ABSTRACT

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Hepatitis E virus (HEV) causes several outbreaks of hepatitis in humans. Many aspects of HEV pathogenesis are not well understood. The HEV ORF3 product (henceforth known as vp13) is a multifunctional protein essential for infection of animals. To better understand the vp13 functions, this study was performed. We observed that vp13 protein was associated with the microtubules (MT) in transfected cells. Mutational studies revealed that both hydrophobic domains at the N-terminal region of vp13 are required for the vp13-MT interaction. Our studies also showed that HEV vp13 protein increased the stability of the MT, activated the apoptotic pathway, and, increased the levels of tumor suppressor gene p53 and its downstream effector $p21^{Cip/WAF1}$ in the transfected cells. However, no noticeable effect on cell survival was observed. These results indicated that HEV vp13 protein may act as a viral regulatory protein.

FUNCTIONAL CHARACTERIZATION OF THE INTERACTION OF HEPATITIS E VIRUS ORF3 PRODUCT WITH THE CYTOSKELETON

By

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Dedication

I wholeheartedly dedicate this work to my parents, Mr. T. K. Kannan and Mrs. Radha Kannan, my husband Govindarajan Dhanasekaran and my son Rishi who, through their unconditional love, support and many sacrifices, have helped me achieve this great accomplishment.

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List of Abbreviations

aa	Amino acid
ALT	Alanine transaminase
DMEM	Dulbecco's Minimum Essential Medium
FBS	Fetal bovine serum
FHF	Fulminant hepatic failure
FITC	Fluorescein isothiocyanate
HEV	Hepatitis E virus
kb	Kilo-bases
kDa	Kilo Dalton
MAP	Microtubule-associated protein
MAPK	Mitogen-activated protein kinase
MT	Microtubule
MTS	Microtubule stabilization
NOC	Nocodazole
nt	Nucleotide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PARP-1	Poly(ADP-ribose) polymerase-1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
SDS	Sodium dodecyl sulphate

TCA	Trichloroacetic acid
UTR	Untranslated region

Chapter 1: General Background

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus that is classified as the sole member of the genus *Hepevirus* (Emerson *et al.*, 2004; Emerson & Purcell, 2003). HEV is the causative agent of hepatitis outbreaks in developing countries and sporadic cases of acute hepatitis in developed countries (Emerson *et al.*, 2004; Emerson & Purcell, 2003). It is transmitted by feco-oral route, mainly through contaminated water, although perinatal and parenteral routes have been documented. HEV infection is more common among young adults and the mortality rate ranges from 0.5 to 3%. A fulminant form of hepatitis E that occurs in pregnant women, especially in their third trimester of gestation, is a striking feature of HEV infection and can lead to up to 20% mortality rate for the infected woman. (Purcell & Emerson, 2001).

There are four major genotypes of HEV strains and a single known serotype. HEV is a zoonotic agent and animal HEV strains in swine and chicken are found in the U.S. (Haqshenas *et al.*, 2001; Meng *et al.*, 1997). Swine HEV was isolated in US, Japan and many other countries and their sequences were identified to be similar to locally-characterized human HEV isolates. HEV was also isolated from wild deer and undercooked deer meat (Li *et al.*, 2005; Tei *et al.*, 2003). These data show that HEV is indeed a zoonotic virus and domestic swine, wild deer and boars are potential reservoirs of HEV in nature (Goens & Perdue, 2004). Other species with reported susceptibility to HEV infection include sheep (Usmanov *et al.*, 1994), rat (Maneerat *et al.*, 1996) and mouse (Karetnyi Iu *et al.*, 1993).

The genome of HEV is about 7.2 kb in length and consists of three open reading frames (ORF) (Tam *et al.*, 1991). ORF1 encodes a nonstructural polyprotein that includes the RNA-dependent RNA polymerase. ORF2 encodes the capsid protein – a major structural protein in the virion. ORF3 encodes a phosphoprotein that was found to be essential for experimental HEV infection in macaques and pigs (Graff *et al.*, 2005; Huang *et al.*, 2007). The exact functions of vp13 in HEV infection remain unknown though a number of studies have found that it may play a role in cellular signaling pathways (Kar-Roy *et al.*, 2004; Korkaya *et al.*, 2001; Moin *et al.*, 2007; Tyagi *et al.*, 2002; Tyagi *et al.*, 2005; Tyagi *et al.*, 2004; Zafrullah *et al.*, 1997).

Attempts to gain more insights into HEV infection, pathogenesis or basic viral replication mechanisms have been hindered by several reasons that include lack of an effective in vitro cell culture system and a suitable animal model. Therefore, propagation of HEV and studies of virus replication still rely upon non-human primates or cells transfected with cloned replicons of HEV (Emerson *et al.*, 2001; Panda *et al.*, 2000). To specifically study the function of individual HEV proteins, scientists rely on overexpression of these proteins in mammalian cells transiently or stably. Due to lack of efficient cell culture for HEV propagation, functional study of ORF3 in HEV biology and infection has been limited, and therefore, the function of the protein is not yet clearly defined. Hence, this study was performed in an attempt to gain insights into the roles of HEV vp13 protein in viral pathogenesis.

In the following sections, literature on HEV is reviewed, background for this study is introduced, results and discussion are presented accordingly.

Chapter 2: Literature Review

Hepatitis, or inflammation of the liver, is a serious illness that often leads to very high mortality in human beings. There are several etiologies for hepatitis among which infectious agents play a significant role. Several bacteria and viruses directly or indirectly lead to liver damage. Common etiological agents of viral hepatitis include: Hepatitis A virus (*Picornaviridae*), Hepatitis B virus (*Hepadnaviridae*), Hepatitis C virus (*Flaviviridae*), Hepatitis D virus (unclassified agent) and Hepatitis E virus (*Hepeviridae*).

