to enhance interferon production induced by polyIC, a synthetic analog of dsRNA. PolyIC treatment induced higher level of IFN-β mRNA in HeLa cells stably expressing vp13 than control cells. Using a luciferase reporter construct driven by IFN-β promoter, we demonstrated that vp13 enhanced RIG-I-dependent luciferase expression. This enhancement was found to be because of both increased RIG-I protein level and its activation. The levels of both endogenous and exogenous RIG-I were increased by vp13 due to extension of RIG-I half-life. Additionally, vp13 interacts with RIG-I N-terminal domain and enhances its ubiquitination, which is essential for RIG-I activation. Analysis of vp13 deletion constructs suggested that C-terminal domain of vp13 was essential for the enhancement of RIG-I signaling. In HEV-infected hepatoma cells, wild type HEV led to a higher level of RIG-I and more polyIC-induced IFN-β expression than ORF3-null mutants. Analysis of vp13 from four HEV genotypes showed that vp13 from type I and type III strains boosted RIG-I signaling while vp13 from type II and IV strains had minimal effect. These results indicate that vp13 enhances RIG-I signaling, which may play a role to HEV invasion.

INTRODUCTION

HEV, a single-stranded positive-sense RNA virus, is the sole member of the genus *Hepevirus* in *Hepeviridae* family (70). The HEV genome is approximately 7.2 kb in length and consists of ORFs (393). HEV ORF3 encodes a phosphoprotein with a molecular mass of approximately 13 kDa (vp13) (393). A number of studies showed that vp13 plays roles in cellular signaling pathways (7) and interacts with microtubules (154). Moreover, vp13 is essential for establishment of HEV infection in macaques and pigs under experimental conditions (98, 134). ORF3-null mutants of HEV failed to establish a productive infection in Rhesus monkeys suggesting an essential role for vp13 *in vivo* (98). These data indicate that vp13 may play an important role in HEV virus-cell interactions. Yet the exact role of vp13 in HEV infection remains unknown. It is also not known whether vp13 has any effect on host innate immune responses.

Host PRRs for RNA viruses include RLR pathway and TLR pathway. TLRs that can detect viral RNA are TLR3, TLR7, and TLR8 (111). All TLRs except TLR3 signal through the adaptor molecule MyD88 (242). TLR3 signals solely via the adaptor TIR-domain-containing adaptor inducing IFN-β (TRIF) (120). The RLR family of PRRs is comprised of RIG-I and MDA5 (164). Both RIG-I and MDA5 signal through adaptor IPS-1 (also known as MAVS, Cardif, VISA) on the outer membrane of the mitochondria (169). Both RLR and TLR3 can recognize dsRNA of viral genome or the replication intermediates of RNA viruses. Activation of RLR and TLR signaling leads to activation of two IκB kinase (IKK)-related kinases, TBK1 and IKKε, which phosphorylate IRF3 and IRF7 (81, 365). These transcription factors translocate into the nucleus and result in

induction of type I IFNs, which not only leads to an antiviral state in the neighboring uninfected cells, but also serve as key regulators to evoke adaptive immune response.

In this study, vp13 was discovered to enhance interferon expression induced by polyIC, a synthetic analog of dsRNA. vp13 expression led to increased level of RIG-I via extension of half-life of the protein. Immunoprecipitation assay indicated that vp13 interacted with RIG-I N-terminal domain and increased its ubiquitination. In HEV-infected hepatoma cells, wild type HEV led to higher levels of RIG-I and increased expression of polyIC-induced IFN-β compared to ORF3-null HEV mutants. These results indicate that vp13 enhanced IFN induction via RIG-I signaling.

MATERIALS AND METHODS

Cells, viruses and replicons. HEK293T cells and S10-3 cell were maintained as same as Chapter 4. HeLa were maintained in DMEM-Reduced Serum (DMEM-RS) medium supplemented with 3% FBS. Transfection of HeLa, HEK293T, and S10-3 cells was described in Chapter 4. HeLa cells stably expressing vp13 were established by transfection of the cells with VenusN1-H3 or VenusC1-vp13 (154) and selecting for resistance to G418 (500 µg/ml). Cell cloning was done by limited dilution. PolyIC (Invivogen, San Diego, CA) was used to induce interferon production at a concentration of 10 µg/ml for direct addition to the cultured cells or at 1 µg/ml for transfection of the cells. Avirulent LaSota Newcastle disease virus with the inserted gene of green fluorescence protein (NDV-GFP) was used as an indicator of polyIC-induced interferon production, as previously described (265).

Obtaining of Full-length RNAs of HEV, HEV-ORF3-null, HEV-GFP and HEV-luciferase by in vitro transcription from replicon plasmids pSK-E2, pSK-E2ΔORF3, pSK-E2-GFP and pE2-Luc (71, 75, 97) were described in Chapter 4. Transfection of S10-3 cells with RNAs was described in Chapter 4 as well.

Plasmids. HEV ORF3 plasmids, VenusN1-vp13 and VenusC1-vp13 used in this study were reported previously (154). HEV replicons, pSK-E2, pSK-E2-GFP, pE2-Luc and ORF3-null pSK-E2 were described previously (71, 75, 97, 98). ORF3 sequences from strains of HEV genotype II (GenBank accession# M74506) and IV (accession# AB074915) were synthesized (Genscript, Piscataway, NJ) and cloned to *Xho*I and *Eco*RI sites in the VenusC1 vector as described (154). ORF3 of HEV genotype III isolate Kernow (369) was similarly cloned into VenusC1 vectors with primers listed in Table

6.1. ORF3 truncation mutants were constructed using VenusC1 vector with primers listed in Table 6.1.

Full length RIG-I was cloned into *Kpn*I site in pCMV-Flag-MAT-1 vector. Construction of Myc-RIG-I(N) (191), MDA5(N) (441), FLAG-TBK1and FLAG-IKKε (81) plasmids were described previously. The pCDNA3-TRIF-CFP (82) and pRK5-HA-Ubiquitin-K63 (209) were obtained from Addgene.

Immunofluorescence assay (IFA) and live cell fluorescence microscopy. An immunofluorescence assay (IFA) was carried out as described previously in Chapter 4 using chimpanzee antibody against HEV. GFP expression in live cells transfected with RNA from HEV replicon containing GFP gene was similarly observed.

Western blot analysis. Whole cell lysates were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously (154, 455). Antibodies against GFP (Santa Cruz Biotechnology, Santa Cruz, CA), FLAG (Sigma, St. Louis, MO), HA (Thermo Fisher Scientific, Waltham, MA), Myc (Rockland Immunochemicals, Inc., Gilbertsville, PA), vp13, RIG-I (Santa Cruz) and tubulin (Sigma) were used in the blotting. The Recording and quantification of chemiluminescence signal were described in Chapter 4. To determine RIG-I half-life, cycloheximide (Sigma) was used at a final concentration of 100 µg/ml to inhibit protein translation. Cell lysate samples harvested at indicated time points after cycloheximide treatment were subjected to Western blotting.

Reverse transcription and real-time PCR (RT-qPCR). Total RNA RNA isolation, DNase treatment and Reverse transcription was conducted as same as described in Chapter 4. The primers for detection of IFN-β (265) and RIG-I (188) cDNA were

described previously. Real-time PCR with SYBR Green, detection for transcripts of house-keeping gene RPL32 and quantification of gene expression was conducted as same as Chapter 4.

Immunoprecipitation (IP). IP was conducted as same as Chapter 4. Antibodies against Myc or GFP were added to the supernatant. IP with protein G agarose (KPL Inc, Gaithersburg, MD) was done following the manufacturer's instructions. The samples of IP with Myc antibody were subjected to Western blotting with vp13 antibody. The samples of IP with GFP antibody were subjected to Western blotting with RIG-I antibody.

For detection of ubiquitinated RIG-I, N-Ethylmaleimide (NEM) (Thermo Scientific, Rockford, IL) was included in the lysis buffer at a final concentration of 50 μM. After lysate clarification, the supernatant was moved to a fresh tube and SDS was added to a final concentration of 1%. The supernatant was then boiled for 5 min and cooled down on ice for IP with Myc antibody. The IP samples were subjected to Western blotting with ubiquitin antibody.

Luciferase reporter assay. Transfection of HEK293T cells with firefly luciferase reporter plasmid of IFN-β promoter, the indicated plasmids and Renilla luciferase vector pGL4.74 hRL-TK (Promega) was described in Chapter 4. VenusC1 Empty vectors of testing plasmids were included as control. Measurement of Firefly and Renilla luciferase assays were described in Chapter 4. Firefly luciferase levels were normalized with renilla expression. Values representing the fold change in luciferase activity compared to control cells are shown.

Statistical analysis. Statistical analysis between treatment samples, such as IFN-β mRNA level between the groups was conducted the same way as Chapter 4.

RESULTS

vp13 enhances polyIC-induced interferon production

In our studies of vp13, we established HeLa cells stably expressing vp13 with the plasmids encoding vp13 fused to the N- or C-terminal end of GFP, VenusN1-vp13 or VenuC1-vp13, respectively (154). HeLa-VenusN1-vp13 was first used in an assay to assess the effect of vp13 on polyIC-induction of type I interferons. NDV is sensitive to type I IFNs and was used as an indicator of IFN induction. HeLa-VenusN1-vp13 cells were inoculated with NDV-GFP 12 h post treatment with polyIC and monitored for the expression of GFP. The cell line had a low level VenusN1-vp13 expression that did not interfere with observation of NDV-GFP replication. The HeLa-VenusN1-vp13 stable cells were expected to have more or similar number of GFP-positive cells than the control cells, if vp13 had inhibitory or no effect on IFN induction. However, substantially fewer GFP-positive cells were etected in the cells with vp13 expression compared to control cells (Fig. 6. 1A). The vp13 expression in cells without polyIC did not affect NDV-GFP replication. The result suggested that vp13 enhanced the polyIC-induced IFN production.

To confirm this observation, we examined the IFN-β transcript levels by RT-qPCR. HeLa, HeLa-VenusN1-vp13, and HeLa-VenusC1-vp13 stable cells were treated with polyIC for 12 h and harvested for RNA isolation. The direct addition of polyIC to the cells was supposed to activate the TLR3 signaling pathway to induce IFN production. Both HeLa-VenusN1-vp13 and HeLa-VenusC1-vp13 cells had 5.6 and 10.2-fold more IFN-β mRNA compared to normal HeLa cells after polyIC direct treatment (Fig. 6. 1B). Because polyIC treatment induced higher levels of IFN-β mRNA in HeLa-VenusC1-vp13 cells compared to HeLa-VenusN1-vp13, HeLa-VenusC1-vp13 cells were utilized in the

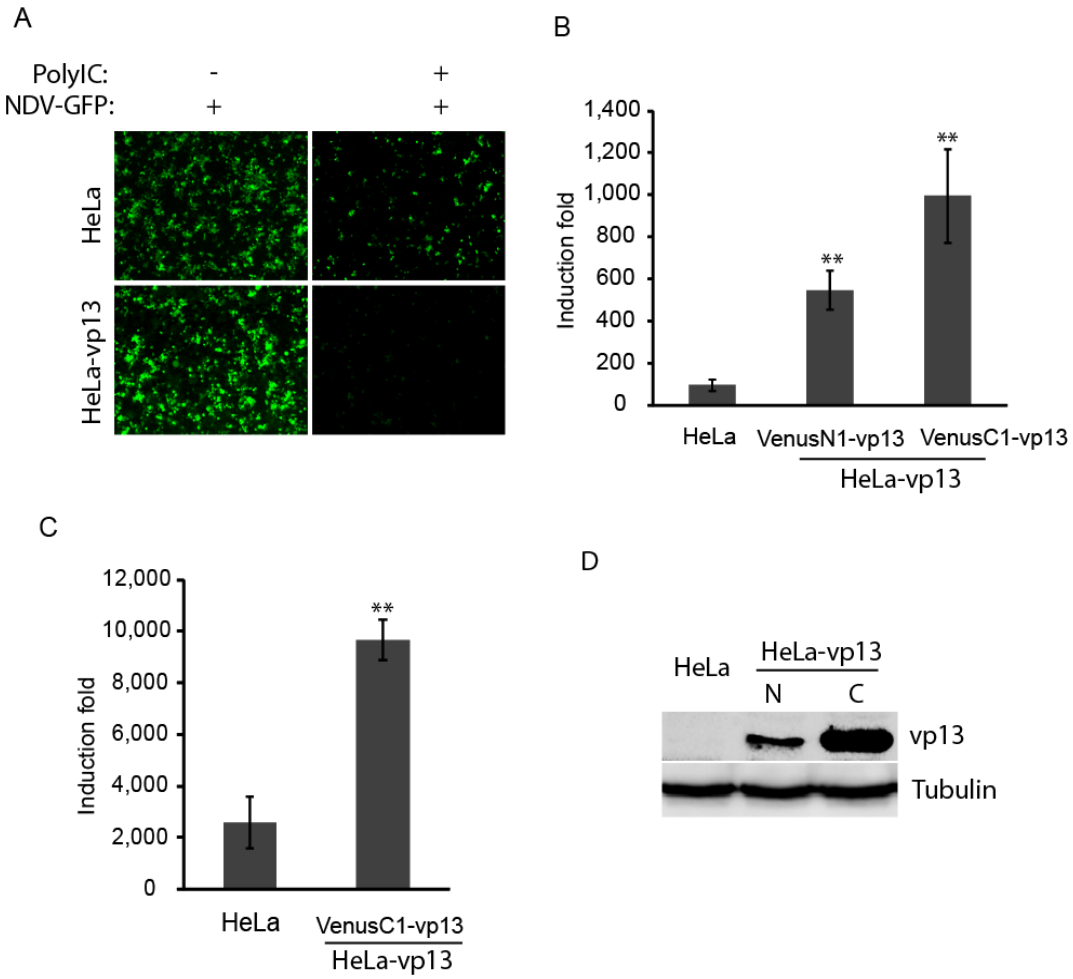

Fig. 6.1 Enhancement of polyIC-induced interferon production by vp13.

A. NDV-GFP replication reduced in HeLa cells stably expressing vp13 (VenusN1-vp13). The cells were treated with polyIC for 12 h and then inoculated with NDV-GFP. The cells were observed under fluorescence microscopy 24 h after NDV inoculation. **B**. Increase of polyIC-induced IFN-β mRNA in HeLa cells with vp13 expression detected by RT-qPCR. The HeLa cells stably express with VenusN1-vp13 or VenusC1-vp13 and normal HeLa cells were treated with polyIC, which was directly added to the cultured cells. Induction folds of IFN-β mRNA level in comparison to non-treated control cells are shown. Error bars represent standard errors of three repeated experiments. Significant differences from control HeLa cells are shown by "**", which indicates $P < 0.01$. **C**. Increase of polyIC-induced IFN-β mRNA in HeLa cells with vp13 expression. The HeLa cells stably express with VenusC1-vp13 and normal HeLa cells were transfected with polyIC. Relative induction folds of IFN-β mRNA levels are shown. **D**. Detection of Venus-vp13 fusion protein in HeLa-vp13 stable cells. N: VenusN1-vp13; C: VenusC1-vp13. The Western blotting was done with anti-vp13 antibody.

following experiments. Similarly, VenusC1-vp13 plasmid was used for further analysis of the vp13 effect on IFN signaling. To test if vp13 was also able to enhance polyIC-activated RLR pathway, we transfected the cells with polyIC. IFN-β mRNA level in the HeLa-VenusC1-vp13 cells was 3.7-fold higher than HeLa cells after polyIC transfection (Fig. 6. 1C). These results indicated that vp13 enhanced polyIC-induced IFN production in HeLa cells.

The vp13 expression in the stable HeLa cells was detected with anti-vp13 antibody in Western blotting (Fig. 6. 1D). The stable cells with VenusC1-vp13 had higher level of vp13 than the cells with VenusN1-vp13, which may be possibly why the polyIC induced higher level of IFN-β mRNA in the former cells.

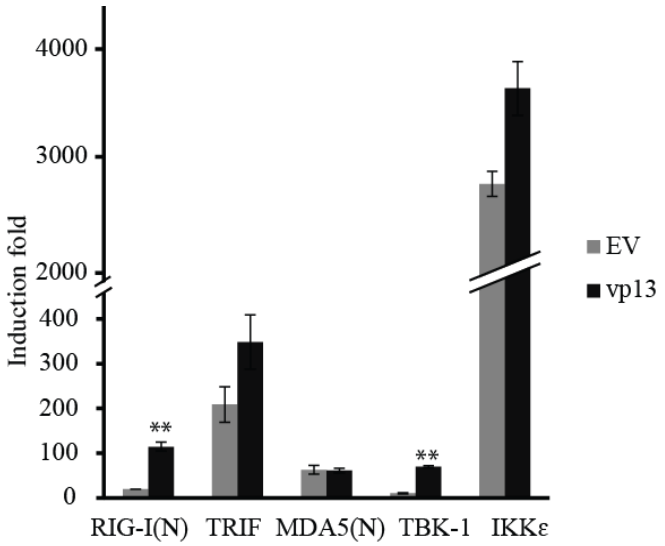

Fig. 6.2 vp13 enhances RIG-I-induced IFN-β expression.

HEK293T cells were co-transfected with IFN-β promoter luciferase reporter, vp13 and one of the following stimulator molecules: RIG-I(N), TRIF, MDA5(N), TBK-1 or IKKε. Empty vector (EV) of vp13 plasmid was included as a control. At 24 h after transfection, the cells were harvested for luciferase activity assay. Relative folds of the luciferase activity are shown. Significant differences in IFN-β promoter activation between vp13 and EV are denoted by "**", which indicates $P < 0.01$.

vp13 enhances RIG-I-induced IFN-β expression in HEK293T cells.

Overexpression of signal molecules from TLR3 and RLR pathways such as RIG-I, MDA5, TRIF, TBK1 and IKKε lead to the activation of IFN-β promoter (32, 88, 223). To find out which signal molecule in the IFN induction pathway was affected by vp13, we examined induction of the IFN-β promoter using a luciferase reporter assay. HEK293T cells were transfected with RIG-I(N), MDA5(N), TRIF, TBK1 or IKKε along with vp13 or empty vector control. Among the molecules tested, IKKε overexpression induced the highest luciferase yield to 2808-fold and TBK1 led to the lowest induction to 10-fold. Compared with empty vector control, the presence of vp13 significantly increased luciferase expression in cells transfected with N-terminal RIG-I or TBK1 by 5.94 and

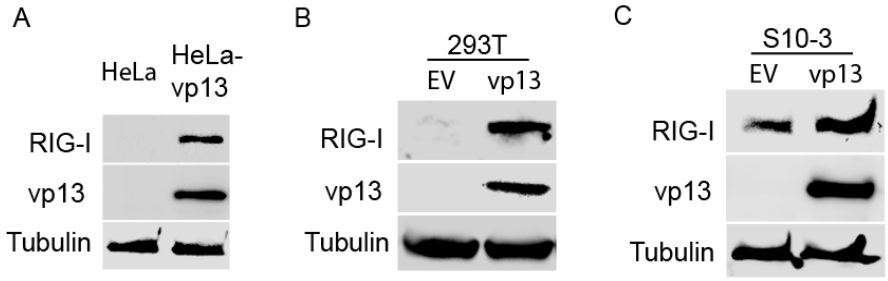

Fig. 6.3 vp13 induces elevation of endogenous RIG-I protein.

Western blotting with antibodies against RIG-I, vp13 and tubulin was conducted. **A**. RIG-I protein level is highly elevated in HeLa-vp13 stable cells. Lysate of normal HeLa cells was included as a control. EV: empty vector. **B**. RIG-I protein level is increased in HEK293T cells transiently transfected with vp13 plasmid. The cells were harvested at 48 h after the transfection. **C**. RIG-I protein level is increased in S10-3 cells transiently transfected with vp13 plasmid. The cells were harvested at 48 h after the transfection.

6.82-fold, respectively (Fig. 6. 2). Co-transfection of vp13 with the other signal molecules did not lead to a significant change in luciferase yield. This result suggested

that vp13 may function at multiple steps within the RIG-I signaling to enhance IFN promoter activation.

vp13 induces elevation of endogenous RIG-I level

To determine the mechanism that vp13 enhances RIG-I-mediated IFN induction, we first tested whether vp13 affected RIG-I expression. While RIG-I was below detection level in control HeLa cells, the endogenous RIG-I was detected in immunoblot analysis in HeLa-VenusC1-vp13 stable cells (Fig. 6. 3A). Likewise, transient expression of vp13 in HEK293T cells increased endogenous RIG-I expression to a detectable level (Fig. 6. 3B). Similar results were detected in S10-3 cells, a cell line capable of supporting HEV replication. The S10-3 cells transiently transfected with VenusC1-vp13 plasmid had considerably higher RIG-I level compared to the cells transfected with an empty vector (Fig. 6. 3C). These results indicated that vp13 expression led to an elevation in the basal level of RIG-I, which was likely one of the reasons for the enhancement of RIG-I-mediated IFN-β induction.

vp13 expression extends the half-life of RIG-I

The increased level of RIG-I in cells with vp13 expression could be due to either higher level of transcription and/or translation or extension of the protein half-life. To distinguish between these possibilities, HEK293T cells were transfected with VenusC1-vp13 or empty vector and RIG-I mRNA levels were assessed by RT-qPCR. Similar levels of RIG-I transcript were detected in the presence or absence of vp13 expression (Fig. 6. 4A), which indicated that the elevation in RIG-I was not due to changes in mRNA levels.