2.1 Introduction on Hepatitis E

Hepatitis E is now recognized as an important public health problem in developing countries. It has been reported that, an estimated one-third of world's population has been infected with Hepatitis E virus on the basis of seroprevalence. It has been a common cause of major epidemics of water-borne hepatitis in Southeast Asia for at least 50 years. The disease was previously referred as enterically transmitted non-A, non-B hepatitis. Hepatitis E is caused by infection with HEV, a non-enveloped, single-stranded, positive-sense RNA virus. Hepatitis E virus is transmitted primarily via the fecal-oral route (Panda *et al.*, 2007). Other documented routes of transmission include parenteral transmission, zoonotic food-borne transmission and vertical transmission. Several reports have suggested the possibility of a parenteral mode of infection/transmission for HEV (Chauhan *et al.*, 1993). Hepatitis E is primarily a water-borne disease, and contaminated water and food supplies have been implicated in major outbreaks (Arankalle *et al.*, 1994).

HEV causes acute and sporadic viral epidemics but usually rare chronic infections. Clinical cases occur predominantly in developing countries in Asia, Africa, and Mexico (Purcell & Emerson, 2001). Recently, hepatitis E has also been recognized frequently in industrialized countries where it was not thought to be endemic. The existence of autochthonous hepatitis E in developed countries like France, Germany and England indicates that hepatitis E is no longer an imported disease (Buisson & Nicand, 2006; Dalton *et al.*, 2007; Preiss *et al.*, 2006). Onset of symptoms usually occurs about 28 to 36 days post-infection (Balayan *et al.*, 1983). Typical signs and symptoms of hepatitis E include jaundice, malaise, anorexia, abdominal pain, vomiting and hepatomegaly. It is usually an acute self-limiting disease, running a course of few weeks. In uncomplicated cases, recovery usually takes place within a month. Recently, more cases of persistent HEV infection with chronic hepatitis and cirrhosis are reported in patients with reduced immune surveillance as induced by chemotherapy or post-transplant immune suppression (Khuroo & Khuroo, 2008).

HEV causes an unusually fulminant form of hepatitis in pregnant women, especially in their third trimesters of pregnancies (Khuroo *et al.*, 2004). Mortality rate in these cases are approximately 20%. HEV-induced damage to Kupffer cells in the liver may allow for damage to the liver from endotoxins produced by intestinal gram-negative bacteria, which is referred as a kind of Schwartzman-like phenomenon and has been postulated as a possible reason for the high incidence of fulminant hepatitis in pregnant women (Goens & Perdue, 2004) . It has also been reported that 30-100% of fetuses or infants acquire the virus from their HEV-infected mothers (Khuroo *et al.*, 1995; Kumar *et al.*, 2001; Singh *et*

al., 2003). HEV is also responsible for sporadic fulminant hepatitis in adults and children (Nanda *et al.*, 1994).

2.2 Biology of Hepatitis E Virus

The unavailability of a suitable culture system for laboratory propagation of HEV has largely hampered better understanding of HEV biology. Conventional cell culture techniques to cultivate this virus have so far not been successful. However, some continuous cell lines such as A549, 2BS, HepG2, KMB17 and BEL7402 have been useful to some extent since they are reported to be slightly susceptible to HEV (Huang *et al.*, 1992; Le *et al.*, 2001; Li *et al.*, 1995), but hardly used or repeated elsewhere due to their extremely low efficiency or other unknown reasons. A549, human lung carcinoma cell line has been used on numerous occasions to isolate and cultivate HEV by some investigators (Huang *et al.*, 1995; Huang *et al.*, 1992; Le *et al.*, 2001; Wei *et al.*, 2000). Recently, a group from Japan reported the propagation of HEV in hepatocarcinoma cell line (PLC/PRF/5) with a very long cultivation time over two months (Takahashi *et al.*, 2008). Many cell lines, including Vero and HeLa cells, do not seem to be sensitive to HEV replication (Le *et al.*, 2001). Several research groups are actively searching for a suitable cell culture system for *in vitro* propagation of HEV. An alternative approach has been widely pursued by several research groups around the world by using HEV replicons or RNA transcripts. In 2004, Emerson *et al* reported the detection of viral antigen and the recovery of infectious virus from transfected PLC/PRF/5 and Huh-7 cells. These findings show that HEV can replicate in a limited number of cell lines of hepatic origin. However, these are not robust systems and HEV still remains an extremely

difficult virus to study *in vitro* (Purcell & Emerson, 2001). The replicon approach has been helpful in studying basic mechanisms of HEV replication and other biological characteristics.

Several species of non-human primates have been utilized in studies involving disease reproduction and transmission and also for amplification of HEV. Experimental infections have been carried out in non-human subjects like chimpanzees, cynomolgus macaques, rhesus macaques, pig tail monkeys, African green monkeys, tamarins, owl monkeys and squirrel monkeys (Ray *et al.*, 1991; Tsarev *et al.*, 1993). The most useful subjects are cynomolgus and rhesus monkeys. Other animals such as pigs (Balayan *et al.*, 1990; Usmanov *et al.*, 1991), rats (Maneerat *et al.*, 1996) and chickens (Haqshenas *et al.*, 2002; Huang *et al.*, 2002a) have also been reported to be susceptible to HEV infections. Though the animals experimentally infected with HEV usually show biochemical and histological evidence of hepatitis, surprisingly, they do not develop clinical disease and therefore do not mimic human infections (Longer *et al.*, 1993).

Extensive sequence divergence is found among HEV strains isolated around the world. Phylogenetic analyses have classified HEV isolates into four major genotypes. Genotype 1 (Asia, North Africa) was initially thought to infect only humans, but a report from Cambodia showed that it was also detected from a pig (Zhang *et al.*, 2008). Genotype 2 is prevalent in Mexico and Southern Africa. Genotype 3 (North and South America, Europe, Asia) is prevalent in swine herds and humans all over the world, and Genotype 4 (Asia) has a wide host range, from human to swine and some other animals

(Schlauder & Mushahwar, 2001). Cross protection between HEV genotype 1 and 4 has been demonstrated in rhesus macaques (Huang *et al.*, 2008).