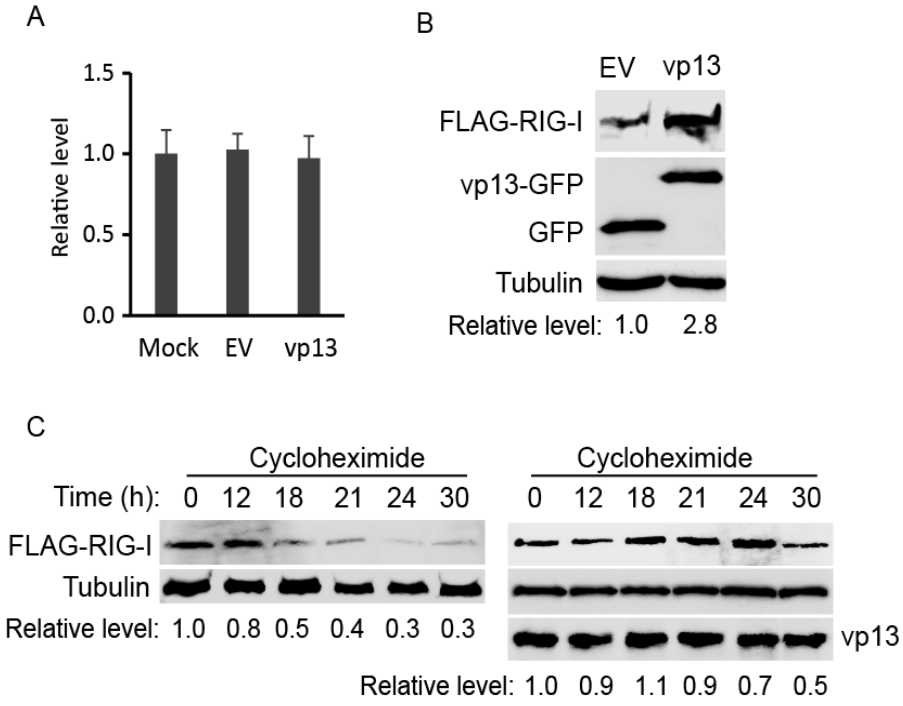

Fig. 6.4 vp13 extends RIG-I half-life in HEK293T cells.

A. RIG-I mRNA level remains stable in cells with vp13 expression. HEK293T cells were transfected with empty vector or vp13 plasmid and harvested 24 h post-transfection. RT-qPCR was performed to detect RIG-I transcripts. EV: empty vector. **B**. vp13 induces elevated expression of exogenous RIG-I. HEK293T cells were transfected with RIG-I and vp13 plasmids. Western blotting with antibodies against FLAG, GFP and tubulin was conducted. **C**. vp13 extends half-life of RIG-I. HEK293T cells were transfected with FLAG-RIG-I and vp13 (right panel) or empty vector (left panel). The cells were treated with cycloheximide 24 h after transfection and harvested at time points indicated above the images. Western blotting with antibodies against FLAG, tubulin and vp13 was conducted.

We next assessed the effect of vp13 expression on translation of RIG-I. HEK293T cells were transfected with FLAG-RIG-I plasmid since endogenous RIG-I is barely detectable by Western blotting. Exogenous RIG-I protein in the cells with vp13 expression was 2.8-fold higher than the cells transfected with an empty vector (Fig. 6.

4B). These results suggest that vp13 is acting at the protein level. We next examined the effect of vp13 on the protein stability of RIG-I. HEK293T cells were transfected with RIG-I and vp13 or an empty vector and treated with cycloheximide to block protein synthesis. The assay showed that RIG-I levels decreased 0.5-fold after 18 h of cycloheximide treatment in cells transfected with empty vector but after 30 h treatment in cells with vp13 expression (Fig. 6. 4C). The RIG-I half-life was extended from 18 h in the cells with empty vector to 30 h in the cells with vp13 expression, indicating that vp13 extended the half-life of RIG-I.

vp13 interacts with the RIG-I N-terminal domain and increases its ubiquitination

RIG-I is composed of two N-terminal caspase recruitment domains (CARDs), a central DexD/H box helicase/ATPase domain and a C-terminal regulatory domain (CTD) (442). N-terminal CARDs are responsible for binding to the adaptor molecule IPS-1 on mitochondria. In resting cells, RIG-I CTD represses the interaction between CARDs of RIG-I and IPS-1 (339). As N-terminal CARDs of RIG-I interact with IPS-1 in RLR-pathway and ubiquitination leads to RIG-I activation , we tested whether vp13 would interact with RIG-I(N) or alter the ubiquitination status of RIG-I N-terminal CARD domains. First we determined if vp13 increased RIG-I(N) expression in a similar way to the full-length RIG-I protein. HEK293T cells were co-transfected with Myc-RIG-I(N) and vp13 plasmids. Western blotting result showed that the cells expressing vp13 had similar level of RIG-I(N) to the cells with empty vector (Fig. 6. 5A), indicating that vp13 did not affect RIG-I(N) expression or stability.

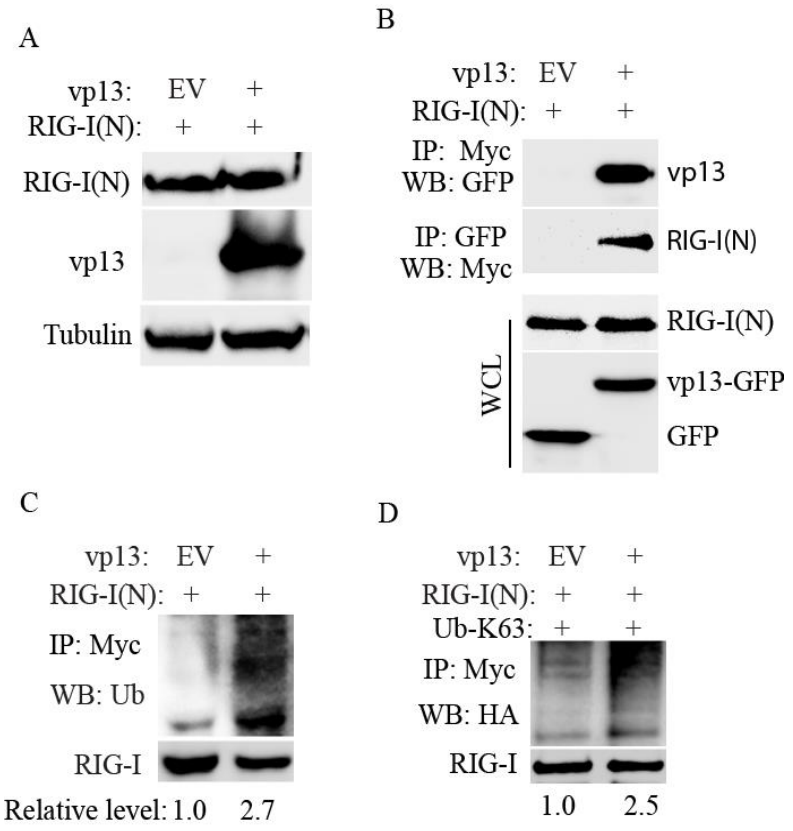

Fig. 6.5 vp13 interacts with RIG-I N-terminal domain and enhances its ubiquitination.

A. vp13 does not enhance expression of RIG-I(N). HEK293T cells were transfected with vp13 and RIG-I(N) plasmids. Western blotting with antibodies against Myc, GFP and tubulin was conducted. **B**. Immunoprecipitation (IP) indicates interaction of vp13 with RIG-I(N). HEK293T cells were transfected with vp13 and RIG-I(N) plasmids. The upper image shows the presence of vp13 in RIG-I(N) IP complex. The second image from top shows the presence of RIG-I(N) in vp13 IP complex. The lower two images shows blotting of whole cell lysate (WCL) with antibodies against Myc and GFP to detect RIG-I(N) and vp13, respectively. **C**. Increase of RIG-I(N) ubiquitination in HEK293T cells with vp13 expression. IP of HEK293T cell lysate was done with Myc antibody. The upper image shows the presence of ubiquitin in the IP complex. The lower image shows the RIG-I(N) pulled down. Relative level of ubiquitination signal in the upper image is shown after normalization with RIG-I. **D.** Increase of RIG-I(N) K-63 ubiquitination in HEK293T cells with vp13 expression. HEK293T cells were transfected with HA-Ubiquitin-K63, RIG-I(N) and vp13.

Next we tested whether vp13 had direct interaction with RIG-I(N). HEK293T cells were co-transfected with Myc-RIG-I(N) and vp13 plasmids. IP with Myc antibody was conducted to pull down Myc-RIG-I(N) and Western blotting with GFP antibody

showed the presence of vp13 in the RIG-I(N) IP samples (Fig. 6. 5B). Similarly, IP with GFP antibody was conducted to pull down GFP-vp13 and Western blotting with Myc antibody showed the presence of RIG-I(N) in the vp13 IP samples. The expression of RIG-I(N) and vp13 was verified in whole cell lysate (Fig. 6. 5B). These results indicate that vp13 is capable of interacting with RIG-I(N). We reasoned that the direct interaction of vp13 and RIG-I(N) could induce RIG-I activation. IP was performed to pull down RIG-I(N) and then Western blotting with antibody against ubiquitin was conducted. The result showed that in the cells with vp13 expression, ubiquitinated RIG-I(N) was significantly elevated to 2.7-fold in comparison to the cells transfected with an empty vector (Fig. 6. 5C). These results suggested that vp13 enhanced RIG-I(N) ubiquitination, which may lead to an increase of IFN-β promoter activation.

It is known that lysine 63 (K63)-linked polyubiquitination of RIG-I by ubiquitin ligase TRIM25 and Riplet causes RIG-I activation (85, 86, 274). We reasoned that the vp13-enhanced ubiquitination of RIG-I should be K63-linked. HEK293T cells were transfected with plasmids of vp13, RIG-I(N), and HA-tagged K63-linked ubiquitin (HA-K63-ubi). IP of RIG-I(N) and Western blotting with antibody against HA were conducted. The K63-linked ubiquitination of RIG-I in the cells with vp13 expression increased by 2.5-fold, compared with control cells (Fig. 6. 5D). The results suggest that

the presence of vp13 promoted RIG-I activation shown by K63-linked ubiquitination.

The C-terminal domain of vp13 is sufficient to enhance RIG-I expression and polyIC-mediated IFN production

To determine which domain of vp13 was associated with the enhancement of IFN production, we constructed three truncation constructs of vp13: D1, D2 and D3 (Fig. 6. 6A). D1 and D3 cover N and C-terminal half of vp13, respectively, and D2 carries the majority of vp13 with a deletion at C-terminal end. HEK293T cells were co-transfected with plasmids of full length RIG-I and full-length vp13 or those three vp13 truncation constructs. Compared to empty vector control, RIG-I levels in the cells transfected with vp13, D1, D2 and D3 plasmids increased 3.2, 1.4, 1.2 and 2.8-fold, respectively (Fig. 6. 6B). Full length vp13 and D3 was detected with a vp13-specific antibody that was generated with a C-terminal peptide and GFP antibody. The RIG-I level in cells with D3 was similar to full-length vp13, which indicates that C- terminal vp13 contains the domain responsible for the enhancement of polyIC-induced IFN expression.

To confirm this observation, we conducted an IFN-β reporter assay in HEK293T cells. Results showed that the D3 increased IFN-β promoter activation to a level similar to that induced by full-length vp13, while D1 and D2 had minimum effect (Fig. 6. 6C). This indicated that the C-terminal domain of vp13 enhanced RIG-I-induced IFN expression.

Presence of vp13 in HEV-infected S10-3 cells leads to higher levels of polyIC-induced IFN-β expression than ORF3-null mutant

The results above showed vp13 enhanced IFN induction. So we assessed whether vp13 played a similar role during HEV replication. To address the question, we used S10-

3 cells, a subclone of Huh-7 cells, which support HEV replication (369). The cells were transfected with full-length RNAs

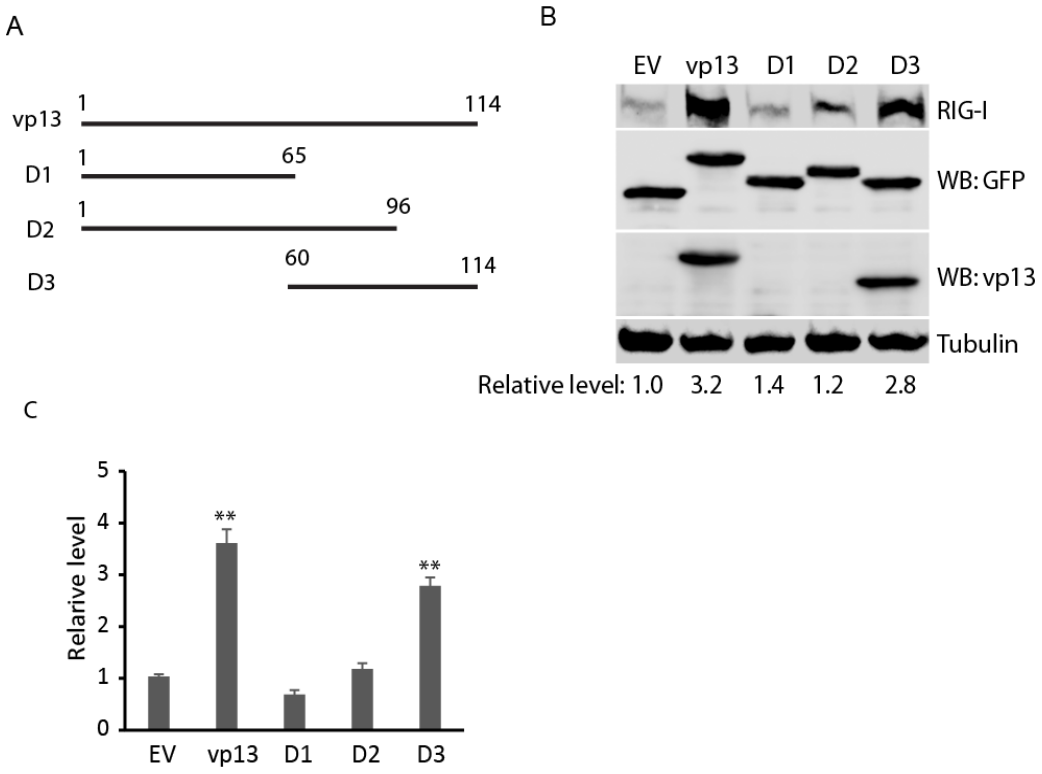

Fig. 6.6. The C-terminal domain of vp13 appears to correlate with enhancement of polyIC-induced IFN-β expression

A. Schematic illustration of cloning vp13 fragments into VemusC vector. The numbers above the lines indicate amino acid position in vp13 (aa1-114). **B**. The C-terminal domain of vp13 correlates with RIG-I elevation. HEK293T cells were co-transfected with RIG-I and vp13 truncation plasmids. Empty vector (EV) was included as a control. Western blotting with antibodies against RIG-I, GFP, vp13 and tubulin was done. D1, D2 and D3 indicate vp13 deletion constructs. The vp13 antibody is against a C-terminal peptide, and thus does not react with D1 and D2. **C**. IFN-β reporter assay. HEK293T cells were co-transfected with RIG-I(N), IFN-β reporter and vp13 plasmids. The cells were harvested for luciferase activity assay 24 h after transfection. Relative luciferase level is shown in comparison with control cells transfected with reporter plasmid and empty vector. Significant differences in IFN-β promoter activation compared to the cells with EV are denoted by "**", which indicates $P < 0.01$.

from HEV replicon pSK-E2 or pSK-E2-GFP. The GFP insertion in pSK-E2 interrupted ORF2 and ORF3 expression, but offered a convenient indicator for direct observation of HEV replication. To determine the RIG-I protein level in the S10-3 cells in the presence

of vp13 expression, Western blotting was conducted. The result showed that endogenous RIG-I in the S10-3 cells with pSK-E2 was considerably higher than the cells with pSK-E2-GFP or pE2-Luc (Fig. 6. 7A). Densitometry analysis showed that the RIG-I level in the cells with pSK-E2 was 2.3-fold higher than the cells with pSK-E2-GFP or pE2-Luc.

Transfection of the cells with polyIC, which was expected to activate RLR pathway, was conducted to induce IFN expression. The cells were harvested 12 h after polyIC treatment for RNA isolation and RT-qPCR. Result showed that the S10-3 cells transfected with pSK-E2 RNA had 1.9-fold higher level of IFN-β transcripts than cells transfected with pSK-E2-GFP RNA in response to polyIC transfection (Fig. 6. 7B). The cells not transfected with polyIC had very low level of IFN-β transcript, as expected.

These results suggested that the presence of vp13 in the S10-3 cells enhanced polyIC-induced IFN expression. To confirm the observation, we used a ORF3-null HEV replicon, pSKE2Δ3, in which a termination codon was introduced to stop ORF3 expression and therefore, no vp13 expression (98). The vp13-null mutant had similar replication rate as wide-type virus in S10-3 cells (72). Compared to the S10-3 cells transfected with wild-type pSKE2 replicon, the cells transfected with vp13-null pSKE2Δ3 mutant had significantly lower levels of IFN-β transcript after polyIC stimulation (Fig. 6. 7C). IFA with antibody against the capsid protein confirmed similar HEV replication in the S10-3 cells transfected with either wild-type or ORF3-null mutant genomic RNAs (Fig. 6. 7D). These data suggest that the presence of vp13 in HEV-infected cells elevates endogenous RIG-I, which subsequently enhances polyIC-induced IFN expression.

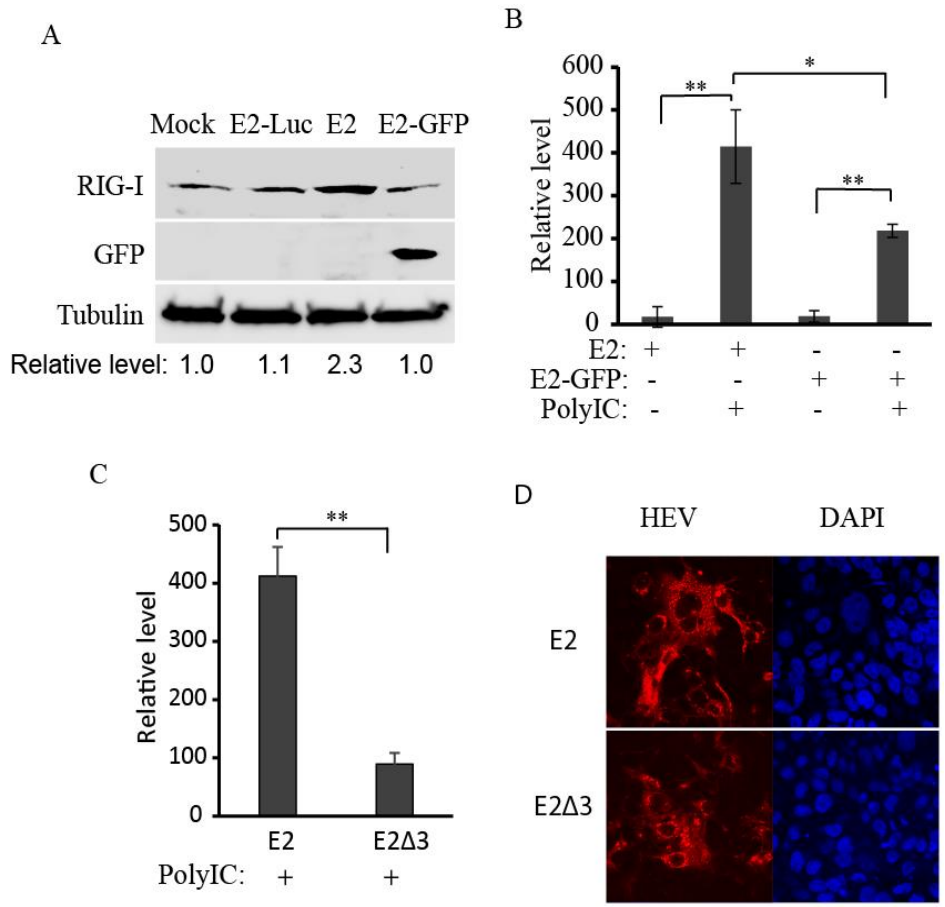

Fig. 6.7 Presence of vp13 in HEV-infected S10-3 cells leads to higher IFN-β expression induced by polyIC

A. Elevation of RIG-I level in HEV-infected cells with full-length HEV RNA. S10-3 cells were transfected with RNA from pSK-E2, pSK-E2-GFP, and pE2-Luc replicons. Western blotting with antibodies against RIG-I, GFP and tubulin was conducted 10 days after the transfection. Relative level of RIG-I after normalization with tubulin is shown below the images. Mock-infected cells were included for comparison. **B**. IFN-β transcript in HEV-infected S-10 cells detected by RT-qPCR. The cells were transfected with polyIC to induce IFN expression. Induction folds of IFN-β mRNA level in comparison to control cells without HEV RNA and polyIC are shown. Significant differences between test sample and control are shown by "*" and "**", which indicate $P < 0.05$ and $P < 0.01$, respectively. **C.** Absence of vp13 in HEV-infected S-10 cells leads to lower IFN-β transcript after polyIC stimulation. **D**. Detection of HEV replication in S10-3 cells. The left panel of images shows immunofluorescence assay of the cells transfected with full length HEV genomic RNA from pSK-E2 or pSK-E2Δ3. Left images of red fluorescence indicates the presence of HEV capsid protein. Nuclear DNA was counterstained using DPAI shown in the right images.