It is believed that an environmental reservoir of HEV exists, especially in the endemic areas, resulting in recurrent epidemic episodes. However, HEV is thermally much less stable than hepatitis A virus (Emerson *et al.*, 2005). HEV is very fragile under conditions of high salt concentrations, freeze thawing and pelleting (Bradley *et al.*, 1992). Individuals who have sporadic or sub-acute/sub-clinical hepatitis E could also be a potential reservoir for HEV. Numerous studies have indicated the presence of HEV in feces of swine, and identified HEV antibodies in sera of pigs (Clayson *et al.*, 1995; Meng *et al.*, 1998b; Meng *et al.*, 1997), cattle (Favorov *et al.*, 2000), sheep (Favorov *et al.*, 2000), goats (Favorov *et al.*, 2000), horses (Saad *et al.*, 2007), and rodents (He *et al.*, 2002; Kabrane-Lazizi *et al.*, 1999a; Karetnyi Iu *et al.*, 1993), further suggesting the possibility of several animal reservoirs. The isolation of Swine HEV (Meng *et al.*, 1997) and Avian HEV (Haqshenas *et al.*, 2001) further confirms the existence of animal reservoirs. Swine isolate and US strain of human HEV shows 99% sequence identity (Meng *et al.*, 1997). HEV isolates of genotype 3 are grouped into three phylogenetic clusters, with the highest nucleotide identity being 94.4-100% between human and swine isolates in each cluster (Takahashi *et al.*, 2003). Experimental evidence of cross-species infection between human and swine HEV isolates have been reported (Meng, 2003). Avian HEV shows 50% sequence identity to human HEV and is considered to be placed in a separate genotype 5 (Haqshenas *et al.*, 2001; Huang *et al.*, 2004).

HEV belongs to the family *Hepeviridae*, genus hepevirus. It shares some similarity in virion size and structure with members of the family *Caliciviridae* and hence was initially classified under that family (Miller, 1995). However, HEV was later removed from this family since its genome organization was found to be very different from that of the members of the Calicivirus family. Based on the presence of homologous regions present across the genome and the production of subgenomic RNA during replication, HEV was once thought to be a non-enveloped alpha-like virus (Purdy *et al.*, 1993). Phylogenetic analysis of the RNA helicase and RNA-dependent RNA polymerase (RdRp) regions of HEV denoted that HEV formed a distinct group, closer to rubella virus (family *Togaviridae*) than to the members of *Caliciviridae* (Koonin *et al.*, 1992). Finally, the virus was assigned into a separate family *Hepeviridae* (Emerson *et al.*, 2004).

Due to lack of efficient cell culture system and a useful animal model, the HEV replication mechanism was proposed on the basis of similarities and sequence homology to other better characterized positive-sense RNA viruses (Reyes *et al.*, 1993). The genomic RNA in HEV replicates through a negative-strand RNA intermediate. The presence of both positive and negative-strand HEV RNA are identified in the liver of rhesus monkeys experimentally infected, while only positive strand RNA was found in the serum and bile, where only mature virions are expected in an experimental study using rhesus monkeys (Nanda *et al.*, 1994). In another study using swine animal model, replicative negative-strand HEV RNA was detected in the small intestines, lymph nodes, colons, and livers indicating the possibility of HEV replication in tissues other than liver (Williams *et al.*, 2001). For initiation of HEV replication, cap-dependant translation has

been shown to be essential. (Kabrane-Lazizi *et al.*, 1999b). It has been demonstrated by RNA-protein interaction study, the specific binding of purified and refolded recombinant HEV RdRp protein to the 3′ end of its RNA genome containing the poly(A) stretch (Agrawal *et al.*, 2001). Though several reports have attempted to elucidate the replication mechanism of HEV, the many unknowns remain.

2.3 Molecular Virology

HEV genome is a positive-sense, single-stranded RNA genome that is approximately 7 kb long (Fig. 1). It is a non-enveloped virus that has a diameter of 27-34 nm. The virus particles were first visualized in stool samples using immune electron microscopy (Balayan *et al.*, 1983). Virus preparations from stool samples revealed a virion density of 1.29 g/ml in potassium tartarate and glycerol gradient (Bradley *et al.*, 1992).

The first full-length genome sequence of HEV was determined in 1991 for a strain from Burma (Tam *et al.*, 1991). HEV genome is 7.2 kb in size with three open reading frames (Aye *et al.*, 1992; Kabrane-Lazizi *et al.*, 1999b; Purdy *et al.*, 1993; Tam *et al.*, 1991; Yin *et al.*, 1994). At the 5′ terminus of the genome is a 28-nucleotide (nt) long small untranslated region (UTR) which forms a hairpin structure (Huang *et al.*, 1992). The genomic RNA of HEV also possesses a methylated cap at its 5′ terminus (Kabrane-Lazizi *et al.*, 1999b; Zhang *et al.*, 2001). The 3′ end of HEV is polyadenylated and a 68

nt long UTR precedes the poly A tail (Tam *et al.*, 1991). These 5′ and 3′ *cis* acting elements are believed to play important roles in viral replication and transcription (Agrawal *et al.*, 2001; Purdy *et al.*, 1993). In infected liver cells of an experimentally infected monkey, two subgenomic viral RNA of 3.7 and 2.0 kb in length were detected, in addition to full-length genomic RNA (Fig. 1B). These subgenomic RNA share the same 3' terminus.

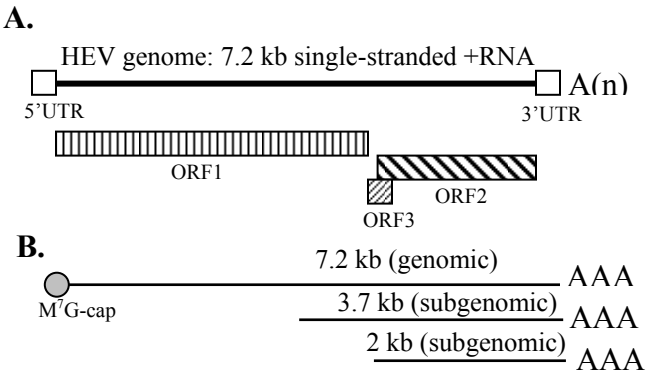

Fig. 1 Genome organization of hepatitis E virus (A) and viral RNAs (B). A. The HEV genome encodes three open reading frames. B. The genomic RNA is capped. Three viral RNAs were found in the liver of experimentally infected monkey.

ORF1, that begins approximately 30 nt downstream of the 5′ end of the RNA, is 5073-5124 nt long and encodes the non-structural proteins (Purcell & Emerson, 2001). The coding region of ORF1 contains a methyltransferase domain, Y domain, papain-like cysteine protease domain, proline-rich hinge or spacer region, X domain, helicase and RNA-dependent RNA polymerase (RdRp) domains (Koonin *et al.*, 1992). The methyltransferase and RdRp activities of ORF1-encoded protein have been demonstrated (Agrawal *et al.*, 2001; Magden *et al.*, 2001). Helicase and protease activities, however, have not been shown yet. The functions of the Y and X domains also remain unknown. The methyltransferase domain is believed to be responsible for RNA capping as

evidenced by the presence of a 5' cap structure on the viral genome (Kabrane-Lazizi *et al.*, 1999b; Zhang *et al.*, 2001). The RNA helicase of HEV is ~38 kDa (Panda, 2000) and found to contain all the conserved motifs of helicase superfamily I. The HEV helicase, as other helicases, probably promotes the unwinding of RNA-RNA duplexes during the process of genomic replication and transcription. The RdRp of HEV is about 36 kDa and is probably involved in early stages of viral replication (Panda, 2000). HEV RdRp possesses characteristics similar to that of other positive sense animal and plant viruses (Fry *et al.*, 1992; Koonin *et al.*, 1992). It was shown that the HEV RdRP specifically binds to the 3' end of the viral RNA and directs the synthesis of complementary RNA (Agrawal *et al.*, 2001).

ORF 2 encodes the viral major capsid protein - the major viral immunogenic protein. The capsid protein has high basic amino acid (aa) content (Jameel *et al.*, 1992; Tam *et al.*, 1991). Both non-glycosylated (~74 kDa) and glycosylated (~88 kDa) forms of the capsid protein were observed in transfected cells (Jameel *et al.*, 1992; Tam *et al.*, 1991). However, the functional role of the glycosylation is not known. A signal sequence is present at the N-terminal region of the protein (Zafrullah *et al.*, 1999). Major epitopes exist in the capsid protein and ORF2 is highly conserved (Khudyakov Yu *et al.*, 1994). Therefore, most serological tests are based on ORF2 product. ORF2-endcoded protein has been expressed in several protein expression systems namely, *E. coli* (Li *et al.*, 1997; Panda *et al.*, 1995); insect cells (He *et al.*, 1993; Li *et al.*, 1997; Robinson *et al.*, 1998), and mammalian cells (Carl *et al.*, 1994; Jameel *et al.*, 1996; Torresi *et al.*, 1999).

Graff et al demonstrated that a bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 products (Graff *et al.*, 2006). The subgenomic RNA is capped and initiated at nt 5122 downstream of the first two methionine codons in ORF3 and two closely spaced methionine codons in different reading frames were used for the initiation of ORF3 and ORF2 translation. In this report, they showed that the sizes of ORF2 and ORF3 products of genotype 4 do not differ in size from those of other three genotypes as it was previously thought.

ORF3-encoded protein (vp13) is a phosphoprotein that plays a major role in cell signaling pathways (Sehgal *et al.*, 2006). vp13 was shown to be not necessary for infection of hepatoma cells in vitro (Emerson *et al.*, 2006b). However, a recent study by Huang and colleagues showed that an intact ORF3 is required for infectivity in pigs (Huang *et al.*, 2007). The latter group also observed that a single mutation at the third in-frame AUG of ORF3 completely abolishes virus infectivity *in vivo,* indicating that the third in-frame AUG in the junction region is required for virus infection and is the initiation site for vp13. A cis-acting element at the start of the ORF is shown to be required for replication and this sequence is highly conserved across all four mammalian genotypes of HEV (Graff *et al.*, 2006).

2.4 Animal HEV and epidemiology

HEV RNA or HEV antibodies have been found in various animal species. HEV in swine and in avian species have been isolated and relatively well studied. Hence, literature of swine and avian HEV are reviewed in the following sections.

2.4.1 Swine HEV

HEV infection among domestic pigs was first reported in Nepal (Clayson *et al.*, 1995). Subsequently, a novel swine HEV was identified and characterized(Meng *et al.*, 1997). Swine HEV is closely related to the human HEV. Their studies also showed that swine HEV is a ubiquitous agent and most of the pigs, in the Midwestern part of US that are 3 months or older are seropositive for swine HEV. Currently, swine HEV is endemic to the North American commercial swine population (Goens & Perdue, 2004). Epidemiological studies have also shown that swine HEV is present in various parts of the world (Chandler *et al.*, 1999; Hsieh *et al.*, 1999; Wu *et al.*, 2000).