Enhancement of IFN induction by vp13 appears to be genotype-specific.

HEV genomic sequences are divergent. There are at least four genotypes of HEV across the world. The above experiments were done with vp13 from Sar55, a strain of genotype I HEV, which can cause acute hepatitis. We wondered whether vp13 from strains of the other three genotypes would similarly enhance IFN production. ORF3 from strains of genotype II, III and IV were cloned. To determine the vp13 effect on RIG-I(N)-induced IFN production, we performed IFN-β promoter reporter assay. HEK293T cells were transfected with IFN-β promoter reporter and vp13 plasmids. The result showed that the genotype III vp13 enhanced RIG-I(N)-induced luciferase expression by 2.1-fold compared with cells with empty vector (Fig. 6. 8A). vp13 from the genotype II and genotype IV HEV strains had slightly lower level of luciferase activity than the empty vector. vp13 of genotype I enhanced luciferase activity, as expected.

RIG-I protein levels in HEK293T cells co-transfected with full length RIG-I and one of the four vp13 plasmids were determined by Western blotting. Results showed that compared to the cells with empty vector, the cells with vp13 of the genotype III strain had 2.4-fold higher level RIG-I expression, which was similar to the cells transfected with vp13 from the genotype I strain (Fig. 6. 8B). Blotting with a GFP antibody confirmed the similar level expression of vp13 from the four genotypes in the cells. However, the cells with vp13 from the genotype II and genotype IV HEV strains led to minimal changes of RIG-I level. The results indicate that vp13 from the strains of different genotypes had variable effects on RLR pathway

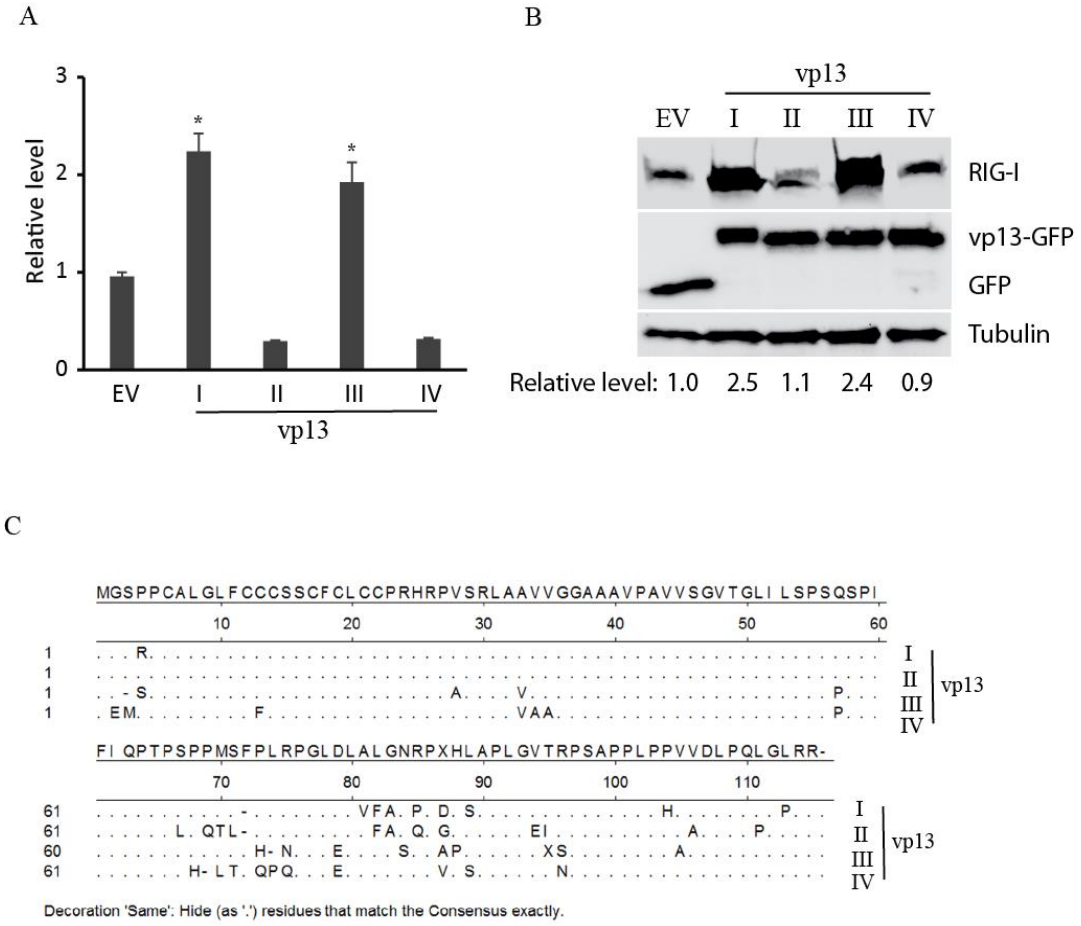

Fig. 6.8 vp13 from HEV genotype I and III enhance IFN induction.

A. vp13 from strains of HEV genotype I and III induce higher IFN-β promoter expression. HEK293T cells were co-transfected with vp13 and IFN-β promoter reporter plasmids for luciferase reporter assay. Relative luciferase level is shown. Significant differences from cells transfected with empty vector are shown by "*", which indicate $P < 0.05$. **B**. vp13 from strains of HEV genotype I and III induce RIG-I elevation. HEK293T cells were co-transfected with vp13 and RIG-I plasmids. Western blotting with antibodies against FLAG, GFP and tubulin was conducted. **C**. Alignment of amino acid sequence of vp13 from the four genotypes (Genotype I (GenBank accession # AF444002), genotype II (accession# M74506), genotype III (accession# HQ709170), and genotype IV (accession# AB074915). Those residues that are the same as consensus sequence are shown as ".".

.Amino acid sequence alignment of HEV vp13 of the four genotypes shows there are more variations in its C-terminal than N-terminal domain (Fig. 6. 8C). As the C-terminal domain correlated with the enhancement of IFN expression, the variation in C-terminal sequence could mean functional divergence. The amino acid sequence identity is 86% between genotype I and II, 79% between genotype I and III, 78% between genotype I and IV. Together, these data shows vp13 enhancement of IFN induction appears to be genotype-specific or strain-specific.

DISCUSSION

This study demonstrated that HEV vp13 enhances polyIC-induced interferon expression by increasing RIG-I protein level, interacting with RIG-I(N) and increasing RIG-I(N) ubiquitination. The vp13-mediated elevation of RIG-I protein level is possibly due to the extension of RIG-I half-life. The C-terminal domain of vp13 was found to be sufficient for the enhancement of IFN induction. The vp13-mediated enhancement of IFN induction was also observed in HEV-infected S10-3 liver cells.

We observed that polyIC treatment of HeLa-vp13 stable cells induced stronger inhibition of NDV-GFP replication than polyIC-treated normal HeLa cells. The result indicates that vp13 plays the role in enhancing polyIC-induced antiviral response. Direct addition of polyIC to the cultured cells activates TLR3 pathway and transfection of the cells activates RLR pathway. In our experiment, the direct addition induced lower IFN-β expression than transfection. So we used the transfection method in this study to delineate the mechanism of vp13 enhancement of IFN induction. In addition, our results also provide potential answers to the vp13 augmentation of polyIC-induced TLR3 pathway: the presence of vp13 enhances TBK1-induced IFN-β expression as TLR3 activation induces phosphorylation of IRF3 by TBK1.

Our screening of signal molecules in the IFN induction pathways identified that vp13 could enhance IFN induction by RIG-I and TBK1. The vp13-enhanced RIG-I signaling was examined in this study. The mechanism of the vp13 enhancement of IFN induction via TBK1 is yet unknown. Up-regulating expression of TBK1 by vp13 might be a possible reason, but unlikely, because IFN induction by upstream molecules such as TRIF and MDA5(N) was not augmented by vp13 co-transfection.

The mechanism for vp13-mediated enhancement of RIG-I induction of IFN was delineated in this study. Elevation of basal level of RIG-I was discovered in HeLa-vp13 stable cells, and transiently transfected HEK293T and S10-3 cells. The increase of RIG-I in S10-3 cells could be more meaningful as they are liver-derived HEV-susceptible cells.

The up-regulation of RIG-I appears to be mainly because of vp13-mediated extension of half-life of the protein. Presence of vp13 extends half-life of exogenous RIG-I from 18 h to 30 h. The possible mechanisms for this extension might be inhibition of RIG-I degradation by ubiquitin-proteasome pathway or increase of the protein translation. The former speculation sounds more reasonable as cycloheximide treatment blocks protein translation. The observations that expression of RIG-I(N) was not affected by vp13 and that half-life of the full-length RIG-I protein was extended in the presence of vp13 is consistent with this speculation.

The other reason for vp13-ehancement of IFN induction could be that vp13 interacts with RIG-I(N) and enhances its ubiquitination. RIG-I(N) IP pulled down vp13, vice versa, vp13 IP pulled down RIG-I(N). As RIG-I(N) contains the CARD domains to interact with IPS-1 to transmit the signal to induce IFN production, the up-regulation of RIG-I(N) ubiquitination by vp13 further enhances the signaling. Two ubiquitin ligases TRIM25 (85, 86) and Riplet (274) mediates K63-linked polyubiquitination of RIG-I, leading to its interaction with IPS-1. This study shows that vp13 increases K63-linked RIG-I(N) polyubiquitination. The increase of K63-linked polyubiquitination is consistent with the vp13 enhancement of RIG-I(N) activation. As RIG-I CTD interacts with N-terminal CARDs in resting cells, vp13 is not able to interact with the whole RIG-I protein. Once the N-terminal CARDs are exposed after RIG-I undergoes conformational

change when helicase domain of RIG-I protein senses viral RNA (339), vp13 would be able to interact with the CARDs and further augment the activation signaling.

The active domain of vp13 in this function locates in the C-terminal portion of this protein, as identified by truncation analysis. The C-terminal vp13 contains proline-rich sequence that interacts with SH3 domain in cellular proteins (180). The PSAP motif of avian HEV was found to play a role in virus release *in vivo* though not essential for the virus infectivity (170). We constructed mutant vp13 with PSAP mutations and our preliminary study with the mutants indicates that the PSAP motif appears not correlate with the vp13 enhancement of IFN induction (data not shown). Further study is needed to identify the active motif involved in the enhancement of IFN induction found in this study. Our finding is consistent with the multi-function characteristic of vp13.

The vp13-mediated enhancement of IFN induction appears to be true in HEV-infected hepatoma cells, as polyIC induced higher levels of IFN-β mRNA in HEV-infected S10-3 cells with vp13 expression. The elevation of RIG-I protein level in the HEV-infected S10-3 cells expressing vp13 further substantiates the observation, and is consistent with the data that vp13 increases RIG-I protein level in stably or transiently transfected cells. Compared to the ORF3 –null mutants, in HEV-infected S10-3 cells, the presence of vp13 caused a significant increase in IFN-β mRNA level induced by polyIC. The small magnitude of change could be due to the low rate of HEV-positive cells and virus-mediated inhibition of the IFN induction. We noticed that HEV ORF1 product inhibited polyIC-induced IFN production (manuscript submitted). Therefore, it appears to be a balance between IFN induction and inhibition, so that the vp13-induced enhancement might be under control by the other viral products. In addition to

enhancement of IFN induction, vp13 also enhances RIG-I mediated NF-κB promoter activation and leads to expression of NF-κB activated cytokines in HeLa-vp13 stable cells stimulated by polyIC (data not shown). Among the cytokines elevated, some are proinflammatory and may contribute to HEV-mediated inflammation and pathogenesis during HEV infection. A recent report observed that higher levels of TNF-α, IL-6, IFN-γ and TGF-β1, as well as higher HEV viral load were in pregnant women with acute viral hepatitis and fulminant hepatic failure(183).

It is generally expected that a virus would inhibit the interferon induction and signaling to gain time for its own replication. Why vp13, a viral protein, enhances RIG-I-mediated IFN production is an intriguing question. In addition to the possibilities explained above, the vp13-mediated enhancement of IFN induction could possibly play a role in the context of HEV infection, for example, stages of virus replication. We hypothesize that during early stage of HEV infection, ORF1 is more expressed and IFN induction is inhibited, and during late stage, viral RNA replication is completed and ORF3 expression is increased to promote HEV egress and spreading to other cells.

Our data also showed vp13-mediated IFN enhancement is different among the four HEV genotypes. vp13 from a genotype III strain is also able to enhance IFN induction in our study, but a recent report showed that vp13 from a strain of genotype III inhibits IFN-α activated signaling in HEV-stable A549 cells (61). The vp13 of genotype I strain appears to have no such effect on IFN-activated signaling because NDV-GFP replication was inhibited in HeLa-vp13 stable cells after polyIC treatment. The inconsistency might be because of different strains tested and different cells used in our study.

On the other hand, the finding that vp13 enhances interferon induction would be useful in different applications. For example, the C-terminal domain of vp13 can be inserted into genomes of attenuated live virus to induce a stronger innate immune response for a better protective immunity. The insertion of C-terminal domain of vp13 into the recombinant virus also makes vp13 act as a marker to differentiate from the wide type virus infection. Further characterization of vp13 and its active domain in this application is warranted.

Table 6.1 List of primers used in chapter 6

Primer[a]	Sequences (5' to 3')[b]	Target gene
RIG-I-F1	TTA*GGTACC*ATGACCACCGAGCAG CGAC	Cloning of RIG-I
RIG-I-F2	GGA*GGTACC*TCATTTGGACATTTCT GCTG	
H3F11	CGC*CTCGAG*TGGGTTCGCGACCAT GC	Cloning of vp13 D1
H3R12	CA*GAATTC*TTAGGTTGGTTGGATGA ATAT	
H3R24	CA*GAATTC*TTACCTGGTCACGCCAA GCGG	Cloning of vp13 D2
H3F8	GC*GAATTC*ATGTTCATCCAACCAAC CC	Cloning of vp13 D3
H3R23	CA*GAATTC*TTAGCGGCGCGGCCCCA GCTGTG	
T2H3F2	GC*CTCGAG*GTTCGCCACCATGCGCC CTAG	Cloning ORF3 of genotype II
T2H3R2	GC*GAATTC*TCAGCGCCGCAGCCCCG GCTG	
KH3F2	CG*CTCGAG*GATCACCATGTGCCCTA G	Cloning ORF3 of Kenow HEV
KH3R3	GC*GAATTC*TCAACGGCGCAGCCCCA GC	
T4H3F2	GC*CTCGAG*AGATGCCACCATGCGC TCTCG	Cloning ORF3 of genotype IV
T4H3R2	GC*GAATTC*TCAACGGCGCAGCCCCA GCTG	

a. F: forward primer, R: reverse primer. The "H3" before a primer name indicates the primer is based on sequences of HEV ORF3.

b. The italicized letters indicate restriction enzyme cleavage sites for cloning.

Conclusion

In this study, we examined the mechanisms of HEV interference with host innate immune system, specifically IFN induction and IFN-activated signaling. Interestingly, our data indicate that HEV modulates the IFN pathways in both a positive and negative way. On the one hand, the macro domain and PCP domain from HEV ORF1 polyprotein are able to inhibit host IFN induction via block IRF3 phosphorylation and ubiquitination of RIG-I and TBK1, which resulted in inhibition of IFN induction. Moreover, IFN-activated signaling can be inhibited by Met domain and ProX domain of the ORF1 product as well. This indicates HEV is able to antagonize host innate immunity to facilitate virus replication.

On the other hand, vp13, the product of HEV ORF3, plays an active role in IFN induction that is reverse to the role of ORF1 product described above. Our data indicate vp13 is able to elevate RIG-I expression and activation to enhance host IFN induction. Although data from this study indicate functions of ORF1 and ORF3 products seems to be antagonizing each other, considering temporal order of ORF1 and ORF3 expression in HEV-infected cells, we hypothesize that HEV modulates host innate immune response differently at different stages of viral replication.

In the early stage of HEV replication, it inhibits host innate immune response to facilitate its replication. In the middle and late stage of its replication, the capsid protein and vp13 are translated from HEV subgenomic RNA, followed by virion packaging and egress. During this late stage, innate immune response, specifically, proinflammatory cytokines, may be enhanced by vp13 and the enhancement may be related with liver

injury and virion release. This assumption is corroborated by a recent report that indicates that higher cytokine level in HEV-infected pregnant women correlates with fulminant liver failure and liver injury.

The interesting data from this study provide us with more insight on HEV-cell interactions and its molecular pathogenesis. The data can also facilitate the development of specific antiviral therapeutics against HEV infection, which should benefit HEV-mediated acute liver injury in immunocompetent individuals or chronic infection in immunocompromised patients. Further work is warranted to elucidate the mechanisms of HEV modulation during different stages of viral replication and/or infection.

Future Directions

In this study, we firstly demonstrated that HEV inhibit host IFN induction via PCP and X domain from ORF1 product. Although PCP domain had been identified as deubiquitinase for RIG-I and TBK1, it is possible that PCP domain may cause deubiquitination for more molecules such as TRAF6 and NEMO to inhibit NF-κB mediated cytokine induction. We will test this hypothesis in future works. Also, the mechanism of inhibition of IRF3 activation by X domain is not known. Further work will elucidate how X domain inhibits the phosphorylation of IRF3 and the essential motif of X in this interference.

On the other hand, we expect X domain may play more roles than inhibition of IRF3 activation. We had observed that X domain can bind chromosome tightly when expressed in HEK293T. It is possible that X domain may able to regulate a wide range of host gene expression and this could be a common function for X domain homologues in other viruses. We will examine this speculation in future works as well.

In the Chapter 5, we demonstrated HEV Met domain and ProX domain from ORF1 product inhibit IFN signaling as well. We speculated the Met domain may result in promoter methylation which is downstream of STAT1 phosphorylation and nuclear translocation to dampen JAK/STAT activated gene expression. On the other hand, as X domain is part of the ProX fragment, it is possible that X domain could bind with ISRE promoter region to inhibit transcription initiation. These speculations will be examined further in future.

The vp13 of HEV has been demonstrated as a multifunctional protein. In addition to previous knowledge, we further demonstrated that vp13 can enhance RIG-I mediated

signaling. Along this line, the detail mechanisms of the interference are not known. Further work will be done to figure out how vp13 extends the half-life of RIG-I and what the consequence is for the vp13 enhancement of RIG-I signaling in pro-inflammatory cytokine induction.

In addition, vp13 can be explored to enhance vaccine efficacy by increasing innate immune response. Currently, we try to insert vp13 into the genome of modified live virus (MLV) vaccine strain of PRRSV to see whether vp13 can be utilized to enhance vaccine efficiency of the MLV. Taken together, our study generated valuable information that can be further explored for studies on HEV pathogenesis and for the vaccine and antiviral drug development for HEV and PRRSV.

Bibliography

1. **Abreu, M. T.** 2010. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nature reviews. Immunology **10:**131-144.
2. **Accola, M. A., B. Huang, A. Al Masri, and M. A. McNiven.** 2002. The antiviral dynamin family member, MxA, tubulates lipids and localizes to the smooth endoplasmic reticulum. J Biol Chem **277:**21829-21835.
3. **Adhikari, A., M. Xu, and Z. J. Chen.** 2007. Ubiquitin-mediated activation of TAK1 and IKK. Oncogene **26:**3214-3226.
4. **Aggarwal, R., R. Shukla, S. Jameel, S. Agrawal, P. Puri, V. K. Gupta, A. P. Patil, and S. Naik.** 2007. T-cell epitope mapping of ORF2 and ORF3 proteins of human hepatitis E virus. J Viral Hepat **14:**283-292.
5. **Agrawal, S., D. Gupta, and S. K. Panda.** 2001. The 3' end of hepatitis E virus (HEV) genome binds specifically to the viral RNA-dependent RNA polymerase (RdRp). Virology **282:**87-101.
6. **Aguiar, R. C., K. Takeyama, C. He, K. Kreinbrink, and M. A. Shipp.** 2005. B-aggressive lymphoma family proteins have unique domains that modulate transcription and exhibit poly(ADP-ribose) polymerase activity. J Biol Chem **280:**33756-33765.
7. **Ahmad, I., R. P. Holla, and S. Jameel.** 2011. Molecular virology of hepatitis E virus. Virus Res.
8. **Akhtar, L. N., and E. N. Benveniste.** 2011. Viral exploitation of host SOCS protein functions. J Virol **85:**1912-1921.
9. **Akira, S., Y. Nishio, M. Inoue, X. J. Wang, S. Wei, T. Matsusaka, K. Yoshida, T. Sudo, M. Naruto, and T. Kishimoto.** 1994. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. Cell **77:**63-71.
10. **Akira, S., and K. Takeda.** 2004. Toll-like receptor signalling. Nature reviews. Immunology **4:**499-511.
11. **Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell.** 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature **413:**732-738.
12. **Allen, M. D., A. M. Buckle, S. C. Cordell, J. Lowe, and M. Bycroft.** 2003. The crystal structure of AF1521 a protein from Archaeoglobus fulgidus with homology to the non-histone domain of macroH2A. J Mol Biol **330:**503-511.
13. **Amerik, A. Y., and M. Hochstrasser.** 2004. Mechanism and function of deubiquitinating enzymes. Biochimica et biophysica acta **1695:**189-207.
14. **Andrejeva, J., K. S. Childs, D. F. Young, T. S. Carlos, N. Stock, S. Goodbourn, and R. E. Randall.** 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci U S A **101:**17264-17269.
15. **Andrejeva, J., D. F. Young, S. Goodbourn, and R. E. Randall.** 2002. Degradation of STAT1 and STAT2 by the V proteins of simian virus 5 and

human parainfluenza virus type 2, respectively: consequences for virus replication in the presence of alpha/beta and gamma interferons. J Virol **76:**2159-2167.

16. **Angelov, D., A. Molla, P. Y. Perche, F. Hans, J. Cote, S. Khochbin, P. Bouvet, and S. Dimitrov.** 2003. The histone variant macroH2A interferes with transcription factor binding and SWI/SNF nucleosome remodeling. Mol Cell **11:**1033-1041.
17. **Ansari, I. H., S. K. Nanda, H. Durgapal, S. Agrawal, S. K. Mohanty, D. Gupta, S. Jameel, and S. K. Panda.** 2000. Cloning, sequencing, and expression of the hepatitis E virus (HEV) nonstructural open reading frame 1 (ORF1). J Med Virol **60:**275-283.
18. **Apte, R. N., O. Ascher, and D. H. Pluznik.** 1977. Genetic analysis of generation of serum interferon by bacterial lipopolysaccharide. J Immunol **119:**1898-1902.
19. **Arnheiter, H., S. Skuntz, M. Noteborn, S. Chang, and E. Meier.** 1990. Transgenic mice with intracellular immunity to influenza virus. Cell **62:**51-61.
20. **Ashour, J., M. Laurent-Rolle, P. Y. Shi, and A. Garcia-Sastre.** 2009. NS5 of dengue virus mediates STAT2 binding and degradation. J Virol **83:**5408-5418.
21. **Bacon, C. M., E. F. Petricoin, 3rd, J. R. Ortaldo, R. C. Rees, A. C. Larner, J. A. Johnston, and J. J. O'Shea.** 1995. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. Proc Natl Acad Sci U S A **92:**7307-7311.
22. **Balachandran, S., E. Thomas, and G. N. Barber.** 2004. A FADD-dependent innate immune mechanism in mammalian cells. Nature **432:**401-405.
23. **Baoprasertkul, P., P. Xu, E. Peatman, H. Kucuktas, and Z. Liu.** 2007. Divergent Toll-like receptors in catfish (Ictalurus punctatus): TLR5S, TLR20, TLR21. Fish & shellfish immunology **23:**1218-1230.
24. **Barral, P. M., J. M. Morrison, J. Drahos, P. Gupta, D. Sarkar, P. B. Fisher, and V. R. Racaniello.** 2007. MDA-5 is cleaved in poliovirus-infected cells. J Virol **81:**3677-3684.
25. **Barral, P. M., D. Sarkar, Z. Z. Su, G. N. Barber, R. DeSalle, V. R. Racaniello, and P. B. Fisher.** 2009. Functions of the cytoplasmic RNA sensors RIG-I and MDA-5: key regulators of innate immunity. Pharmacol Ther **124:**219-234.
26. **Batts, W., S. Yun, R. Hedrick, and J. Winton.** 2011. A novel member of the family Hepeviridae from cutthroat trout (Oncorhynchus clarkii). Virus Res **158:**116-123.
27. **Bauhofer, O., A. Summerfield, Y. Sakoda, J. D. Tratschin, M. A. Hofmann, and N. Ruggli.** 2007. Classical swine fever virus Npro interacts with interferon regulatory factor 3 and induces its proteasomal degradation. J Virol **81:**3087-3096.
28. **Baum, A., and A. Garcia-Sastre.** 2010. Induction of type I interferon by RNA viruses: cellular receptors and their substrates. Amino acids **38:**1283-1299.
29. **Belvin, M. P., and K. V. Anderson.** 1996. A conserved signaling pathway: the Drosophila toll-dorsal pathway. Annual review of cell and developmental biology **12:**393-416.
30. **Berke, T., and D. O. Matson.** 2000. Reclassification of the Caliciviridae into distinct genera and exclusion of hepatitis E virus from the family on the basis of comparative phylogenetic analysis. Arch Virol **145:**1421-1436.

31. **Best, S. M., K. L. Morris, J. G. Shannon, S. J. Robertson, D. N. Mitzel, G. S. Park, E. Boer, J. B. Wolfinbarger, and M. E. Bloom.** 2005. Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist. J Virol **79:**12828-12839.
32. **Beura, L. K., S. N. Sarkar, B. Kwon, S. Subramaniam, C. Jones, A. K. Pattnaik, and F. A. Osorio.** 2010. Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. J Virol **84:**1574-1584.
33. **Beutler, B.** 2004. Innate immunity: an overview. Mol Immunol **40:**845-859.
34. **Borgen, K., T. Herremans, E. Duizer, H. Vennema, S. Rutjes, A. Bosman, A. M. de Roda Husman, and M. Koopmans.** 2008. Non-travel related Hepatitis E virus genotype 3 infections in the Netherlands; a case series 2004 - 2006. BMC Infect Dis **8:**61.
35. **Bose, P. D., B. C. Das, R. K. Hazam, A. Kumar, S. Medhi, and P. Kar.** 2014. Evidence of extrahepatic replication of hepatitis E virus in human placenta. J Gen Virol **95:**1266-1271.
36. **Bowie, A., E. Kiss-Toth, J. A. Symons, G. L. Smith, S. K. Dower, and L. A. O'Neill.** 2000. A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. Proc Natl Acad Sci U S A **97:**10162-10167.
37. **Bowman, T., R. Garcia, J. Turkson, and R. Jove.** 2000. STATs in oncogenesis. Oncogene **19:**2474-2488.
38. **Boyapalle, S., T. Wong, J. Garay, M. Teng, H. San Juan-Vergara, and S. Mohapatra.** 2012. Respiratory syncytial virus NS1 protein colocalizes with mitochondrial antiviral signaling protein MAVS following infection. PLoS ONE **7:**e29386.
39. **Chandra, V., P. Holla, D. Ghosh, D. Chakrabarti, M. Padigaru, and S. Jameel.** 2011. The hepatitis E virus ORF3 protein regulates the expression of liver-specific genes by modulating localization of hepatocyte nuclear factor 4. PLoS ONE **6:**e22412.
40. **Chandra, V., M. Kalia, K. Hajela, and S. Jameel.** The ORF3 protein of hepatitis E virus delays degradation of activated growth factor receptors by interacting with CIN85 and blocking formation of the Cbl-CIN85 complex. J Virol **84:**3857-3867.
41. **Chandra, V., A. Kar-Roy, S. Kumari, S. Mayor, and S. Jameel.** 2008. The hepatitis E virus ORF3 protein modulates epidermal growth factor receptor trafficking, STAT3 translocation, and the acute-phase response. J Virol **82:**7100-7110.
42. **Chen, F., D. Bhatia, Q. Chang, and V. Castranova.** 2006. Finding NEMO by K63-linked polyubiquitin chain. Cell death and differentiation **13:**1835-1838.
43. **Chen, L., L. Hu, T. H. Chan, G. S. Tsao, D. Xie, K. K. Huo, L. Fu, S. Ma, B. J. Zheng, and X. Y. Guan.** 2009. Chromodomain helicase/adenosine triphosphatase DNA binding protein 1-like (CHD1l) gene suppresses the nucleus-to-mitochondria translocation of nur77 to sustain hepatocellular carcinoma cell survival. Hepatology **50:**122-129.

44. **Chiang, C., and P. A. Beachy.** 1994. Expression of a novel Toll-like gene spans the parasegment boundary and contributes to hedgehog function in the adult eye of Drosophila. Mechanisms of development **47:**225-239.
45. **Chin, K. C., and P. Cresswell.** 2001. Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. Proc Natl Acad Sci U S A **98:**15125-15130.
46. **Ching, R. W., G. Dellaire, C. H. Eskiw, and D. P. Bazett-Jones.** 2005. PML bodies: a meeting place for genomic loci? Journal of cell science **118:**847-854.
47. **Chiu, Y. H., J. B. Macmillan, and Z. J. Chen.** 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell **138:**576-591.
48. **Chuang, T., and R. J. Ulevitch.** 2001. Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells. Biochimica et biophysica acta **1518:**157-161.
49. **Chuang, T. H., and R. J. Ulevitch.** 2000. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. European cytokine network **11:**372-378.
50. **Colonna, M., G. Trinchieri, and Y. J. Liu.** 2004. Plasmacytoid dendritic cells in immunity. Nature immunology **5:**1219-1226.
51. **Costanzi, C., and J. R. Pehrson.** 1998. Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. Nature **393:**599-601.
52. **Cui, X. F., T. Imaizumi, H. Yoshida, E. C. Borden, and K. Satoh.** 2004. Retinoic acid-inducible gene-I is induced by interferon-gamma and regulates the expression of interferon-gamma stimulated gene 15 in MCF-7 cells. Biochemistry and cell biology = Biochimie et biologie cellulaire **82:**401-405.
53. **Cui, Y., M. Li, K. D. Walton, K. Sun, J. A. Hanover, P. A. Furth, and L. Hennighausen.** 2001. The Stat3/5 locus encodes novel endoplasmic reticulum and helicase-like proteins that are preferentially expressed in normal and neoplastic mammary tissue. Genomics **78:**129-134.
54. **Darnell, J. E., Jr., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415-1421.
55. **de Veer, M. J., M. Holko, M. Frevel, E. Walker, S. Der, J. M. Paranjape, R. H. Silverman, and B. R. Williams.** 2001. Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol **69:**912-920.
56. **Decker, T., D. J. Lew, Y. S. Cheng, D. E. Levy, and J. E. Darnell, Jr.** 1989. Interactions of alpha- and gamma-interferon in the transcriptional regulation of the gene encoding a guanylate-binding protein. The EMBO journal **8:**2009-2014.
57. **Decker, T., D. J. Lew, J. Mirkovitch, and J. E. Darnell, Jr.** 1991. Cytoplasmic activation of GAF, an IFN-gamma-regulated DNA-binding factor. The EMBO journal **10:**927-932.
58. **Dement'eva, E. V., A. Semiletov Iu, T. L. Iashina, and V. A. Shibnev.** 1997. [Analysis of the organization of immunodominant epitopes of hepatitis virus E orf2 and orf3 proteins using synthetic peptides]. Vopr Virusol **42:**102-105.
59. **Deng, L., C. Wang, E. Spencer, L. Yang, A. Braun, J. You, C. Slaughter, C. Pickart, and Z. J. Chen.** 2000. Activation of the IkappaB kinase complex by

TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell **103:**351-361.

60. **Der, S. D., A. Zhou, B. R. Williams, and R. H. Silverman.** 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A **95:**15623-15628.
61. **Dong, C., M. Zafrullah, T. Mixson-Hayden, X. Dai, J. Liang, J. Meng, and S. Kamili.** 2012. Suppression of interferon-alpha signaling by hepatitis E virus. Hepatology **55:**1324-1332.
62. **Donnelly, R. P., F. Sheikh, S. V. Kotenko, and H. Dickensheets.** 2004. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. J Leukoc Biol **76:**314-321.
63. **Dosztanyi, Z., J. Chen, A. K. Dunker, I. Simon, and P. Tompa.** 2006. Disorder and sequence repeats in hub proteins and their implications for network evolution. J Proteome Res **5:**2985-2995.
64. **Drexler, J. F., A. Seelen, V. M. Corman, A. Fumie Tateno, V. Cottontail, R. Melim Zerbinati, F. Gloza-Rausch, S. M. Klose, Y. Adu-Sarkodie, S. K. Oppong, E. K. Kalko, A. Osterman, A. Rasche, A. Adam, M. A. Muller, R. G. Ulrich, E. M. Leroy, A. N. Lukashev, and C. Drosten.** 2012. Bats worldwide carry hepatitis E virus-related viruses that form a putative novel genus within the family Hepeviridae. J Virol **86:**9134-9147.
65. **Drobeniuc, J., J. Meng, G. Reuter, T. Greene-Montfort, N. Khudyakova, Z. Dimitrova, S. Kamili, and C. G. Teo.** 2010. Serologic assays specific to immunoglobulin M antibodies against hepatitis E virus: pangenotypic evaluation of performances. Clin Infect Dis **51:**e24-27.
66. **Du, Y., J. Bi, J. Liu, X. Liu, X. Wu, P. Jiang, D. Yoo, Y. Zhang, J. Wu, R. Wan, X. Zhao, L. Guo, W. Sun, X. Cong, L. Chen, and J. Wang.** 2014. 3Cpro of Foot-and-Mouth Disease Virus Antagonizes the Interferon Signaling Pathway by Blocking STAT1/STAT2 Nuclear Translocation. J Virol **88:**4908-4920.
67. **Dunker, A. K., C. J. Oldfield, J. Meng, P. Romero, J. Y. Yang, J. W. Chen, V. Vacic, Z. Obradovic, and V. N. Uversky.** 2008. The unfoldomics decade: an update on intrinsically disordered proteins. BMC genomics **9 Suppl 2:**S1.
68. **Dushay, M. S., and E. D. Eldon.** 1998. Drosophila immune responses as models for human immunity. American journal of human genetics **62:**10-14.
69. **Egloff, M. P., H. Malet, A. Putics, M. Heinonen, H. Dutartre, A. Frangeul, A. Gruez, V. Campanacci, C. Cambillau, J. Ziebuhr, T. Ahola, and B. Canard.** 2006. Structural and functional basis for ADP-ribose and poly(ADP-ribose) binding by viral macro domains. J Virol **80:**8493-8502.
70. **Emerson, S., D. Anderson, V. A. Arankalle, X.-J. Meng, M. Purdy, G. G. Schlauder, and S. Tsarev.** 2004. Presented at the Virus Taxonomy, VIIIth report of the ICTV, London, UK.
71. **Emerson, S. U., H. Nguyen, J. Graff, D. A. Stephany, A. Brockington, and R. H. Purcell.** 2004. In vitro replication of hepatitis E virus (HEV) genomes and of an HEV replicon expressing green fluorescent protein. J Virol **78:**4838-4846.
72. **Emerson, S. U., H. Nguyen, U. Torian, and R. H. Purcell.** 2006. ORF3 protein of hepatitis E virus is not required for replication, virion assembly, or infection of hepatoma cells in vitro. J Virol **80:**10457-10464.

73. **Emerson, S. U., H. T. Nguyen, U. Torian, D. Burke, R. Engle, and R. H. Purcell.** 2010. Release of genotype 1 hepatitis E virus from cultured hepatoma and polarized intestinal cells depends on open reading frame 3 protein and requires an intact PXXP motif. J Virol **84:**9059-9069.
74. **Emerson, S. U., and R. H. Purcell.** 2003. Hepatitis E virus. Rev Med Virol **13:**145-154.
75. **Emerson, S. U., M. Zhang, X. J. Meng, H. Nguyen, M. St Claire, S. Govindarajan, Y. K. Huang, and R. H. Purcell.** 2001. Recombinant hepatitis E virus genomes infectious for primates: importance of capping and discovery of a cis-reactive element. Proc Natl Acad Sci U S A **98:**15270-15275.
76. **Erker, J. C., S. M. Desai, G. G. Schlauder, G. J. Dawson, and I. K. Mushahwar.** 1999. A hepatitis E virus variant from the United States: molecular characterization and transmission in cynomolgus macaques. J Gen Virol **80 (Pt 3):**681-690.
77. **Farhat, K., S. Riekenberg, H. Heine, J. Debarry, R. Lang, J. Mages, U. Buwitt-Beckmann, K. Roschmann, G. Jung, K. H. Wiesmuller, and A. J. Ulmer.** 2008. Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling. J Leukoc Biol **83:**692-701.
78. **Fensterl, V., D. Grotheer, I. Berk, S. Schlemminger, A. Vallbracht, and A. Dotzauer.** 2005. Hepatitis A virus suppresses RIG-I-mediated IRF-3 activation to block induction of beta interferon. J Virol **79:**10968-10977.
79. **Fensterl, V., and G. C. Sen.** 2009. Interferons and viral infections. Biofactors **35:**14-20.
80. **Fensterl, V., and G. C. Sen.** 2011. The ISG56/IFIT1 gene family. J Interferon Cytokine Res **31:**71-78.
81. **Fitzgerald, K. A., S. M. McWhirter, K. L. Faia, D. C. Rowe, E. Latz, D. T. Golenbock, A. J. Coyle, S. M. Liao, and T. Maniatis.** 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nature immunology **4:**491-496.
82. **Fitzgerald, K. A., D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock.** 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med **198:**1043-1055.
83. **Forsbach, A., J. G. Nemorin, C. Montino, C. Muller, U. Samulowitz, A. P. Vicari, M. Jurk, G. K. Mutwiri, A. M. Krieg, G. B. Lipford, and J. Vollmer.** 2008. Identification of RNA sequence motifs stimulating sequence-specific TLR8-dependent immune responses. J Immunol **180:**3729-3738.
84. **Foy, E., K. Li, C. Wang, R. Sumpter, Jr., M. Ikeda, S. M. Lemon, and M. Gale, Jr.** 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science **300:**1145-1148.
85. **Gack, M. U., A. Kirchhofer, Y. C. Shin, K. S. Inn, C. Liang, S. Cui, S. Myong, T. Ha, K. P. Hopfner, and J. U. Jung.** 2008. Roles of RIG-I N-terminal tandem CARD and splice variant in TRIM25-mediated antiviral signal transduction. Proc Natl Acad Sci U S A **105:**16743-16748.

86. **Gack, M. U., Y. C. Shin, C. H. Joo, T. Urano, C. Liang, L. Sun, O. Takeuchi, S. Akira, Z. Chen, S. Inoue, and J. U. Jung.** 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature **446:**916-920.
87. **Gale, M., Jr., and E. M. Foy.** 2005. Evasion of intracellular host defence by hepatitis C virus. Nature **436:**939-945.
88. **Gallatin, W. M., M. J. Gale, Jr., P. A. Hoffman, D. M. Willerford, K. E. Draves, R. E. Benveniste, W. R. Morton, and E. A. Clark.** 1989. Selective replication of simian immunodeficiency virus in a subset of CD4+ lymphocytes. Proc Natl Acad Sci U S A **86:**3301-3305.
89. **Gay, N. J., and F. J. Keith.** 1991. Drosophila Toll and IL-1 receptor. Nature **351:**355-356.
90. **Geng, Y., C. Zhao, A. Song, J. Wang, X. Zhang, T. J. Harrison, Y. Zhou, W. Wang, and Y. Wang.** 2011. The serological prevalence and genetic diversity of hepatitis E virus in farmed rabbits in China. Infect Genet Evol **11:**476-482.
91. **Gerolami, R., P. Borentain, F. Raissouni, A. Motte, C. Solas, and P. Colson.** 2011. Treatment of severe acute hepatitis E by ribavirin. J Clin Virol **52:**60-62.
92. **Gerondakis, S., R. J. Grumont, and A. Banerjee.** 2007. Regulating B-cell activation and survival in response to TLR signals. Immunology and cell biology **85:**471-475.
93. **Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna.** 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci U S A **103:**8459-8464.
94. **Gorbalenya, A. E., E. V. Koonin, A. P. Donchenko, and V. M. Blinov.** 1989. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucleic Acids Res **17:**4713-4730.
95. **Gorbalenya, A. E., E. V. Koonin, and M. M. Lai.** 1991. Putative papain-related thiol proteases of positive-strand RNA viruses. Identification of rubi- and aphthovirus proteases and delineation of a novel conserved domain associated with proteases of rubi-, alpha- and coronaviruses. FEBS Lett **288:**201-205.
96. **Gottipati, K., N. Ruggli, M. Gerber, J. D. Tratschin, M. Benning, H. Bellamy, and K. H. Choi.** 2013. The structure of classical swine fever virus N(pro): a novel cysteine Autoprotease and zinc-binding protein involved in subversion of type I interferon induction. PLoS Pathog **9:**e1003704.
97. **Graff, J., H. Nguyen, C. Kasorndorkbua, P. G. Halbur, M. St Claire, R. H. Purcell, and S. U. Emerson.** 2005. In vitro and in vivo mutational analysis of the 3'-terminal regions of hepatitis e virus genomes and replicons. J Virol **79:**1017-1026.
98. **Graff, J., H. Nguyen, C. Yu, W. R. Elkins, M. St Claire, R. H. Purcell, and S. U. Emerson.** 2005. The open reading frame 3 gene of hepatitis E virus contains a cis-reactive element and encodes a protein required for infection of macaques. J Virol **79:**6680-6689.