Pigs infected with swine HEV do not exhibit clinical signs of the disease. The characteristics of infection such as route of infection, transmission, virus tropism, clinical course, virus shedding etc. have been studied in pigs that were either naturally or experimentally-infected (Choi & Chae, 2003; Kasorndorkbua *et al.*, 2004; Kasorndorkbua *et al.*, 2003; Meng *et al.*, 1998a; Meng *et al.*, 1998b; Williams *et al.*, 2001). Experimentally-infected animals did not show any clinical disease or show abnormal levels of liver enzyme (Meng *et al.*, 1998a). Some of these studies also showed that swine HEV replicates in tissues other than the liver (Williams *et al.*, 2001). In an attempt to determine if pregnant pigs suffer from fulminant hepatitis, like pregnant women, pregnant gilts were infected with swine HEV through the intravenous route (Kasorndorkbua *et al.*, 2003). However, the inoculated animals did not exhibit any clinical symptoms. On the other hand, studies have shown that swine HEV can infect non-human primates and vice versa (Meng *et al.*, 1998b), indicating a zoonotic potential

of swine HEV. Indeed, some studies have also shown that swine handlers are at an increased risk of getting HEV infection (Meng *et al.*, 1999; Meng *et al.*, 2002).

Swine HEV is widely present in commercial pig populations around the world. And, swine HEV is genetically and antigenically closely-related to human HEV genotype 3. Collectively, these findings not only emphasize the zoonotic potential of swine HEV but also its adverse implications in xenotransplantations. Hence, there is an increased need for research on swine HEV, especially with respect to its zoonotic potential. Since swine HEV is ubiquitous and the infected-animals appear apparently normal without much decrease in their productivity, there is no immediate demand for development of diagnostics or vaccines.

2.4.2 Avian HEV

A novel HEV was isolated from US chickens suffering from hepatitis-splenomegaly syndrome (Haqshenas *et al.*, 2001). The virus was found to be closely related to, but distinct from, other HEVs and was named avian HEV. The individual genes of avian HEV were found to show 47-61% amino acid identities with those of other HEVs (Haqshenas *et al.*, 2001). The complete genomic sequence of an avian HEV was first published in 2004 and found to be 6654 nt long, 600 nt shorter than human and swine HEVs (Huang *et al.*, 2004). The isolates of avian HEVs also exhibit considerable sequence divergence among themselves (Billam *et al.*, 2007; Sun *et al.*, 2004a). Unlike swine HEV, avian HEV was unable to infect and produce disease in non-human primates (Huang *et al.*, 2004). However, avian HEV was able to infect and seroconvert turkeys (Sun *et al.*, 2004b). It would be interesting to test if swine HEV can infect chickens or

vice versa. Serological studies show that avian HEV is widespread in the US poultry population (Huang *et al.*, 2002b). Avian HEV is also detected in a layer flock in Ontario, Canada (Agunos *et al.*, 2006).

It is believed that as in the case of swine HEV, avian HEV might also cause inapparent subclinical infections in chickens. Avian HEV seems to produce disease in older birds, especially broiler breeders, from 30 to 72 weeks of age. Affected birds showed enlarged liver and spleen, regressed ovaries and red abdominal fluid (Riddell, 1997). Infected young birds, on the other hand, usually did not show any clinical symptoms (Sun *et al.*, 2004a; Sun *et al.*, 2004b). A systematic study was conducted to assess the pathogenesis and replication of avian HEV in experimentally-infected birds (Billam *et al.*, 2005). The authors observed that the virus was able to produce gross and microscopic lesions in the liver and was able to produce slight increase in liver enzyme levels (Billam *et al.*, 2005). A recent attempt to study vertical transmission of avian HEV has shown that though the virus is present in the egg white, there is no evidence of vertical transmission for avian HEV (Guo *et al.*, 2007a). It is interesting to note that though human HEV has been observed to exhibit vertical transmission, both swine and avian HEV do not show such transmission. Since avian HEV is a newly identified agent and does not seem to cause clinically important disease in birds, very few research efforts have been taken for developing vaccines or other control measures. The only study till date was using a recombinant capsid protein to provide protection in chicken (Guo *et al.*, 2007b).

2.5 Pathogenesis and pathology

Most of the knowledge currently gained regarding the pathogenesis and pathology of HEV has largely been through experimental infections of animals. Infection is primarily through the feco-oral route. However, parenteral routes of infection have also been postulated (Arankalle & Chobe, 1999; Khuroo *et al.*, 2004; Wang *et al.*, 1993). HEV infections are usually acute with no chronic states. The virus is presumed to replicate primarily in the liver (Tsarev *et al.*, 1992) . With fecal-oral transmission, jaundice occurs after a short viremic period and elevated serum alanine transaminase (ALT) levels (Jameel, 1999). Clinical symptoms occurs at this stage and includes jaundice, anorexia, hepatomegaly, abdominal pain and tenderness, nausea and vomiting and fever (Goens & Perdue, 2004). The incubation period ranges from 3 to 8 weeks with an average of 40 days (Purcell & Emerson, 2001; Yarbough, 1999). The virus is excreted in bile and passed out in feces, which then serves as a source of infection for new individuals.

Experimental infection of non-human primates with HEV has also led to important information about the course of illness, virus excretion, changes in liver enzyme profile, histological changes in liver etc. (Arankalle *et al.*, 1995). HEV infections have very low mortality rate of about 1% in normal individuals, but higher than those with HAV infections, 0.2% (Goens & Perdue, 2004). However, in pregnant women the mortality can reach as high as 20%, which is believed to be due to an abnormal immune reaction in these subjects (Jameel, 1999; Purcell & Emerson, 2001).