99. **Graff, J., U. Torian, H. Nguyen, and S. U. Emerson.** 2006. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. J Virol **80:**5919-5926.
100. **Graff, J., Y. H. Zhou, U. Torian, H. Nguyen, M. St Claire, C. Yu, R. H. Purcell, and S. U. Emerson.** 2008. Mutations within potential glycosylation sites in the capsid protein of hepatitis E virus prevent the formation of infectious virus particles. J Virol **82:**1185-1194.
101. **Guiducci, C., M. Gong, A. M. Cepika, Z. Xu, C. Tripodo, L. Bennett, C. Crain, P. Quartier, J. J. Cush, V. Pascual, R. L. Coffman, and F. J. Barrat.** 2013. RNA recognition by human TLR8 can lead to autoimmune inflammation. J Exp Med **210:**2903-2919.
102. **Guo, J. T., J. Hayashi, and C. Seeger.** 2005. West Nile virus inhibits the signal transduction pathway of alpha interferon. J Virol **79:**1343-1350.
103. **Han, W., X. Li, and X. Fu.** 2011. The macro domain protein family: structure, functions, and their potential therapeutic implications. Mutat Res **727:**86-103.
104. **Haqshenas, G., F. F. Huang, M. Fenaux, D. K. Guenette, F. W. Pierson, C. T. Larsen, H. L. Shivaprasad, T. E. Toth, and X. J. Meng.** 2002. The putative capsid protein of the newly identified avian hepatitis E virus shares antigenic epitopes with that of swine and human hepatitis E viruses and chicken big liver and spleen disease virus. J Gen Virol **83:**2201-2209.
105. **Haqshenas, G., H. L. Shivaprasad, P. R. Woolcock, D. H. Read, and X. J. Meng.** 2001. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. J Gen Virol **82:**2449-2462.
106. **Hartman, A. L., B. H. Bird, J. S. Towner, Z. A. Antoniadou, S. R. Zaki, and S. T. Nichol.** 2008. Inhibition of IRF-3 activation by VP35 is critical for the high level of virulence of ebola virus. J Virol **82:**2699-2704.
107. **Hasan, U., C. Chaffois, C. Gaillard, V. Saulnier, E. Merck, S. Tancredi, C. Guiet, F. Briere, J. Vlach, S. Lebecque, G. Trinchieri, and E. E. Bates.** 2005. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol **174:**2942-2950.
108. **Havell, E. A., B. Berman, C. A. Ogburn, K. Berg, K. Paucker, and J. Vilcek.** 1975. Two antigenically distinct species of human interferon. Proc Natl Acad Sci U S A **72:**2185-2187.
109. **Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem.** 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature **410:**1099-1103.
110. **He, S., J. Miao, Z. Zheng, T. Wu, M. Xie, M. Tang, J. Zhang, M. H. Ng, and N. Xia.** 2008. Putative receptor-binding sites of hepatitis E virus. J Gen Virol **89:**245-249.
111. **Heil, F., P. Ahmad-Nejad, H. Hemmi, H. Hochrein, F. Ampenberger, T. Gellert, H. Dietrich, G. Lipford, K. Takeda, S. Akira, H. Wagner, and S. Bauer.** 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine

uncovers a strong relationship within the TLR7, 8 and 9 subfamily. European journal of immunology **33:**2987-2997.
112. **Helbig, K. J., and M. R. Beard.** 2014. The role of viperin in the innate antiviral response. J Mol Biol **426:**1210-1219.
113. **Hemmi, H., and S. Akira.** 2005. TLR signalling and the function of dendritic cells. Chemical immunology and allergy **86:**120-135.
114. **Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira.** 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nature immunology **3:**196-200.
115. **Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira.** 2000. A Toll-like receptor recognizes bacterial DNA. Nature **408:**740-745.
116. **Hennessy, E. J., A. E. Parker, and L. A. O'Neill.** 2010. Targeting Toll-like receptors: emerging therapeutics? Nature reviews. Drug discovery **9:**293-307.
117. **Herold, J., S. G. Siddell, and A. E. Gorbalenya.** 1999. A human RNA viral cysteine proteinase that depends upon a unique Zn2+-binding finger connecting the two domains of a papain-like fold. J Biol Chem **274:**14918-14925.
118. **Herremans, M., J. Bakker, E. Duizer, H. Vennema, and M. P. Koopmans.** 2007. Use of serological assays for diagnosis of hepatitis E virus genotype 1 and 3 infections in a setting of low endemicity. Clin Vaccine Immunol **14:**562-568.
119. **Ho, L. J., L. F. Hung, C. Y. Weng, W. L. Wu, P. Chou, Y. L. Lin, D. M. Chang, T. Y. Tai, and J. H. Lai.** 2005. Dengue virus type 2 antagonizes IFN-alpha but not IFN-gamma antiviral effect via down-regulating Tyk2-STAT signaling in the human dendritic cell. J Immunol **174:**8163-8172.
120. **Hoebe, K., X. Du, P. Georgel, E. Janssen, K. Tabeta, S. O. Kim, J. Goode, P. Lin, N. Mann, S. Mudd, K. Crozat, S. Sovath, J. Han, and B. Beutler.** 2003. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature **424:**743-748.
121. **Holla, R. P., I. Ahmad, Z. Ahmad, and S. Jameel.** 2013. Molecular Virology of Hepatitis E Virus. Seminars in Liver Disease **33:**3-14.
122. **Honda, K., H. Yanai, T. Mizutani, H. Negishi, N. Shimada, N. Suzuki, Y. Ohba, A. Takaoka, W. C. Yeh, and T. Taniguchi.** 2004. Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. Proc Natl Acad Sci U S A **101:**15416-15421.
123. **Hoofnagle, J. H., K. E. Nelson, and R. H. Purcell.** 2012. Hepatitis E. N Engl J Med **367:**1237-1244.
124. **Horng, T., G. M. Barton, R. A. Flavell, and R. Medzhitov.** 2002. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature **420:**329-333.
125. **Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann.** 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science **314:**994-997.
126. **Horvath, C. M.** 2000. STAT proteins and transcriptional responses to extracellular signals. Trends in biochemical sciences **25:**496-502.

127. **Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira.** 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol **162:**3749-3752.
128. **Hou, F., L. Sun, H. Zheng, B. Skaug, Q. X. Jiang, and Z. J. Chen.** 2011. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell **146:**448-461.
129. **Hou, J., U. Schindler, W. J. Henzel, T. C. Ho, M. Brasseur, and S. L. McKnight.** 1994. An interleukin-4-induced transcription factor: IL-4 Stat. Science **265:**1701-1706.
130. **Huang, F., J. Adelman, H. Jiang, N. I. Goldstein, and P. B. Fisher.** 1999. Differentiation induction subtraction hybridization (DISH): a strategy for cloning genes displaying differential expression during growth arrest and terminal differentiation. Gene **236:**125-131.
131. **Huang, F., J. Adelman, H. Jiang, N. I. Goldstein, and P. B. Fisher.** 1999. Identification and temporal expression pattern of genes modulated during irreversible growth arrest and terminal differentiation in human melanoma cells. Oncogene **18:**3546-3552.
132. **Huang, F. F., Z. F. Sun, S. U. Emerson, R. H. Purcell, H. L. Shivaprasad, F. W. Pierson, T. E. Toth, and X. J. Meng.** 2004. Determination and analysis of the complete genomic sequence of avian hepatitis E virus (avian HEV) and attempts to infect rhesus monkeys with avian HEV. J Gen Virol **85:**1609-1618.
133. **Huang, R., N. Nakazono, K. Ishii, D. Li, O. Kawamata, R. Kawaguchi, and Y. Tsukada.** 1995. Hepatitis E virus (87A strain) propagated in A549 cells. J Med Virol **47:**299-302.
134. **Huang, Y. W., T. Opriessnig, P. G. Halbur, and X. J. Meng.** 2007. Initiation at the third in-frame AUG codon of open reading frame 3 of the hepatitis E virus is essential for viral infectivity in vivo. J Virol **81:**3018-3026.
135. **Ichiyama, K., K. Yamada, T. Tanaka, S. Nagashima, Jirintai, M. Takahashi, and H. Okamoto.** 2009. Determination of the 5'-terminal sequence of subgenomic RNA of hepatitis E virus strains in cultured cells. Arch Virol **154:**1945-1951.
136. **Imaizumi, T., S. Aratani, T. Nakajima, M. Carlson, T. Matsumiya, K. Tanji, K. Ookawa, H. Yoshida, S. Tsuchida, T. M. McIntyre, S. M. Prescott, G. A. Zimmerman, and K. Satoh.** 2002. Retinoic acid-inducible gene-I is induced in endothelial cells by LPS and regulates expression of COX-2. Biochem Biophys Res Commun **292:**274-279.
137. **Jacobs, S. R., and B. Damania.** 2011. The viral interferon regulatory factors of KSHV: immunosuppressors or oncogenes? Frontiers in immunology **2:**19.
138. **Jaitin, D. A., L. C. Roisman, E. Jaks, M. Gavutis, J. Piehler, J. Van der Heyden, G. Uze, and G. Schreiber.** 2006. Inquiring into the differential action of interferons (IFNs): an IFN-alpha2 mutant with enhanced affinity to IFNAR1 is functionally similar to IFN-beta. Mol Cell Biol **26:**1888-1897.
139. **Jameel, S.** 1999. Molecular biology and pathogenesis of hepatitis E virus. Exp. Rev. Mol. Med.

140. **Jameel, S., M. Zafrullah, M. H. Ozdener, and S. K. Panda.** 1996. Expression in animal cells and characterization of the hepatitis E virus structural proteins. J Virol **70:**207-216.
141. **Janeway, C. A., Jr.** 1989. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol **54 Pt 1:**1-13.
142. **Jin, M. S., S. E. Kim, J. Y. Heo, M. E. Lee, H. M. Kim, S. G. Paik, H. Lee, and J. O. Lee.** 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell **130:**1071-1082.
143. **John, L., S. Thomas, O. Herchenroder, B. M. Putzer, and S. Schaefer.** 2011. Hepatitis E virus ORF2 protein activates the pro-apoptotic gene CHOP and anti-apoptotic heat shock proteins. PLoS One **6:**e25378.
144. **Johne, R., A. Plenge-Bonig, M. Hess, R. G. Ulrich, J. Reetz, and A. Schielke.** 2010
Detection of a novel hepatitis E-like virus in faeces of wild rats using a nested broad-spectrum RT-PCR. J Gen Virol **91:**750-758.
145. **Kabrane-Lazizi, Y., M. Zhang, R. H. Purcell, K. D. Miller, R. T. Davey, and S. U. Emerson.** 2001. Acute hepatitis caused by a novel strain of hepatitis E virus most closely related to United States strains. J Gen Virol **82:**1687-1693.
146. **Kadare, G., and A. L. Haenni.** 1997. Virus-encoded RNA helicases. J Virol **71:**2583-2590.
147. **Kagan, J. C., and R. Medzhitov.** 2006. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell **125:**943-955.
148. **Kalie, E., D. A. Jaitin, Y. Podoplelova, J. Piehler, and G. Schreiber.** 2008. The stability of the ternary interferon-receptor complex rather than the affinity to the individual subunits dictates differential biological activities. J Biol Chem **283:**32925-32936.
149. **Kamar, N., R. Bendall, F. Legrand-Abravanel, N. S. Xia, S. Ijaz, J. Izopet, and H. R. Dalton.** 2012. Hepatitis E. Lancet **379:**2477-2488.
150. **Kamar, N., R. P. Bendall, J. M. Peron, P. Cintas, L. Prudhomme, J. M. Mansuy, L. Rostaing, F. Keane, S. Ijaz, J. Izopet, and H. R. Dalton.** 2011. Hepatitis E virus and neurologic disorders. Emerg Infect Dis **17:**173-179.
151. **Kamar, N., H. R. Dalton, F. Abravanel, and J. Izopet.** 2014. Hepatitis E virus infection. Clinical microbiology reviews **27:**116-138.
152. **Kamar, N., L. Rostaing, F. Abravanel, C. Garrouste, S. Lhomme, L. Esposito, G. Basse, O. Cointault, D. Ribes, M. B. Nogier, L. Alric, J. M. Peron, and J. Izopet.** 2010. Ribavirin therapy inhibits viral replication on patients with chronic hepatitis e virus infection. Gastroenterology **139:**1612-1618.
153. **Kang, D. C., R. V. Gopalkrishnan, Q. Wu, E. Jankowsky, A. M. Pyle, and P. B. Fisher.** 2002. mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. Proc Natl Acad Sci U S A **99:**637-642.
154. **Kannan, H., S. Fan, D. Patel, I. Bossis, and Y. J. Zhang.** 2009. The hepatitis E virus open reading frame 3 product interacts with microtubules and interferes with their dynamics. J Virol **83:**6375-6382.

155. **Kapur, N., D. Thakral, H. Durgapal, and S. K. Panda.** 2012. Hepatitis E virus enters liver cells through receptor-dependent clathrin-mediated endocytosis. J Viral Hepat **19:**436-448.
156. **Kar-Roy, A., H. Korkaya, R. Oberoi, S. K. Lal, and S. Jameel.** 2004. The hepatitis E virus open reading frame 3 protein activates ERK through binding and inhibition of the MAPK phosphatase. J Biol Chem **279:**28345-28357.
157. **Karpe, Y. A., and K. S. Lole.** 2011. Deubiquitination activity associated with hepatitis E virus putative papain-like cysteine protease. J Gen Virol **92:**2088-2092.
158. **Karpe, Y. A., and K. S. Lole.** 2011. Deubiquitination activity associated with hepatitis E virus putative papain like cysteine protease. J Gen Virol.
159. **Karpe, Y. A., and K. S. Lole.** 2010. NTPase and 5' to 3' RNA duplex-unwinding activities of the hepatitis E virus helicase domain. J Virol **84:**3595-3602.
160. **Karpe, Y. A., and K. S. Lole.** 2010. RNA 5'-triphosphatase activity of the hepatitis E virus helicase domain. J Virol **84:**9637-9641.
161. **Kato, H., O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T. S. Dermody, T. Fujita, and S. Akira.** 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med **205:**1601-1610.
162. **Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C. S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira.** 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature **441:**101-105.
163. **Katze, M. G., Y. He, and M. Gale, Jr.** 2002. Viruses and interferon: a fight for supremacy. Nature reviews. Immunology **2:**675-687.
164. **Kawai, T., and S. Akira.** 2006. Innate immune recognition of viral infection. Nature immunology **7:**131-137.
165. **Kawai, T., and S. Akira.** 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature immunology **11:**373-384.
166. **Kawai, T., and S. Akira.** 2007. Signaling to NF-kappaB by Toll-like receptors. Trends in molecular medicine **13:**460-469.
167. **Kawai, T., and S. Akira.** 2008. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci **1143:**1-20.
168. **Kawai, T., S. Sato, K. J. Ishii, C. Coban, H. Hemmi, M. Yamamoto, K. Terai, M. Matsuda, J. Inoue, S. Uematsu, O. Takeuchi, and S. Akira.** 2004. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. Nature immunology **5:**1061-1068.
169. **Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira.** 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nature immunology **6:**981-988.
170. **Kenney, S. P., R. S. Pudupakam, Y. W. Huang, F. W. Pierson, T. LeRoith, and X. J. Meng.** 2012. The PSAP motif within the ORF3 protein of an avian strain of the hepatitis E virus is not critical for viral infectivity in vivo but plays a role in virus release. J Virol **86:**5637-5646.

171. **Khuroo, M. S.** 2011. Discovery of hepatitis E: The epidemic non-A, non-B hepatitis 30 years down the memory lane. Virus Res.
172. **Kim, D. H., M. Longo, Y. Han, P. Lundberg, E. Cantin, and J. J. Rossi.** 2004. Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. Nature biotechnology **22:**321-325.
173. **Kisseleva, T., S. Bhattacharya, J. Braunstein, and C. W. Schindler.** 2002. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene **285:**1-24.
174. **Kochs, G., and O. Haller.** 1999. Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids. Proc Natl Acad Sci U S A **96:**2082-2086.
175. **Koizumi, Y., N. Isoda, Y. Sato, T. Iwaki, K. Ono, K. Ido, K. Sugano, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2004. Infection of a Japanese patient by genotype 4 hepatitis e virus while traveling in Vietnam. J Clin Microbiol **42:**3883-3885.
176. **Komuro, A., and C. M. Horvath.** 2006. RNA- and virus-independent inhibition of antiviral signaling by RNA helicase LGP2. J Virol **80:**12332-12342.
177. **Koonin, E. V.** 1991. The phylogeny of RNA-dependent RNA polymerases of positive-strand RNA viruses. J Gen Virol **72 (Pt 9):**2197-2206.
178. **Koonin, E. V., and V. V. Dolja.** 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Critical reviews in biochemistry and molecular biology **28:**375-430.
179. **Koonin, E. V., A. E. Gorbalenya, M. A. Purdy, M. N. Rozanov, G. R. Reyes, and D. W. Bradley.** 1992. Computer-assisted assignment of functional domains in the nonstructural polyprotein of hepatitis E virus: delineation of an additional group of positive-strand RNA plant and animal viruses. Proc Natl Acad Sci U S A **89:**8259-8263.
180. **Korkaya, H., S. Jameel, D. Gupta, S. Tyagi, R. Kumar, M. Zafrullah, M. Mazumdar, S. K. Lal, L. Xiaofang, D. Sehgal, S. R. Das, and D. Sahal.** 2001. The ORF3 protein of hepatitis E virus binds to Src homology 3 domains and activates MAPK. J Biol Chem **276:**42389-42400.
181. **Kotenko, S. V., and J. A. Langer.** 2004. Full house: 12 receptors for 27 cytokines. International immunopharmacology **4:**593-608.
182. **Kubota, T., M. Matsuoka, T. H. Chang, P. Tailor, T. Sasaki, M. Tashiro, A. Kato, and K. Ozato.** 2008. Virus infection triggers SUMOylation of IRF3 and IRF7, leading to the negative regulation of type I interferon gene expression. J Biol Chem **283:**25660-25670.
183. **Kumar, A., S. G. Devi, P. Kar, S. Agarwal, S. A. Husain, R. K. Gupta, and S. Sharma.** 2014. Association of cytokines in hepatitis E with pregnancy outcome. Cytokine **65:**95-104.
184. **Kumthip, K., P. Chusri, N. Jilg, L. Zhao, D. N. Fusco, H. Zhao, K. Goto, D. Cheng, E. A. Schaefer, L. Zhang, C. Pantip, S. Thongsawat, A. O'Brien, L. F. Peng, N. Maneekarn, R. T. Chung, and W. Lin.** 2012. Hepatitis C virus NS5A disrupts STAT1 phosphorylation and suppresses type I interferon signaling. J Virol **86:**8581-8591.

185. **Kwo, P. Y., G. G. Schlauder, H. A. Carpenter, P. J. Murphy, J. E. Rosenblatt, G. J. Dawson, E. E. Mast, K. Krawczynski, and V. Balan.** 1997. Acute hepatitis E by a new isolate acquired in the United States. Mayo Clin Proc **72:**1133-1136.
186. **Larner, A. C., G. Jonak, Y. S. Cheng, B. Korant, E. Knight, and J. E. Darnell, Jr.** 1984. Transcriptional induction of two genes in human cells by beta interferon. Proc Natl Acad Sci U S A **81:**6733-6737.
187. **Lauw, F. N., D. R. Caffrey, and D. T. Golenbock.** 2005. Of mice and man: TLR11 (finally) finds profilin. Trends in immunology **26:**509-511.
188. **Le Goffic, R., J. Pothlichet, D. Vitour, T. Fujita, E. Meurs, M. Chignard, and M. Si-Tahar.** 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. J Immunol **178:**3368-3372.
189. **Leaman, D. W., M. Chawla-Sarkar, B. Jacobs, K. Vyas, Y. Sun, A. Ozdemir, T. Yi, B. R. Williams, and E. C. Borden.** 2003. Novel growth and death related interferon-stimulated genes (ISGs) in melanoma: greater potency of IFN-beta compared with IFN-alpha2. J Interferon Cytokine Res **23:**745-756.
190. **Lee, J., N. Sayed, A. Hunter, K. F. Au, W. H. Wong, E. S. Mocarski, R. R. Pera, E. Yakubov, and J. P. Cooke.** 2012. Activation of innate immunity is required for efficient nuclear reprogramming. Cell **151:**547-558.
191. **Lei, X., X. Liu, Y. Ma, Z. Sun, Y. Yang, Q. Jin, B. He, and J. Wang.** 2010. The 3C protein of enterovirus 71 inhibits retinoid acid-inducible gene I-mediated interferon regulatory factor 3 activation and type I interferon responses. J Virol **84:**8051-8061.
192. **Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann.** 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86:**973-983.
193. **Lengyel, P.** 1982. Biochemistry of Interferons and Their Actions. Annual Review of Biochemistry **51:**251-282.
194. **Lenschow, D. J., N. V. Giannakopoulos, L. J. Gunn, C. Johnston, A. K. O'Guin, R. E. Schmidt, B. Levine, and H. W. t. Virgin.** 2005. Identification of interferon-stimulated gene 15 as an antiviral molecule during Sindbis virus infection in vivo. J Virol **79:**13974-13983.
195. **Levy, D. E., and J. E. Darnell, Jr.** 2002. Stats: transcriptional control and biological impact. Nature reviews **3:**651-662.
196. **Levy, D. E., D. S. Kessler, R. Pine, N. Reich, and J. E. Darnell, Jr.** 1988. Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control. Genes Dev **2:**383-393.
197. **Lew, D. J., T. Decker, and J. E. Darnell, Jr.** 1989. Alpha interferon and gamma interferon stimulate transcription of a single gene through different signal transduction pathways. Mol Cell Biol **9:**5404-5411.
198. **Lhomme, S., C. Garrouste, N. Kamar, K. Saune, F. Abravanel, J. M. Mansuy, M. Dubois, L. Rostaing, and J. Izopet.** 2014. Influence of polyproline region and macro domain genetic heterogeneity on HEV persistence in immunocompromised patients. J Infect Dis **209:**300-303.

199. **Li, H., Z. Zheng, P. Zhou, B. Zhang, Z. Shi, Q. Hu, and H. Wang.** 2010. The cysteine protease domain of porcine reproductive and respiratory syndrome virus non-structural protein 2 antagonizes interferon regulatory factor 3 activation. J Gen Virol **91:**2947-2958.
200. **Li, S. W., J. Zhang, Z. Q. He, Y. Gu, R. S. Liu, J. Lin, Y. X. Chen, M. H. Ng, and N. S. Xia.** 2005. Mutational analysis of essential interactions involved in the assembly of hepatitis E virus capsid. J Biol Chem **280:**3400-3406.
201. **Li, S. W., J. Zhang, Y. M. Li, S. H. Ou, G. Y. Huang, Z. Q. He, S. X. Ge, Y. L. Xian, S. Q. Pang, M. H. Ng, and N. S. Xia.** 2005. A bacterially expressed particulate hepatitis E vaccine: antigenicity, immunogenicity and protectivity on primates. Vaccine **23:**2893-2901.
202. **Li, T. C., K. Chijiwa, N. Sera, T. Ishibashi, Y. Etoh, Y. Shinohara, Y. Kurata, M. Ishida, S. Sakamoto, N. Takeda, and T. Miyamura.** 2005. Hepatitis E virus transmission from wild boar meat. Emerg Infect Dis **11:**1958-1960.
203. **Li, T. C., N. Takeda, T. Miyamura, Y. Matsuura, J. C. Wang, H. Engvall, L. Hammar, L. Xing, and R. H. Cheng.** 2005. Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. J Virol **79:**12999-13006.
204. **Li, T. C., Y. Yamakawa, K. Suzuki, M. Tatsumi, M. A. Razak, T. Uchida, N. Takeda, and T. Miyamura.** 1997. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol **71:**7207-7213.
205. **Li, X., C. T. Ranjith-Kumar, M. T. Brooks, S. Dharmaiah, A. B. Herr, C. Kao, and P. Li.** 2009. The RIG-I-like receptor LGP2 recognizes the termini of double-stranded RNA. J Biol Chem **284:**13881-13891.
206. **Li, X. D., L. Sun, R. B. Seth, G. Pineda, and Z. J. Chen.** 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci U S A **102:**17717-17722.
207. **Li, Y., C. Li, P. Xue, B. Zhong, A. P. Mao, Y. Ran, H. Chen, Y. Y. Wang, F. Yang, and H. B. Shu.** 2009. ISG56 is a negative-feedback regulator of virus-triggered signaling and cellular antiviral response. Proc Natl Acad Sci U S A **106:**7945-7950.
208. **Liang, Y., J. Yao, and S. Gillam.** 2000. Rubella virus nonstructural protein protease domains involved in trans- and cis-cleavage activities. J Virol **74:**5412-5423.
209. **Lim, K. L., K. C. Chew, J. M. Tan, C. Wang, K. K. Chung, Y. Zhang, Y. Tanaka, W. Smith, S. Engelender, C. A. Ross, V. L. Dawson, and T. M. Dawson.** 2005. Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. J Neurosci **25:**2002-2009.
210. **Lin, J. X., T. S. Migone, M. Tsang, M. Friedmann, J. A. Weatherbee, L. Zhou, A. Yamauchi, E. T. Bloom, J. Mietz, S. John, and et al.** 1995. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity **2:**331-339.

211. **Lin, R. J., C. L. Liao, E. Lin, and Y. L. Lin.** 2004. Blocking of the alpha interferon-induced Jak-Stat signaling pathway by Japanese encephalitis virus infection. J Virol **78:**9285-9294.
212. **Linder, P., P. F. Lasko, M. Ashburner, P. Leroy, P. J. Nielsen, K. Nishi, J. Schnier, and P. P. Slonimski.** 1989. Birth of the D-E-A-D box. Nature **337:**121-122.
213. **Lindner, H. A., N. Fotouhi-Ardakani, V. Lytvyn, P. Lachance, T. Sulea, and R. Menard.** 2005. The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme. J Virol **79:**15199-15208.
214. **Liu, H., Y. Fu, D. Jiang, G. Li, J. Xie, Y. Peng, X. Yi, and S. A. Ghabrial.** 2009. A novel mycovirus that is related to the human pathogen hepatitis E virus and rubi-like viruses. J Virol **83:**1981-1991.
215. **Liu, T. X., J. W. Zhang, J. Tao, R. B. Zhang, Q. H. Zhang, C. J. Zhao, J. H. Tong, M. Lanotte, S. Waxman, S. J. Chen, M. Mao, G. X. Hu, L. Zhu, and Z. Chen.** 2000. Gene expression networks underlying retinoic acid-induced differentiation of acute promyelocytic leukemia cells. Blood **96:**1496-1504.
216. **Liu, X., Q. Wang, W. Chen, and C. Wang.** 2013. Dynamic regulation of innate immunity by ubiquitin and ubiquitin-like proteins. Cytokine & growth factor reviews **24:**559-570.
217. **Liu, Y. J.** 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annual review of immunology **23:**275-306.
218. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, CA) **25:**402-408.
219. **Loeb, K. R., and A. L. Haas.** 1992. The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins. J Biol Chem **267:**7806-7813.
220. **Loo, Y. M., D. M. Owen, K. Li, A. K. Erickson, C. L. Johnson, P. M. Fish, D. S. Carney, T. Wang, H. Ishida, M. Yoneyama, T. Fujita, T. Saito, W. M. Lee, C. H. Hagedorn, D. T. Lau, S. A. Weinman, S. M. Lemon, and M. Gale, Jr.** 2006. Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc Natl Acad Sci U S A **103:**6001-6006.
221. **Lu, G., J. T. Reinert, I. Pitha-Rowe, A. Okumura, M. Kellum, K. P. Knobeloch, B. Hassel, and P. M. Pitha.** 2006. ISG15 enhances the innate antiviral response by inhibition of IRF-3 degradation. Cell Mol Biol (Noisy-le-grand) **52:**29-41.
222. **Lu, L., C. Li, and C. H. Hagedorn.** 2005. Phylogenetic analysis of global hepatitis E virus sequences: genetic diversity, subtypes and zoonosis. Rev Med Virol.
223. **Lu, L. L., M. Puri, C. M. Horvath, and G. C. Sen.** 2008. Select paramyxoviral V proteins inhibit IRF3 activation by acting as alternative substrates for inhibitor of kappaB kinase epsilon (IKKe)/TBK1. J Biol Chem **283:**14269-14276.
224. **Lukavsky, P. J.** 2009. Structure and function of HCV IRES domains. Virus Res **139:**166-171.

225. **Luthra, P., D. Sun, R. H. Silverman, and B. He.** 2011. Activation of IFN-β expression by a viral mRNA through RNase L and MDA5. Proc Natl Acad Sci U S A **108:**2118-2123.
226. **Maelfait, J., and R. Beyaert.** 2012. Emerging role of ubiquitination in antiviral RIG-I signaling. Microbiology and molecular biology reviews : MMBR **76:**33-45.
227. **Magden, J., N. Takeda, T. Li, P. Auvinen, T. Ahola, T. Miyamura, A. Merits, and L. Kaariainen.** 2001. Virus-specific mRNA capping enzyme encoded by hepatitis E virus. J Virol **75:**6249-6255.
228. **Malakhova, O. A., and D. E. Zhang.** 2008. ISG15 inhibits Nedd4 ubiquitin E3 activity and enhances the innate antiviral response. J Biol Chem **283:**8783-8787.
229. **Malathi, K., B. Dong, M. Gale, Jr., and R. H. Silverman.** 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. Nature **448:**816-819.
230. **Mallet, V., E. Nicand, P. Sultanik, C. Chakvetadze, S. Tesse, E. Thervet, L. Mouthon, P. Sogni, and S. Pol.** Brief communication: case reports of ribavirin treatment for chronic hepatitis E. Ann Intern Med **153:**85-89.
231. **Mao, A. P., S. Li, B. Zhong, Y. Li, J. Yan, Q. Li, C. Teng, and H. B. Shu.** 2010. Virus-triggered ubiquitination of TRAF3/6 by cIAP1/2 is essential for induction of interferon-beta (IFN-beta) and cellular antiviral response. J Biol Chem **285:**9470-9476.
232. **Marr, L. D., C. Y. Wang, and T. K. Frey.** 1994. Expression of the rubella virus nonstructural protein ORF and demonstration of proteolytic processing. Virology **198:**586-592.
233. **Martzen, M. R., S. M. McCraith, S. L. Spinelli, F. M. Torres, S. Fields, E. J. Grayhack, and E. M. Phizicky.** 1999. A biochemical genomics approach for identifying genes by the activity of their products. Science **286:**1153-1155.
234. **Mast, E. E., M. J. Alter, P. V. Holland, and R. H. Purcell.** 1998. Evaluation of assays for antibody to hepatitis E virus by a serum panel. Hepatitis E Virus Antibody Serum Panel Evaluation Group. Hepatology **27:**857-861.
235. **Matsuda, T., and C. L. Cepko.** 2004. Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc Natl Acad Sci U S A **101:**16-22.
236. **Matsumiya, T., and D. M. Stafforini.** 2010. Function and regulation of retinoic acid-inducible gene-I. Crit Rev Immunol **30:**489-513.
237. **McAtee, C. P., Y. Zhang, P. O. Yarbough, T. Bird, and T. R. Fuerst.** 1996. Purification of a soluble hepatitis E open reading frame 2-derived protein with unique antigenic properties. Protein Expr Purif **8:**262-270.
238. **McWhirter, S. M., B. R. Tenoever, and T. Maniatis.** 2005. Connecting mitochondria and innate immunity. Cell **122:**645-647.
239. **Medzhitov, R.** 2007. Recognition of microorganisms and activation of the immune response. Nature **449:**819-826.
240. **Medzhitov, R., and C. A. Janeway, Jr.** 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell **91:**295-298.
241. **Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr.** 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature **388:**394-397.

242. **Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway, Jr.** 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell **2:**253-258.
243. **Melroe, G. T., L. Silva, P. A. Schaffer, and D. M. Knipe.** 2007. Recruitment of activated IRF-3 and CBP/p300 to herpes simplex virus ICP0 nuclear foci: Potential role in blocking IFN-beta induction. Virology **360:**305-321.
244. **Meng, J., X. Dai, J. C. Chang, E. Lopareva, J. Pillot, H. A. Fields, and Y. E. Khudyakov.** 2001. Identification and characterization of the neutralization epitope(s) of the hepatitis E virus. Virology **288:**203-211.
245. **Meng, X. J.** 2010. Recent advances in Hepatitis E virus. J Viral Hepat **17:**153-161.
246. **Meng, X. J., R. H. Purcell, P. G. Halbur, J. R. Lehman, D. M. Webb, T. S. Tsareva, J. S. Haynes, B. J. Thacker, and S. U. Emerson.** 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci U S A **94:**9860-9865.
247. **Meylan, E., J. Curran, K. Hofmann, D. Moradpour, M. Binder, R. Bartenschlager, and J. Tschopp.** 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature **437:**1167-1172.
248. **Mhaindarkar, V., K. Sharma, and K. S. Lole.** 2014. Mutagenesis of hepatitis E virus helicase motifs: effects on enzyme activity. Virus Res **179:**26-33.
249. **Mibayashi, M., L. Martinez-Sobrido, Y. M. Loo, W. B. Cardenas, M. Gale, Jr., and A. Garcia-Sastre.** 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J Virol **81:**514-524.
250. **Michalek, S. M., R. N. Moore, J. R. McGhee, D. L. Rosenstreich, and S. E. Mergenhagen.** 1980. The primary role of lymphoreticular cells in the mediation of host responses to bacterial endotoxim. J Infect Dis **141:**55-63.
251. **Miyoshi, K., J. M. Shillingford, G. H. Smith, S. L. Grimm, K. U. Wagner, T. Oka, J. M. Rosen, G. W. Robinson, and L. Hennighausen.** 2001. Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. The Journal of cell biology **155:**531-542.
252. **Mizuo, H., K. Suzuki, Y. Takikawa, Y. Sugai, H. Tokita, Y. Akahane, K. Itoh, Y. Gotanda, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2002. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J Clin Microbiol **40:**3209-3218.
253. **Moin, S. M., V. Chandra, R. Arya, and S. Jameel.** 2009. The hepatitis E virus ORF3 protein stabilizes HIF-1alpha and enhances HIF-1-mediated transcriptional activity through p300/CBP. Cell Microbiol **11:**1409-1421.
254. **Moin, S. M., M. Panteva, and S. Jameel.** 2007. The hepatitis E virus Orf3 protein protects cells from mitochondrial depolarization and death. J Biol Chem **282:**21124-21133.
255. **Monastyrskaia, G. S., M. B. Kostina, O. B. Filiukova, E. V. Protopopova, S. N. Konovalova, A. V. Kachko, L. G. Nikolaev, V. B. Loktev, and E. D. Sverdlov.** 2004. [Activation of the RIG-I gene, coding for DEXH/D-protein in infection of RH cells by tick-borne encephalitis virus]. Bioorg Khim **30:**146-150.

256. **Moraga, I., D. Harari, G. Schreiber, G. Uze, and S. Pellegrini.** 2009. Receptor density is key to the alpha2/beta interferon differential activities. Mol Cell Biol **29:**4778-4787.
257. **Mori, Y., and Y. Matsuura.** 2011. Structure of hepatitis E viral particle. Virus Res **161:**59-64.
258. **Morisato, D., and K. V. Anderson.** 1994. The spatzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the Drosophila embryo. Cell **76:**677-688.
259. **Moynagh, P. N.** 2005. TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. Trends in immunology **26:**469-476.
260. **Mukherjee, A., S. A. Morosky, E. Delorme-Axford, N. Dybdahl-Sissoko, M. S. Oberste, T. Wang, and C. B. Coyne.** 2011. The coxsackievirus B 3C protease cleaves MAVS and TRIF to attenuate host type I interferon and apoptotic signaling. PLoS Pathog **7:**e1001311.
261. **Mulla, M. J., K. Myrtolli, S. Tadesse, N. L. Stanwood, A. Gariepy, S. Guller, E. R. Norwitz, and V. M. Abrahams.** 2013. Cutting-edge report: TLR10 plays a role in mediating bacterial peptidoglycan-induced trophoblast apoptosis. Am J Reprod Immunol **69:**449-453.
262. **Nagashima, S., M. Takahashi, D. Jirintai, T. Tanaka, K. Yamada, T. Nishizawa, and H. Okamoto.** A PSAP motif in the ORF3 protein of hepatitis E virus is necessary for virion release from infected cells. J Gen Virol.
263. **Nagashima, S., M. Takahashi, S. Jirintai, T. Tanaka, T. Nishizawa, J. Yasuda, and H. Okamoto.** 2011. Tumour susceptibility gene 101 and the vacuolar protein sorting pathway are required for the release of hepatitis E virions. J Gen Virol **92:**2838-2848.
264. **Nan, Y., Z. Ma, R. Wang, Y. Yu, H. Kannan, B. Fredericksen, and Y. J. Zhang.** 2014. Enhancement of Interferon Induction by ORF3 Product of Hepatitis E Virus. J Virol.
265. **Nan, Y., R. Wang, M. Shen, K. S. Faaberg, S. K. Samal, and Y. J. Zhang.** 2012. Induction of type I interferons by a novel porcine reproductive and respiratory syndrome virus isolate. Virology **432:**261-270.
266. **Nardozzi, J., N. Wenta, N. Yasuhara, U. Vinkemeier, and G. Cingolani.** 2010. Molecular basis for the recognition of phosphorylated STAT1 by importin alpha5. J Mol Biol **402:**83-100.
267. **Nelson, E. A., S. R. Walker, E. Weisberg, M. Bar-Natan, R. Barrett, L. B. Gashin, S. Terrell, J. L. Klitgaard, L. Santo, M. R. Addorio, B. L. Ebert, J. D. Griffin, and D. A. Frank.** 2011. The STAT5 inhibitor pimozide decreases survival of chronic myelogenous leukemia cells resistant to kinase inhibitors. Blood **117:**3421-3429.
268. **Neuvonen, M., and T. Ahola.** 2009. Differential activities of cellular and viral macro domain proteins in binding of ADP-ribose metabolites. J Mol Biol **385:**212-225.
269. **Nusslein-Volhard, C., and E. Wieschaus.** 1980. Mutations affecting segment number and polarity in Drosophila. Nature **287:**795-801.

270. **O'Reilly, E. K., and C. C. Kao.** 1998. Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure. Virology **252:**287-303.
271. **O'Shea, J. J.** 1997. Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet? Immunity **7:**1-11.
272. **Okamoto, H.** 2011. Efficient cell culture systems for hepatitis E virus strains in feces and circulating blood. Rev Med Virol **21:**18-31.
273. **Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya.** 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nature immunology **4:**161-167.
274. **Oshiumi, H., M. Matsumoto, S. Hatakeyama, and T. Seya.** 2009. Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-beta induction during the early phase of viral infection. J Biol Chem **284:**807-817.
275. **Oshiumi, H., M. Sasai, K. Shida, T. Fujita, M. Matsumoto, and T. Seya.** 2003. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem **278:**49751-49762.
276. **Osiak, A., O. Utermohlen, S. Niendorf, I. Horak, and K. P. Knobeloch.** 2005. ISG15, an interferon-stimulated ubiquitin-like protein, is not essential for STAT1 signaling and responses against vesicular stomatitis and lymphocytic choriomeningitis virus. Mol Cell Biol **25:**6338-6345.
277. **Paliwal, D., S. K. Panda, N. Kapur, S. P. Varma, and H. Durgapal.** 2014. Hepatitis E Virus (HEV) Protease: a Chymotrypsin-like Enzyme that Processes both Non-structural (pORF1) and Capsid (pORF2) Protein. J Gen Virol.
278. **Palti, Y.** 2011. Toll-like receptors in bony fish: from genomics to function. Developmental and comparative immunology **35:**1263-1272.
279. **Panda, S. K., I. H. Ansari, H. Durgapal, S. Agrawal, and S. Jameel.** 2000. The in vitro-synthesized RNA from a cDNA clone of hepatitis E virus is infectious. J Virol **74:**2430-2437.
280. **Papon, L., A. Oteiza, T. Imaizumi, H. Kato, E. Brocchi, T. G. Lawson, S. Akira, and N. Mechti.** 2009. The viral RNA recognition sensor RIG-I is degraded during encephalomyocarditis virus (EMCV) infection. Virology **393:**311-318.
281. **Parisien, J. P., J. F. Lau, J. J. Rodriguez, B. M. Sullivan, A. Moscona, G. D. Parks, R. A. Lamb, and C. M. Horvath.** 2001. The V protein of human parainfluenza virus 2 antagonizes type I interferon responses by destabilizing signal transducer and activator of transcription 2. Virology **283:**230-239.
282. **Parvez, M. K.** 2013. Molecular characterization of hepatitis E virus ORF1 gene supports a papain-like cysteine protease (PCP)-domain activity. Virus Res **178:**553-556.
283. **Pasdeloup, D., M. McElwee, F. Beilstein, M. Labetoulle, and F. J. Rixon.** 2013. Herpesvirus tegument protein pUL37 interacts with dystonin/BPAG1 to promote capsid transport on microtubules during egress. J Virol **87:**2857-2867.
284. **Patel, D., Y. Nan, M. Shen, K. Ritthipichai, X. Zhu, and Y. J. Zhang.** 2010. Porcine reproductive and respiratory syndrome virus inhibits type I interferon

signaling by blocking STAT1/STAT2 nuclear translocation. J Virol **84:**11045-11055.