Major histopathological changes that occur during HEV infections are focal necrosis, similar to those in drug-associated toxicity, with minimal infiltration of inflammatory cells (Purcell & Emerson, 2001; Tsarev *et al.*, 1994). The liver lesions are assumed to be immunologically mediated rather than virus-induced damage (Emerson and Purcell, 2006). Two important histological features of HEV infection considered to differentiate hepatitis E from hepatitis A are the development of chronic cholestasis and, the tendency of liver parenchymal cells to organize in pseudoglandular formations (Goens & Perdue, 2004; Purcell & Emerson, 2001). As it is presumed that the virus replicates in the liver, HEV is subsequently found in bile, in large quantities (Purcell & Emerson, 2001; Tsarev *et al.*, 1992). From the bile, it is passed out in feces, which then serves as a source of infection for new individuals. The shedding precedes the onset of clinical signs, coinciding with increasing serum ALT and the progression of clinical signs (Jameel, 1999; Tsarev *et al.*, 1992). In experimental infections of macaques, anti-HEV IgM is detected approximately 3-4 weeks after infection and continued to be detectable up to 3 months (Tsarev *et al.*, 1994). Further, these findings are consistent with reports in humans (Emerson & Purcell, 2003; Jameel, 1999; Purcell & Emerson, 2001). Following IgM, anti-HEV IgG is detected which peaks several weeks later and can be detected for many months and years after infection (Arankalle *et al.*, 1999; Yarbough, 1999). In chronic liver disease patients, acute HEV infection can trigger severe liver decompensation, which can lead to hepatic encephalopathy and renal failure (Kumar *et al.*, 2004).

The characteristic feature of HEV infection when compared to other hepatitis viral infections is the increase in incidence of fulminant hepatic failure (FHF) complicated by encephalopathy and disseminated intravascular coagulation in HEV-infected pregnant women (Jameel, 1999; Khuroo & Kamili, 2003; Madan *et al.*, 1998). The disease severity increases with the gestation and is reflected by increased mortality rate (Khuroo & Kamili, 2003; Singh *et al.*, 2003). Vertical transmission rates range between 30-100% (Khuroo *et al.*, 1995; Kumar *et al.*, 2001; Singh *et al.*, 2003). FHF could not be reproduced in pregnant monkey models, however (Purcell & Emerson, 2001). A Schwartzman-like phenomenon refers to the disseminated intravascular coagulation occuring from a second endotoxin assault. The phenomenon is worsened by the reticuloendothelial blockage and has been accounted for the FHF in HEV-infected pregnant women. It has been shown that pregnant women are sensitive to such an endotoxin-mediated effect (Goens & Perdue, 2004). Recently, it has been reported that the high mortality rate in pregnant women is associated with immunological changes, which include downregulation of the p65 component of nuclear factor kappa B and a predominant T-helper type 2 (Th2) bias in the T cell response (Navaneethan *et al.*, 2008). Understanding the biology of HEV is needed to know about the detailed pathogenesis of FHF in pregnant women.

2.6 Public health concern

Water-borne transmission of HEV is considered one of the major routes of transmission of HEV and, most surveys have shown that fecally-contaminated drinking water was implicated in outbreaks and cluster of cases (Mushahwar, 2008). Findings

from a study on the prevalence of HEV in water showed that one of five pretreatment sewage samples collected from Washington, DC was positive for HEV by RT-PCR (Mushahwar, 2008). Recent reports have also shown that transmission is not only through fecal-oral but also through ingestion of undercooked infected meat (swine or wild animals) or through infected blood products (Bihl & Negro, 2008).

It is now strongly believed that HEV is a zoonotic pathogen. The possibility that swine hepatitis E virus can infect humans poses risks in xenotransplantations. In Japan, the first direct evidence of zoonotic transmission of HEV was documented in two outbreaks involving human consumption of raw wild boar liver and raw deer meat; one patient died (Tei et al., 2003; Yazaki et al., 2003). HEV was found in 1.9 percent of 363 packages of raw pork liver from grocery stores in Japan and one HEV isolate from packaged liver was identical in sequence to the HEV isolated from a patient with hepatitis (Yazaki et al., 2003). It was also demonstrated that Hepatitis E was transmitted by blood transfusion from a donor infected via the zoonotic food-borne route (Matsubayashi et al., 2004). Anti-HEV antibodies have been detected in a number of animal species including pigs (Clayson et al., 1995; Favorov et al., 2000; Meng et al., 1998a; Meng et al., 1997), cattle (Favorov et al., 2000), sheep (Favorov et al., 2000), goats (Favorov et al., 2000), horses (Saad et al., 2007), rodents (He et al., 2002; Kabrane-Lazizi et al., 1999a; Karetnyi Iu et al., 1993), and cats (Okamoto et al., 2004), suggesting that there could be a large animal reservoir of HEV. Meng et al. (Meng et al., 2002) reported that swine veterinarians were one and a half times more likely to seroconvert to HEV infection, although clearly multiple sources of exposure can exist. A recent study showed that there

was a 5.4 times increased risk to people exposed to swine than unexposed for HEV infection (Galiana et al., 2008). Workers in wastewater treatment plants were also found to have antibodies to HEV (El-Esnawy et a., 1998). All these data demonstrate the zoonotic implications of HEV infection. Moreover, autochthonous HEV infections in developed countries may be due to zoonotic infection (Christensen et al., 2008; Matsubayashi et al., 2004; Wichmann et al., 2008). Normally, HEV infections are acute and self-limiting, but several reports have shown persistent HEV infection with chronic hepatitis and cirrhosis in patients with reduced immune surveillance due to chemotherapy or post-transplant immunosuppression (Bihl & Negro, 2008; Gerolami *et al.*, 2008; Haagsma *et al.*, 2008; Schildgen *et al.*, 2008).

2.7 Diagnosis

Diagnosis of HEV is usually based on epidemiological characteristics of the outbreak and by exclusion of other liver diseases such as hepatitis A and B. Also, if any travel to HEV endemic region was involved, HEV should be suspected (Goens & Perdue, 2004). Similarly, increased incidence of fulminant hepatitis during pregnancy and water-borne outbreaks probably indicates HEV infection (Hamid et al., 1996). Commercial test kits to detect IgG and IgM antibodies to HEV are available in Europe, Asia and Canada, but not in USA (Emerson *et al.*, 2006a). Though immunoelectron microscopy is an ideal method to detect virus particles in fecal specimens, it is still not a very sensitive test (Ticehurst *et al.*, 1992). Molecular methods such as RT-PCR using specific primers are also useful in detecting viral RNA in serum, fecal or environmental samples such as water etc. The existing assays to detect antibodies to HEV are enzyme immunoassays that

use recombinant proteins or synthetic peptides representing antigenic domains from ORF2 and ORF3. Ahn et al. (Ahn *et al.*, 2006) observed that real-time RT-PCR was more sensitive than conventional RT-PCR. Recently, a microarray-based nano-amplification technique was developed as a rapid means to detect HEV (Liu et al., 2006). However, serological methods still remain the best choice to detect HEV (Emerson *et al.*, 2006a), given the low sensitivity and laborious nature of immunoelectron microscopy (Goens & Perdue, 2004). And the sensitivity of the real time RT-PCR technique depends on a proper match between the HEV strain and the PCR primers used in the assay.