285. **Patel, D., T. Opriessnig, D. A. Stein, P. G. Halbur, X. J. Meng, P. L. Iversen, and Y. J. Zhang.** 2008. Peptide-conjugated morpholino oligomers inhibit porcine reproductive and respiratory syndrome virus replication. Antiviral Res **77:**95-107.
286. **Pehrson, J. R., and V. A. Fried.** 1992. MacroH2A, a core histone containing a large nonhistone region. Science **257:**1398-1400.
287. **Pehrson, J. R., and R. N. Fuji.** 1998. Evolutionary conservation of histone macroH2A subtypes and domains. Nucleic Acids Res **26:**2837-2842.
288. **Perche, P. Y., C. Vourc'h, L. Konecny, C. Souchier, M. Robert-Nicoud, S. Dimitrov, and S. Khochbin.** 2000. Higher concentrations of histone macroH2A in the Barr body are correlated with higher nucleosome density. Curr Biol **10:**1531-1534.
289. **Perttila, J., P. Spuul, and T. Ahola.** 2013. Early secretory pathway localization and lack of processing for hepatitis E virus replication protein pORF1. J Gen Virol **94:**807-816.
290. **Pestka, S.** 2007. The interferons: 50 years after their discovery, there is much more to learn. J Biol Chem **282:**20047-20051.
291. **Pestka, S., C. D. Krause, D. Sarkar, M. R. Walter, Y. Shi, and P. B. Fisher.** 2004. Interleukin-10 and related cytokines and receptors. Annual review of immunology **22:**929-979.
292. **Pestka, S., C. D. Krause, and M. R. Walter.** 2004. Interferons, interferon-like cytokines, and their receptors. Immunological reviews **202:**8-32.
293. **Peters, R. T., S. M. Liao, and T. Maniatis.** 2000. IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex. Mol Cell **5:**513-522.
294. **Pichlmair, A., O. Schulz, C. P. Tan, T. I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa.** 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science **314:**997-1001.
295. **Pisetsky, D. S.** 2012. The origin and properties of extracellular DNA: from PAMP to DAMP. Clin Immunol **144:**32-40.
296. **Platanias, L. C.** 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nature reviews. Immunology **5:**375-386.
297. **Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler.** 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science **282:**2085-2088.
298. **Precious, B., K. Childs, V. Fitzpatrick-Swallow, S. Goodbourn, and R. E. Randall.** 2005. Simian virus 5 V protein acts as an adaptor, linking DDB1 to STAT2, to facilitate the ubiquitination of STAT1. J Virol **79:**13434-13441.
299. **Precious, B., D. F. Young, L. Andrejeva, S. Goodbourn, and R. E. Randall.** 2005. In vitro and in vivo specificity of ubiquitination and degradation of STAT1 and STAT2 by the V proteins of the paramyxoviruses simian virus 5 and human parainfluenza virus type 2. J Gen Virol **86:**151-158.

300. **Prins, K. C., W. B. Cardenas, and C. F. Basler.** 2009. Ebola virus protein VP35 impairs the function of interferon regulatory factor-activating kinases IKKepsilon and TBK-1. J Virol **83:**3069-3077.
301. **Prokunina-Olsson, L., B. Muchmore, W. Tang, R. M. Pfeiffer, H. Park, H. Dickensheets, D. Hergott, P. Porter-Gill, A. Mumy, I. Kohaar, S. Chen, N. Brand, M. Tarway, L. Liu, F. Sheikh, J. Astemborski, H. L. Bonkovsky, B. R. Edlin, C. D. Howell, T. R. Morgan, D. L. Thomas, B. Rehermann, R. P. Donnelly, and T. R. O'Brien.** 2013. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nature genetics **45:**164-171.
302. **Pudupakam, R. S., Y. W. Huang, T. Opriessnig, P. G. Halbur, F. W. Pierson, and X. J. Meng.** 2009. Deletions of the hypervariable region (HVR) in open reading frame 1 of hepatitis E virus do not abolish virus infectivity: evidence for attenuation of HVR deletion mutants in vivo. J Virol **83:**384-395.
303. **Pudupakam, R. S., S. P. Kenney, L. Cordoba, Y. W. Huang, B. A. Dryman, T. Leroith, F. W. Pierson, and X. J. Meng.** 2011. Mutational analysis of the hypervariable region of hepatitis e virus reveals its involvement in the efficiency of viral RNA replication. J Virol **85:**10031-10040.
304. **Purcell, R. H., and S. U. Emerson.** 2008. Hepatitis E: an emerging awareness of an old disease. J Hepatol **48:**494-503.
305. **Purdy, M. A.** 2012. Evolution of the hepatitis E virus polyproline region: order from disorder. J Virol **86:**10186-10193.
306. **Purdy, M. A., J. Lara, and Y. E. Khudyakov.** 2012. The hepatitis e virus polyproline region is involved in viral adaptation. PLoS One **7:**e35974.
307. **Putics, A., W. Filipowicz, J. Hall, A. E. Gorbalenya, and J. Ziebuhr.** 2005. ADP-ribose-1"-monophosphatase: a conserved coronavirus enzyme that is dispensable for viral replication in tissue culture. J Virol **79:**12721-12731.
308. **Putics, A., A. E. Gorbalenya, and J. Ziebuhr.** 2006. Identification of protease and ADP-ribose 1"-monophosphatase activities associated with transmissible gastroenteritis virus non-structural protein 3. J Gen Virol **87:**651-656.
309. **Raj, V. S., S. L. Smits, S. D. Pas, L. B. Provacia, H. Moorman-Roest, A. D. Osterhaus, and B. L. Haagmans.** 2012. Novel hepatitis E virus in ferrets, the Netherlands. Emerg Infect Dis **18:**1369-1370.
310. **Ratia, K., S. Pegan, J. Takayama, K. Sleeman, M. Coughlin, S. Baliji, R. Chaudhuri, W. Fu, B. S. Prabhakar, M. E. Johnson, S. C. Baker, A. K. Ghosh, and A. D. Mesecar.** 2008. A noncovalent class of papain-like protease/deubiquitinase inhibitors blocks SARS virus replication. Proc Natl Acad Sci U S A **105:**16119-16124.
311. **Ratia, K., K. S. Saikatendu, B. D. Santarsiero, N. Barretto, S. C. Baker, R. C. Stevens, and A. D. Mesecar.** 2006. Severe acute respiratory syndrome coronavirus papain-like protease: structure of a viral deubiquitinating enzyme. Proc Natl Acad Sci U S A **103:**5717-5722.
312. **Ratra, R., A. Kar-Roy, and S. K. Lal.** 2008. The ORF3 Protein of Hepatitis E Virus Interacts with Hemopexin by Means of Its 26 Amino Acid N-Terminal Hydrophobic Domain II. Biochemistry.

313. **Ratra, R., A. Kar-Roy, and S. K. Lal.** 2009. ORF3 protein of hepatitis E virus interacts with the Bbeta chain of fibrinogen resulting in decreased fibrinogen secretion from HuH-7 cells. J Gen Virol **90:**1359-1370.
314. **Rebouillat, D., and A. G. Hovanessian.** 1999. The human 2',5'-oligoadenylate synthetase family: interferon-induced proteins with unique enzymatic properties. J Interferon Cytokine Res **19:**295-308.
315. **Rehman, S., N. Kapur, H. Durgapal, and S. K. Panda.** 2008. Subcellular localization of hepatitis E virus (HEV) replicase. Virology **370:**77-92.
316. **Reich, N. C., and L. M. Pfeffer.** 1990. Evidence for involvement of protein kinase C in the cellular response to interferon alpha. Proc Natl Acad Sci U S A **87:**8761-8765.
317. **Reid, S. P., L. W. Leung, A. L. Hartman, O. Martinez, M. L. Shaw, C. Carbonnelle, V. E. Volchkov, S. T. Nichol, and C. F. Basler.** 2006. Ebola virus VP24 binds karyopherin alpha1 and blocks STAT1 nuclear accumulation. J Virol **80:**5156-5167.
318. **Reis e Sousa, C.** 2004. Toll-like receptors and dendritic cells: for whom the bug tolls. Seminars in immunology **16:**27-34.
319. **Reyes, G. R., C. C. Huang, A. W. Tam, and M. A. Purdy.** 1993. Molecular organization and replication of hepatitis E virus (HEV). Arch Virol Suppl **7:**15-25.
320. **Reyes, G. R., M. A. Purdy, J. P. Kim, K. C. Luk, L. M. Young, K. E. Fry, and D. W. Bradley.** 1990. Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science **247:**1335-1339.
321. **Reyes, G. R., P. O. Yarbough, A. W. Tam, M. A. Purdy, C. C. Huang, J. S. Kim, D. W. Bradley, and K. E. Fry.** 1991. Hepatitis E virus (HEV): the novel agent responsible for enterically transmitted non-A, non-B hepatitis. Gastroenterol Jpn **26 Suppl 3:**142-147.
322. **Reymond, A., G. Meroni, A. Fantozzi, G. Merla, S. Cairo, L. Luzi, D. Riganelli, E. Zanaria, S. Messali, S. Cainarca, A. Guffanti, S. Minucci, P. G. Pelicci, and A. Ballabio.** 2001. The tripartite motif family identifies cell compartments. The EMBO journal **20:**2140-2151.
323. **Roach, J. C., G. Glusman, L. Rowen, A. Kaur, M. K. Purcell, K. D. Smith, L. E. Hood, and A. Aderem.** 2005. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci U S A **102:**9577-9582.
324. **Roberts, W. K., A. Hovanessian, R. E. Brown, M. J. Clemens, and I. M. Kerr.** 1976. Interferon-mediated protein kinase and low-molecular-weight inhibitor of protein synthesis. Nature **264:**477-480.
325. **Robinson, R. A., W. H. Burgess, S. U. Emerson, R. S. Leibowitz, S. A. Sosnovtseva, S. Tsarev, and R. H. Purcell.** 1998. Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. Protein Expr Purif **12:**75-84.
326. **Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. Bazan.** 1998. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A **95:**588-593.

327. **Rolls, A., R. Shechter, A. London, Y. Ziv, A. Ronen, R. Levy, and M. Schwartz.** 2007. Toll-like receptors modulate adult hippocampal neurogenesis. Nat Cell Biol **9:**1081-1088.
328. **Ronco, L. V., A. Y. Karpova, M. Vidal, and P. M. Howley.** 1998. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. Genes Dev **12:**2061-2072.
329. **Ropp, S. L., A. W. Tam, B. Beames, M. Purdy, and T. K. Frey.** 2000. Expression of the hepatitis E virus ORF1. Arch Virol **145:**1321-1337.
330. **Rosenstreich, D. L., L. M. Glode, and S. E. Mergenhagen.** 1977. Action of endotoxin on lymphoid cells. J Infect Dis **136 Suppl:**S239-245.
331. **Rosetto, M., Y. Engstrom, C. T. Baldari, J. L. Telford, and D. Hultmark.** 1995. Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line. Biochem Biophys Res Commun **209:**111-116.
332. **Rothenfusser, S., N. Goutagny, G. DiPerna, M. Gong, B. G. Monks, A. Schoenemeyer, M. Yamamoto, S. Akira, and K. A. Fitzgerald.** 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. J Immunol **175:**5260-5268.
333. **Rozanov, M. N., E. V. Koonin, and A. E. Gorbalenya.** 1992. Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. J Gen Virol **73 (Pt 8):**2129-2134.
334. **Rubinstein, M., S. Rubinstein, P. C. Familletti, R. S. Miller, A. A. Waldman, and S. Pestka.** 1979. Human leukocyte interferon: production, purification to homogeneity, and initial characterization. Proc Natl Acad Sci U S A **76:**640-644.
335. **Sabroe, I., S. K. Dower, and M. K. Whyte.** 2005. The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. Clin Infect Dis **41 Suppl 7:**S421-426.
336. **Sadler, A. J., and B. R. Williams.** 2008. Interferon-inducible antiviral effectors. Nature reviews. Immunology **8:**559-568.
337. **Sadler, G. J., G. F. Mells, N. H. Shah, I. M. Chesner, and R. P. Walt.** 2006. UK acquired hepatitis E-An emerging problem? J Med Virol **78:**473-475.
338. **Saikatendu, K. S., J. S. Joseph, V. Subramanian, T. Clayton, M. Griffith, K. Moy, J. Velasquez, B. W. Neuman, M. J. Buchmeier, R. C. Stevens, and P. Kuhn.** 2005. Structural basis of severe acute respiratory syndrome coronavirus ADP-ribose-1"-phosphate dephosphorylation by a conserved domain of nsP3. Structure **13:**1665-1675.
339. **Saito, T., R. Hirai, Y. M. Loo, D. Owen, C. L. Johnson, S. C. Sinha, S. Akira, T. Fujita, and M. Gale, Jr.** 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci U S A **104:**582-587.
340. **Salomoni, P., and P. P. Pandolfi.** 2002. The role of PML in tumor suppression. Cell **108:**165-170.
341. **Sanford, B. J., T. Opriessnig, S. P. Kenney, B. A. Dryman, L. Cordoba, and X. J. Meng.** 2012. Assessment of the cross-protective capability of recombinant capsid proteins derived from pig, rat, and avian hepatitis E viruses (HEV) against challenge with a genotype 3 HEV in pigs. Vaccine **30:**6249-6255.

342. **Sasai, M., H. Oshiumi, M. Matsumoto, N. Inoue, F. Fujita, M. Nakanishi, and T. Seya.** 2005. Cutting Edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. J Immunol **174:**27-30.
343. **Sato, S., M. Sugiyama, M. Yamamoto, Y. Watanabe, T. Kawai, K. Takeda, and S. Akira.** 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol **171:**4304-4310.
344. **Satoh, T., H. Kato, Y. Kumagai, M. Yoneyama, S. Sato, K. Matsushita, T. Tsujimura, T. Fujita, S. Akira, and O. Takeuchi.** 2010. LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. Proc Natl Acad Sci U S A **107:**1512-1517.
345. **Schindler, C., D. E. Levy, and T. Decker.** 2007. JAK-STAT signaling: from interferons to cytokines. J Biol Chem **282:**20059-20063.
346. **Schindler, C., K. Shuai, V. R. Prezioso, and J. E. Darnell, Jr.** 1992. Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science **257:**809-813.
347. **Schindler, C., and I. Strehlow.** 2000. Cytokines and STAT signaling. Adv Pharmacol **47:**113-174.
348. **Schlauder, G. G., S. M. Desai, A. R. Zanetti, N. C. Tassopoulos, and I. K. Mushahwar.** 1999. Novel hepatitis E virus (HEV) isolates from Europe: evidence for additional genotypes of HEV. J Med Virol **57:**243-251.
349. **Schlee, M.** 2013. Master sensors of pathogenic RNA - RIG-I like receptors. Immunobiology **218:**1322-1335.
350. **Schlee, M., and G. Hartmann.** 2010. The Chase for the RIG-I Ligand-Recent Advances. Molecular Therapy **18:**1254-1262.
351. **Schlee, M., A. Roth, V. Hornung, C. A. Hagmann, V. Wimmenauer, W. Barchet, C. Coch, M. Janke, A. Mihailovic, G. Wardle, S. Juranek, H. Kato, T. Kawai, H. Poeck, K. A. Fitzgerald, O. Takeuchi, S. Akira, T. Tuschl, E. Latz, J. Ludwig, and G. Hartmann.** 2009. Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. Immunity **31:**25-34.
352. **Schmidt, M., B. Raghavan, V. Muller, T. Vogl, G. Fejer, S. Tchaptchet, S. Keck, C. Kalis, P. J. Nielsen, C. Galanos, J. Roth, A. Skerra, S. F. Martin, M. A. Freudenberg, and M. Goebeler.** 2010. Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. Nature immunology **11:**814-819.
353. **Schneider, D. S., K. L. Hudson, T. Y. Lin, and K. V. Anderson.** 1991. Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the Drosophila embryo. Genes Dev **5:**797-807.
354. **Schofield, D. J., J. Glamann, S. U. Emerson, and R. H. Purcell.** 2000. Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. J Virol **74:**5548-5555.

355. **Schoggins, J. W., and C. M. Rice.** 2011. Interferon-stimulated genes and their antiviral effector functions. Current opinion in virology **1:**519-525.
356. **Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning.** 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem **274:**17406-17409.
357. **Sehgal, D., S. Thomas, M. Chakraborty, and S. Jameel.** 2006. Expression and processing of the Hepatitis E virus ORF1 nonstructural polyprotein. Virology journal **3:**38.
358. **Sekimoto, T., N. Imamoto, K. Nakajima, T. Hirano, and Y. Yoneda.** 1997. Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. The EMBO journal **16:**7067-7077.
359. **Semiletov, Y. A., E. V. Dementeva, T. L. Yashina, M. O. Favorov, and V. A. Shibnev.** 1995. Synthesis and Antigenic Activity of Peptides from the Orf3 Protein-Sequence of Hepatitis-E Virus. Bioorg Khim+ **21:**156-157.
360. **Sen, A., L. Rott, N. Phan, G. Mukherjee, and H. B. Greenberg.** 2014. Rotavirus NSP1 protein inhibits interferon-mediated STAT1 activation. J Virol **88:**41-53.
361. **Sen, N., M. Sommer, X. Che, K. White, W. T. Ruyechan, and A. M. Arvin.** 2010. Varicella-zoster virus immediate-early protein 62 blocks interferon regulatory factor 3 (IRF3) phosphorylation at key serine residues: a novel mechanism of IRF3 inhibition among herpesviruses. J Virol **84:**9240-9253.
362. **Seo, J. Y., and P. Cresswell.** 2013. Viperin regulates cellular lipid metabolism during human cytomegalovirus infection. PLoS Pathog **9:**e1003497.
363. **Seth, R. B., L. Sun, C. K. Ea, and Z. J. Chen.** 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell **122:**669-682.
364. **Shanker, V., G. Trincucci, H. M. Heim, and H. T. Duong.** 2013. Protein phosphatase 2A impairs IFNalpha-induced antiviral activity against the hepatitis C virus through the inhibition of STAT1 tyrosine phosphorylation. J Viral Hepat **20:**612-621.
365. **Sharma, S., B. R. tenOever, N. Grandvaux, G. P. Zhou, R. Lin, and J. Hiscott.** 2003. Triggering the interferon antiviral response through an IKK-related pathway. Science **300:**1148-1151.
366. **Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto.** 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med **189:**1777-1782.
367. **Shrestha, M. P., R. M. Scott, D. M. Joshi, M. P. Mammen, Jr., G. B. Thapa, N. Thapa, K. S. Myint, M. Fourneau, R. A. Kuschner, S. K. Shrestha, M. P. David, J. Seriwatana, D. W. Vaughn, A. Safary, T. P. Endy, and B. L. Innis.** 2007. Safety and efficacy of a recombinant hepatitis E vaccine. N Engl J Med **356:**895-903.
368. **Shuai, K., G. R. Stark, I. M. Kerr, and J. E. Darnell, Jr.** 1993. A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. Science **261:**1744-1746.

369. **Shukla, P., H. T. Nguyen, U. Torian, R. E. Engle, K. Faulk, H. R. Dalton, R. P. Bendall, F. E. Keane, R. H. Purcell, and S. U. Emerson.** 2011. Cross-species infections of cultured cells by hepatitis E virus and discovery of an infectious virus-host recombinant. Proc Natl Acad Sci U S A **108:**2438-2443.
370. **Siegal, F. P., N. Kadowaki, M. Shodell, P. A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y. J. Liu.** 1999. The nature of the principal type 1 interferon-producing cells in human blood. Science **284:**1835-1837.
371. **Singleton, M. R., M. S. Dillingham, and D. B. Wigley.** 2007. Structure and mechanism of helicases and nucleic acid translocases. Annu Rev Biochem **76:**23-50.
372. **Smith, D. B., M. A. Purdy, and P. Simmonds.** 2013. Genetic variability and the classification of hepatitis E virus. J Virol **87:**4161-4169.
373. **Sporn, M. B., and A. B. Roberts.** 1985. Autocrine growth factors and cancer. Nature **313:**745-747.
374. **Stack, J., I. R. Haga, M. Schroder, N. W. Bartlett, G. Maloney, P. C. Reading, K. A. Fitzgerald, G. L. Smith, and A. G. Bowie.** 2005. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. J Exp Med **201:**1007-1018.
375. **Stadler, M., M. K. Chelbi-Alix, M. H. Koken, L. Venturini, C. Lee, A. Saib, F. Quignon, L. Pelicano, M. C. Guillemin, C. Schindler, and et al.** 1995. Transcriptional induction of the PML growth suppressor gene by interferons is mediated through an ISRE and a GAS element. Oncogene **11:**2565-2573.
376. **Steen, H. C., and A. M. Gamero.** 2013. STAT2 phosphorylation and signaling. Jak-Stat **2:**e25790.
377. **Stevenson, N. J., N. M. Bourke, E. J. Ryan, M. Binder, L. Fanning, J. A. Johnston, J. E. Hegarty, A. Long, and C. O'Farrelly.** 2013. Hepatitis C virus targets the interferon-alpha JAK/STAT pathway by promoting proteasomal degradation in immune cells and hepatocytes. FEBS Lett **587:**1571-1578.
378. **Strahle, L., D. Garcin, and D. Kolakofsky.** 2006. Sendai virus defective-interfering genomes and the activation of interferon-beta. Virology **351:**101-111.
379. **Sultzer, B. M.** 1968. Genetic control of leucocyte responses to endotoxin. Nature **219:**1253-1254.
380. **Sun, Z., Z. Chen, S. R. Lawson, and Y. Fang.** 2010. The cysteine protease domain of porcine reproductive and respiratory syndrome virus nonstructural protein 2 possesses deubiquitinating and interferon antagonism functions. J Virol **84:**7832-7846.
381. **Suppiah, S., Y. Zhou, and T. K. Frey.** 2011. Lack of Processing of the Expressed ORF1 Gene Product of Hepatitis E Virus. Virology journal **8:**245.
382. **Surjit, M., S. Jameel, and S. K. Lal.** 2004. The ORF2 protein of hepatitis E virus binds the 5' region of viral RNA. J Virol **78:**320-328.
383. **Surjit, M., B. Varshney, and S. K. Lal.** 2012. The ORF2 glycoprotein of hepatitis E virus inhibits cellular NF-kappaB activity by blocking ubiquitination mediated proteasomal degradation of IkappaBalpha in human hepatoma cells. BMC biochemistry **13:**7.
384. **Suthar, M. S., H. J. Ramos, M. M. Brassil, J. Netland, C. P. Chappell, G. Blahnik, A. McMillan, M. S. Diamond, E. A. Clark, M. J. Bevan, and M.**

Gale, Jr. 2012. The RIG-I-like receptor LGP2 controls CD8(+) T cell survival and fitness. Immunity **37:**235-248.

385. **Takahashi, K., N. Kitajima, N. Abe, and S. Mishiro.** 2004. Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer. Virology **330:**501-505.
386. **Takahashi, M., T. Nishizawa, H. Sato, Y. Sato, Jirintai, S. Nagashima, and H. Okamoto.** 2011. Analysis of the full-length genome of a hepatitis E virus isolate obtained from a wild boar in Japan that is classifiable into a novel genotype. J Gen Virol **92:**902-908.
387. **Takahashi, M., K. Yamada, Y. Hoshino, H. Takahashi, K. Ichiyama, T. Tanaka, and H. Okamoto.** 2008. Monoclonal antibodies raised against the ORF3 protein of hepatitis E virus (HEV) can capture HEV particles in culture supernatant and serum but not those in feces. Archives of Virology **153:**1703-1713.
388. **Takahashi, N., Y. Takahashi, and F. W. Putnam.** 1985. Periodicity of leucine and tandem repetition of a 24-amino acid segment in the primary structure of leucine-rich alpha 2-glycoprotein of human serum. Proc Natl Acad Sci U S A **82:**1906-1910.
389. **Takahasi, K., M. Yoneyama, T. Nishihori, R. Hirai, H. Kumeta, R. Narita, M. Gale, Jr., F. Inagaki, and T. Fujita.** 2008. Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. Mol Cell **29:**428-440.
390. **Takaoka, A., and H. Yanai.** 2006. Interferon signalling network in innate defence. Cell Microbiol **8:**907-922.
391. **Takeuchi, O., T. Kawai, H. Sanjo, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, K. Takeda, and S. Akira.** 1999. TLR6: A novel member of an expanding toll-like receptor family. Gene **231:**59-65.
392. **Takeuchi, T., T. Kobayashi, S. Tamura, and H. Yokosawa.** 2006. Negative regulation of protein phosphatase 2Cbeta by ISG15 conjugation. FEBS Lett **580:**4521-4526.
393. **Tam, A. W., M. M. Smith, M. E. Guerra, C. C. Huang, D. W. Bradley, K. E. Fry, and G. R. Reyes.** 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology **185:**120-131.
394. **Tam, A. W., R. White, E. Reed, M. Short, Y. Zhang, T. R. Fuerst, and R. E. Lanford.** 1996. In vitro propagation and production of hepatitis E virus from in vivo-infected primary macaque hepatocytes. Virology **215:**1-9.
395. **Tei, S., N. Kitajima, K. Takahashi, and S. Mishiro.** 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet **362:**371-373.
396. **Terenzi, F., P. Saikia, and G. C. Sen.** 2008. Interferon-inducible protein, P56, inhibits HPV DNA replication by binding to the viral protein E1. The EMBO journal **27:**3311-3321.
397. **Theofilopoulos, A. N., R. Baccala, B. Beutler, and D. H. Kono.** 2005. Type I interferons (alpha/beta) in immunity and autoimmunity. Annual review of immunology **23:**307-336.
398. **Tidswell, M., W. Tillis, S. P. Larosa, M. Lynn, A. E. Wittek, R. Kao, J. Wheeler, J. Gogate, and S. M. Opal.** 2010. Phase 2 trial of eritoran tetrasodium

(E5564), a toll-like receptor 4 antagonist, in patients with severe sepsis. Critical care medicine **38:**72-83.

399. **Tojima, Y., A. Fujimoto, M. Delhase, Y. Chen, S. Hatakeyama, K. Nakayama, Y. Kaneko, Y. Nimura, N. Motoyama, K. Ikeda, M. Karin, and M. Nakanishi.** 2000. NAK is an IkappaB kinase-activating kinase. Nature **404:**778-782.
400. **Tsai, C. J., B. Ma, Y. Y. Sham, S. Kumar, and R. Nussinov.** 2001. Structured disorder and conformational selection. Proteins **44:**418-427.
401. **Tsarev, S. A., S. U. Emerson, G. R. Reyes, T. S. Tsareva, L. J. Legters, I. A. Malik, M. Iqbal, and R. H. Purcell.** 1992. Characterization of a prototype strain of hepatitis E virus. Proc Natl Acad Sci U S A **89:**559-563.
402. **Tsarev, S. A., T. S. Tsareva, S. U. Emerson, A. Z. Kapikian, J. Ticehurst, W. London, and R. H. Purcell.** 1993. ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame-2 protein expressed in insect cells: identification of HEV infection in primates. J Infect Dis **168:**369-378.
403. **Tu, D., Z. Zhu, A. Y. Zhou, C. H. Yun, K. E. Lee, A. V. Toms, Y. Li, G. P. Dunn, E. Chan, T. Thai, S. Yang, S. B. Ficarro, J. A. Marto, H. Jeon, W. C. Hahn, D. A. Barbie, and M. J. Eck.** 2013. Structure and ubiquitination-dependent activation of TANK-binding kinase 1. Cell reports **3:**747-758.
404. **Turan, K., M. Mibayashi, K. Sugiyama, S. Saito, A. Numajiri, and K. Nagata.** 2004. Nuclear MxA proteins form a complex with influenza virus NP and inhibit the transcription of the engineered influenza virus genome. Nucleic Acids Res **32:**643-652.
405. **Tyagi, S., S. Jameel, and S. K. Lal.** 2001. The full-length and N-terminal deletion of ORF2 protein of hepatitis E virus can dimerize. Biochem Biophys Res Commun **286:**214-221.
406. **Tyagi, S., S. Jameel, and S. K. Lal.** 2001. Self-association and mapping of the interaction domain of hepatitis E virus ORF3 protein. J Virol **75:**2493-2498.
407. **Tyagi, S., H. Korkaya, M. Zafrullah, S. Jameel, and S. K. Lal.** 2002. The phosphorylated form of the ORF3 protein of hepatitis E virus interacts with its non-glycosylated form of the major capsid protein, ORF2. J Biol Chem **277:**22759-22767.
408. **Tyagi, S., M. Surjit, and S. K. Lal.** 2005. The 41-amino-acid C-terminal region of the hepatitis E virus ORF3 protein interacts with bikunin, a kunitz-type serine protease inhibitor. J Virol **79:**12081-12087.
409. **Tyagi, S., M. Surjit, A. K. Roy, S. Jameel, and S. K. Lal.** 2004. The ORF3 protein of hepatitis E virus interacts with liver-specific alpha1-microglobulin and its precursor alpha1-microglobulin/bikunin precursor (AMBP) and expedites their export from the hepatocyte. J Biol Chem **279:**29308-29319.
410. **Tzeng, W. P., M. H. Chen, C. A. Derdeyn, and T. K. Frey.** 2001. Rubella virus DI RNAs and replicons: requirement for nonstructural proteins acting in cis for amplification by helper virus. Virology **289:**63-73.
411. **Ullman, A. J., and P. Hearing.** 2008. Cellular proteins PML and Daxx mediate an innate antiviral defense antagonized by the adenovirus E4 ORF3 protein. J Virol **82:**7325-7335.

412. **Uze, G., G. Schreiber, J. Piehler, and S. Pellegrini.** 2007. The receptor of the type I interferon family. Curr Top Microbiol Immunol **316:**71-95.
413. **Valente, G., L. Ozmen, F. Novelli, M. Geuna, G. Palestro, G. Forni, and G. Garotta.** 1992. Distribution of interferon-gamma receptor in human tissues. European journal of immunology **22:**2403-2412.
414. **van der Poel, W. H., F. Verschoor, R. van der Heide, M. I. Herrera, A. Vivo, M. Kooreman, and A. M. de Roda Husman.** 2001. Hepatitis E virus sequences in swine related to sequences in humans, The Netherlands. Emerg Infect Dis **7:**970-976.
415. **van Kasteren, P. B., B. A. Bailey-Elkin, T. W. James, D. K. Ninaber, C. Beugeling, M. Khajehpour, E. J. Snijder, B. L. Mark, and M. Kikkert.** 2013. Deubiquitinase function of arterivirus papain-like protease 2 suppresses the innate immune response in infected host cells. Proc Natl Acad Sci U S A **110:**E838-847.
416. **Veals, S. A., C. Schindler, D. Leonard, X. Y. Fu, R. Aebersold, J. E. Darnell, Jr., and D. E. Levy.** 1992. Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins. Mol Cell Biol **12:**3315-3324.
417. **Venkataraman, T., M. Valdes, R. Elsby, S. Kakuta, G. Caceres, S. Saijo, Y. Iwakura, and G. N. Barber.** 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. J Immunol **178:**6444-6455.
418. **Verpooten, D., Y. Ma, S. Hou, Z. Yan, and B. He.** 2009. Control of TANK-binding kinase 1-mediated signaling by the gamma(1)34.5 protein of herpes simplex virus 1. J Biol Chem **284:**1097-1105.
419. **Wakao, H., F. Gouilleux, and B. Groner.** 1994. Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. The EMBO journal **13:**2182-2191.
420. **Walter, M. R., W. T. Windsor, T. L. Nagabhushan, D. J. Lundell, C. A. Lunn, P. J. Zauodny, and S. K. Narula.** 1995. Crystal structure of a complex between interferon-gamma and its soluble high-affinity receptor. Nature **376:**230-235.
421. **Wang, C., J. Pflugheber, R. Sumpter, Jr., D. L. Sodora, D. Hui, G. C. Sen, and M. Gale, Jr.** 2003. Alpha interferon induces distinct translational control programs to suppress hepatitis C virus RNA replication. J Virol **77:**3898-3912.
422. **Wang, D., L. Fang, P. Li, L. Sun, J. Fan, Q. Zhang, R. Luo, X. Liu, K. Li, H. Chen, Z. Chen, and S. Xiao.** 2011. The leader proteinase of foot-and-mouth disease virus negatively regulates the type I interferon pathway by acting as a viral deubiquitinase. J Virol **85:**3758-3766.
423. **Wang, H., W. Zhang, B. Ni, H. Shen, Y. Song, X. Wang, S. Shao, X. Hua, and L. Cui.** Recombination analysis reveals a double recombination event in hepatitis E virus. Virology journal **7:**129.
424. **Wang, R., Y. Nan, Y. Yu, and Y. J. Zhang.** 2013. Porcine reproductive and respiratory syndrome virus Nsp1beta inhibits interferon-activated JAK/STAT signal transduction by inducing karyopherin-alpha1 degradation. J Virol **87:**5219-5228.
425. **Weber, F., V. Wagner, S. B. Rasmussen, R. Hartmann, and S. R. Paludan.** 2006. Double-stranded RNA is produced by positive-strand RNA viruses and

DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol **80:**5059-5064.

426. **Wedemeyer, H., S. Pischke, and M. P. Manns.** 2012. Pathogenesis and treatment of hepatitis e virus infection. Gastroenterology **142:**1388-1397 e1381.
427. **Wei, S., P. Walsh, R. Huang, and S. S. To.** 2000. 93G, a novel sporadic strain of hepatitis E virus in South China isolated by cell culture. J Med Virol **61:**311-318.
428. **West, A. P., A. A. Koblansky, and S. Ghosh.** 2006. Recognition and signaling by toll-like receptors. Annual review of cell and developmental biology **22:**409-437.
429. **WHO.** 2011. Hepatitis E, Viral hepatitis in the WHO south-east Asia region. World Health Organization, India.
430. **Worm, H. C., G. G. Schlauder, H. Wurzer, and I. K. Mushahwar.** 2000. Identification of a novel variant of hepatitis E virus in Austria: sequence, phylogenetic and serological analysis. J Gen Virol **81:**2885-2890.
431. **Worm, H. C., W. H. van der Poel, and G. Brandstatter.** 2002. Hepatitis E: an overview. Microbes and infection / Institut Pasteur **4:**657-666.
432. **Xiaofang, L., M. Zafrullah, F. Ahmad, and S. Jameel.** 2001. A C-Terminal Hydrophobic Region is Required for Homo-Oligomerization of the Hepatitis E Virus Capsid (ORF2) Protein. J Biomed Biotechnol **1:**122-128.
433. **Xing, L., J. C. Wang, T. C. Li, Y. Yasutomi, J. Lara, Y. Khudyakov, D. Schofield, S. U. Emerson, R. H. Purcell, N. Takeda, T. Miyamura, and R. H. Cheng.** Spatial configuration of hepatitis e virus antigenic domain. J Virol **85:**1117-1124.
434. **Xu, L. G., Y. Y. Wang, K. J. Han, L. Y. Li, Z. Zhai, and H. B. Shu.** 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol Cell **19:**727-740.
435. **Yamada, K., M. Takahashi, Y. Hoshino, H. Takahashi, K. Ichiyama, S. Nagashima, T. Tanaka, and H. Okamoto.** 2009. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. J Gen Virol **90:**1880-1891.
436. **Yamamoto, K., F. W. Quelle, W. E. Thierfelder, B. L. Kreider, D. J. Gilbert, N. A. Jenkins, N. G. Copeland, O. Silvennoinen, and J. N. Ihle.** 1994. Stat4, a novel gamma interferon activation site-binding protein expressed in early myeloid differentiation. Mol Cell Biol **14:**4342-4349.
437. **Yamashita, T., Y. Mori, N. Miyazaki, R. H. Cheng, M. Yoshimura, H. Unno, R. Shima, K. Moriishi, T. Tsukihara, T. C. Li, N. Takeda, T. Miyamura, and Y. Matsuura.** 2009. Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure. Proc Natl Acad Sci U S A **106:**12986-12991.
438. **Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski.** 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. Nature **395:**284-288.
439. **Yang, Z., H. Liang, Q. Zhou, Y. Li, H. Chen, W. Ye, D. Chen, J. Fleming, H. Shu, and Y. Liu.** 2012. Crystal structure of ISG54 reveals a novel RNA binding structure and potential functional mechanisms. Cell Res **22:**1328-1338.

440. **Yazaki, Y., H. Mizuo, M. Takahashi, T. Nishizawa, N. Sasaki, Y. Gotanda, and H. Okamoto.** 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol **84:**2351-2357.
441. **Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y. M. Loo, M. Gale, Jr., S. Akira, S. Yonehara, A. Kato, and T. Fujita.** 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol **175:**2851-2858.
442. **Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nature immunology **5:**730-737.
443. **Yu, C., D. Boon, S. L. McDonald, T. G. Myers, K. Tomioka, H. Nguyen, R. E. Engle, S. Govindarajan, S. U. Emerson, and R. H. Purcell.** 2010. Pathogenesis of hepatitis E virus and hepatitis C virus in chimpanzees: similarities and differences. J Virol **84:**11264-11278.
444. **Yu, S., J. Chen, M. Wu, H. Chen, N. Kato, and Z. Yuan.** 2010. Hepatitis B virus polymerase inhibits RIG-I- and Toll-like receptor 3-mediated beta interferon induction in human hepatocytes through interference with interferon regulatory factor 3 activation and dampening of the interaction between TBK1/IKKepsilon and DDX3. J Gen Virol **91:**2080-2090.
445. **Zafrullah, M., M. H. Ozdener, R. Kumar, S. K. Panda, and S. Jameel.** 1999. Mutational analysis of glycosylation, membrane translocation, and cell surface expression of the hepatitis E virus ORF2 protein. J Virol **73:**4074-4082.
446. **Zafrullah, M., M. H. Ozdener, S. K. Panda, and S. Jameel.** 1997. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. J Virol **71:**9045-9053.
447. **Zeidler, M. P., E. A. Bach, and N. Perrimon.** 2000. The roles of the Drosophila JAK/STAT pathway. Oncogene **19:**2598-2606.
448. **Zeng, W., M. Xu, S. Liu, L. Sun, and Z. J. Chen.** 2009. Key role of Ubc5 and lysine-63 polyubiquitination in viral activation of IRF3. Mol Cell **36:**315-325.
449. **Zhang, D., G. Zhang, M. S. Hayden, M. B. Greenblatt, C. Bussey, R. A. Flavell, and S. Ghosh.** 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. Science **303:**1522-1526.
450. **Zhang, M., R. H. Purcell, and S. U. Emerson.** 2001. Identification of the 5' terminal sequence of the SAR-55 and MEX-14 strains of hepatitis E virus and confirmation that the genome is capped. J Med Virol **65:**293-295.
451. **Zhang, N. N., S. H. Shen, L. J. Jiang, W. Zhang, H. X. Zhang, Y. P. Sun, X. Y. Li, Q. H. Huang, B. X. Ge, S. J. Chen, Z. G. Wang, Z. Chen, and J. Zhu.** 2008. RIG-I plays a critical role in negatively regulating granulocytic proliferation. Proc Natl Acad Sci U S A **105:**10553-10558.
452. **Zhang, W., X. Hua, Q. Shen, S. Yang, H. Yin, and L. Cui.** 2011. Identification of genotype 4 Hepatitis E virus binding proteins on swine liver cells. Virology journal **8:**482.
453. **Zhang, X., C. Wang, L. B. Schook, R. J. Hawken, and M. S. Rutherford.** 2000. An RNA helicase, RHIV -1, induced by porcine reproductive and

respiratory syndrome virus (PRRSV) is mapped on porcine chromosome 10q13. Microbial pathogenesis **28:**267-278.

454. **Zhang, Y., P. McAtee, P. O. Yarbough, A. W. Tam, and T. Fuerst.** 1997. Expression, characterization, and immunoreactivities of a soluble hepatitis E virus putative capsid protein species expressed in insect cells. Clin Diagn Lab Immunol **4:**423-428.
455. **Zhang, Y. J., K. Y. Wang, D. A. Stein, D. Patel, R. Watkins, H. M. Moulton, P. L. Iversen, and D. O. Matson.** 2007. Inhibition of replication and transcription activator and latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus by morpholino oligomers. Antiviral Res **73:**12-23.
456. **Zhao, C., Z. Ma, T. J. Harrison, R. Feng, C. Zhang, Z. Qiao, J. Fan, H. Ma, M. Li, A. Song, and Y. Wang.** 2009. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. J Med Virol **81:**1371-1379.
457. **Zheng, Z. Z., J. Miao, M. Zhao, M. Tang, A. E. Yeo, H. Yu, J. Zhang, and N. S. Xia.** Role of heat-shock protein 90 in hepatitis E virus capsid trafficking. J Gen Virol **91:**1728-1736.
458. **Zhong, Z., Z. Wen, and J. E. Darnell, Jr.** 1994. Stat3 and Stat4: members of the family of signal transducers and activators of transcription. Proc Natl Acad Sci U S A **91:**4806-4810.
459. **Zhou, Y. H., R. H. Purcell, and S. U. Emerson.** 2004. An ELISA for putative neutralizing antibodies to hepatitis E virus detects antibodies to genotypes 1, 2, 3, and 4. Vaccine **22:**2578-2585.
460. **Zhu, F. C., J. Zhang, X. F. Zhang, C. Zhou, Z. Z. Wang, S. J. Huang, H. Wang, C. L. Yang, H. M. Jiang, J. P. Cai, Y. J. Wang, X. Ai, Y. M. Hu, Q. Tang, X. Yao, Q. Yan, Y. L. Xian, T. Wu, Y. M. Li, J. Miao, M. H. Ng, J. W. Shih, and N. S. Xia.** Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. Lancet **376:**895-902.