2.8 Treatment, prevention and control

No specific treatment is available. General treatment is usually supportive. Currently there is no vaccine available for HEV control. ORF2 product contains important epitopes that can induce neutralizing antibodies and has been the focus for vaccine development (Purcell *et al.*, 2003). Tsarev and colleagues found that both passively and actively acquired anti-HEV antibodies can protect cynomolgus monkeys (Tsarev *et al.*, 1994). Two doses of HEV vaccine containing recombinant HEV capsid protein (56 kDa) expressed in insect cells partially protected rhesus monkeys from hepatitis E following intravenous challenge 6 or 12 months after vaccination (Zhang *et al.*, 2002). Different fragments of ORF2 were evaluated in their ability to protect rhesus monkeys against acute hepatitis. All three fragments used in the study were found to protect the challenged animals (Ma *et al.*, 2002). Partial ORF2 was expressed in transgenic tomatoes and the recombinant antigens derived from them were found to have immunoactivity (Ma *et al.*, 2003). Administration of human papillomavirus virus-like

particles containing HEV ORF2 gene into mice intramuscularly induced immune responses to both HEV and human papilloma virus (Renoux *et al.*, 2008). Recently, in a study that used a combined vaccine for HAV and HEV, the inactivated HAV component of the vaccine was observed to increase the immunogenicity of HEV recombinant protein component (Dong et al., 2007). A candidate vaccine containing recombinant ORF2 protein has currently passed phase II trial in Nepal, the region endemic to HEV infection. The vaccine contains the purified polypeptide produced in insect cells infected with a recombinant baculovirus containing a truncated ORF2 protein. It was found to be safe and immunogenic in volunteers in Nepal (Shrestha *et al.*, 2007). It was noted that virus-like particles from HEV capsid protein were not used in this study though the capsid protein tends to form VLP.

Chapter 3: Introduction and Research Objectives

3.1 Introduction on HEV ORF3

HEV ORF3 encodes a phosphoprotein (vp13) with an approximate molecular mass of 13 kDa (Tam *et al.*, 1991). vp13 has not been found in HEV virions. Recently, it was reported that a monoclonal antibody against vp13 reacted with HEV virions released from infected cells, but not the virions in feces (Takahashi *et al.*, 2008), which needs to be confirmed. The translation of ORF3 is initiated at the third in-frame initiation codon AUG, 23 bases downstream of the termination codon of ORF1 (Graff *et al.*, 2006; Huang *et al.*, 2007). ORF3 also contains the cis-reactive element at 5'end, which is essential for infectivity of macaques and pigs. Most of the knowledge about HEV vp13 has been obtained mainly from overexpression of the protein in cells transfected with the vp13 expression plasmid. The exact functions of vp13 in HEV infection remain unclear though a number of studies have found that it may play a role in cellular signaling pathways (Kar-Roy *et al.*, 2004; Korkaya *et al.*, 2001; Moin *et al.*, 2007; Tyagi *et al.*, 2002; Tyagi *et al.*, 2005; Tyagi *et al.*, 2004; Zafrullah *et al.*, 1997).

The HEV vp13 was also detected as a dimeric form of 26-28 kDa in size in *in vitro* expression (Zafrullah et al., 1997). It may be because that vp13 can form homodimers through a C-terminal 43-amino-acid interaction domain (Tyagi et al., 2001). The dimerization domain overlaps with the SH3 binding and phosphorylation site, indicating that it may play a role in signal transduction pathways. The N-terminal portion of the vp13 contains two hydrophobic domains with cysteine rich residues and the C-

terminus contains proline rich residues (Zafrullah *et al.*, 1997). The C-terminal half of vp13 contains a src homology 3 binding domain and MAPK site. vp13 activates MAPK and ERK, and binds but does not activate Src kinase via its proline-rich domain (Kar-Roy *et al.*, 2004; Korkaya *et al.*, 2001). Furthermore, vp13 also interacts with other SH3 domain containing proteins that are upstream modulators of important mitogenic signaling pathways involved in cell survival. A recent report showed that vp13 inhibits epidermal growth factor receptor (EGFR) trafficking and STAT3 nuclear translocation, suggesting a role for vp13 in promoting cell survival (Chandra *et al.*, 2008). In an ORF3-expressing stable Huh-7 cell line, vp13 was able to protect the cells from mitochondrial depolarization and death by upregulation of voltage-dependent anion channel gene and hexokinase I (Moin *et al.*, 2007). However, siRNA-mediated knockdown of ORF3 gene in the stable cells led to only slight reduction of the number of cells that survived staurosporine-induced cell death. Collectively, these data suggest that vp13 may contribute to HEV pathogenesis by promoting cell survival.

Deletion and site-directed mutagenesis studies found that Ser-80 was the phosphorylation site for vp13 by mitogen-activated protein kinase (MAPK) (Zafrullah *et al.*, 1997). The phosphorylated form of vp13 has been shown to interact with non-glycosylated form of the major capsid protein encoded by ORF2 (Tyagi *et al.*, 2002). But it was found that this phosphorylation site was not required for HEV replication in experimental infection of monkeys as a mutant HEV replicon lacking this site still caused viremia and seroconversion (Graff *et al.*, 2005).

Yeast two-hybrid system was used to screen a human liver cDNA library for proteins interacting with ORF3. vp13 was found to interact with Bikunin, a serine protease inhibitor with immunosuppressive property and to hemopexin, an acute phase protein with inflammatory role (Tyagi *et al.*, 2005). One of the other ORF3-interacting partners isolated and identified is hemopexin, a 60 kDa acute-phase plasma glycoprotein with a high binding affinity to heme (Ratra *et al.*, 2008). Hydrophobic domain II was found to be responsible for the interaction. These findings suggest that vp13 may act as a viral regulatory protein. Together, all these data indicate the multifarious roles played by vp13 in HEV pathogenesis and highlight the need to characterize further the functions of vp13.

In addition to its presumed role in cell signaling, HEV vp13 has also been found to be associated with the cytoskeleton. In vp13-transfected COS-7 cells, vp13 was found to partition with the cytoskeletal fraction (Zafrullah *et al.*, 1997). Deletion of N-terminal hydrophobic domain of vp13 abolished this association. However, the nature of interaction and the vp13-binding proteins required for this association are not known.

Due to lack of efficient cell culture for HEV propagation, functional study of vp13 in HEV biology and infection is limited. It was reported that vp13 is essential for HEV infection in Macaques and pigs (Graff *et al.*, 2005; Huang *et al.*, 2007). An infectious cDNA clone of HEV was used to study the roles of ORFs 2 and 3 in HEV replication and infection (Graff *et al.*, 2005). A frame-shift mutation at the 5'terminus of ORF3 in the replicon abolished ORF2 protein production. An ORF3-null mutant did not

produce a detectable infection in rhesus macaques (Graff *et al.*, 2005), indicating the requirement of ORF3 for HEV infection. A mutant with a C-terminally truncated ORF3 of swine HEV infectious clone was not infectious in pigs and a single mutation at the third in-frame AUG of ORF3 abolished the virus infectivity *in vivo*, indicating the ORF3 product is essential for HEV infection (Huang et al., 2007). These experiments demonstrated that HEV vp13 may play an essential role in HEV infection. Further investigation is warranted to elucidate the mechanisms.

Therefore, to further study vp13, we performed preliminary study on vp13 subcellular location and found that vp13 expression co-localizes with cellular microtubules. To further confirm the interaction of vp13 with microtubules and understand the mechanisms and potential consequences, we conducted further experiments to provide more insights on the multifarious roles of vp13 and thereby advance our understanding of HEV pathogenesis. This study was conducted to examine vp13 subcellular location, the functional significance of the vp13 association with the cytoskeletal network, the cellular binding partners required for this association, nature of the interaction and, the effects of this association.

3.2 Research objectives

The specific objectives in the present study on vp13 protein of HEV are:

1. To define the interaction of vp13 protein with microtubules (MT)
2. To determine the effect of vp13 protein-MT interaction in cell growth.

Chapter 4: Materials and Methods

4.1 Cells.

Cell lines HeLa, COS-7, and Huh-7 were maintained at 37°C in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin mixture and L-glutamine. Huh-7 cells are human hepatoma cell line.

4.1.1 Establishment of vp-13 expressing stable cell line

Cells that have stable expression of vp13 would be more suitable for characterizing HEV vp13 than those that only have transient expression of vp13. Therefore, we generated HeLa cells stably-expressing vp13. Briefly, HeLa cells grown to confluence were transfected with VenusN1-H3 plasmid using FuGeneHD (Roche Diagnostics, Indianapolis, IN). After 24 h, the cells were trypsinized and seeded into a 60 mm tissue culture plate at a density of 30,000 cells/ml and were grown under G418 (400 μg/ml) selection. Resistant clones that expressed vp13 fusion protein were selected by live fluorescent microscopy and the positive clones were expanded under G418 selection. Two clones were finally selected for this study. Western blot analysis with GFP- and vp13-specific antibodies showed that both clones had high level expression of vp13 protein. PCR amplification and sequencing confirmed the presence of intact ORF3 sequence in the stable cells.

4.2 Chemicals and antibodies

Microtubule destabilizing drug nocodazole (NOC), dynein inhibitor sodium vanadate (Na3VO4), Taxol and Trichloroacetic acid (TCA) were purchased from Sigma, St Louis, MO. ATP and GTP were purchased from Promega. For all experiments involving NOC treatment, the cells were treated with the drug at a concentration of 10 μM for 4 h. Taxol was used at a concentration of 40 μM. Sodium vanadate was used at a concentration of 100 μM.

Antibody against HEV vp13 was a kind gift from Dr. X-J Meng (Virginia Polytechnic Institute and State University, Blacksburg, VA). Commercially-available antibodies were used for other proteins tested for in this study – β-tubulin, GFP, p53, and FLAG (Sigma); p21Cip1/WAF1 (Invitrogen); PARP, caspase-8 and -9, and acetylated α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); dynein (Millipore).

4.3 Plasmids and vectors

The plasmid pSK-E2 that contains the full length cDNA of HEV genome was a generous gift from Susane Emerson, NIAID, NIH (Emerson *et al.*, 2001). VenusN1 and -C1 vectors that contain an improved version of yellow fluorescent protein named "Venus" were kind gifts from Ioanis Bossis, University of Maryland, College Park (Nagai *et al.*, 2002). The N1 and C1 nomenclature of the Venus vector denotes the presence of the cloning site upstream and downstream of the Venus gene, respectively. The plasmid pCMVTag2 vector, used for the expression of FLAG-tagged fusion protein in mammalian cells, was purchased from Stratagene. A Myc-tagged p50/dynamitin

construct was a kind gift from Richard Vallee, Columbia University, New York, NY (Echeverri *et al.*, 1996).

4.4 Transfection

Transfection experiments were performed on cells grown to 90-95% confluence in 12-well plates. The cells were transfected with 2 µg of the vp13 expression plasmid or a similar amount of empty vector in a volume of 100 µl of Opti-MEM (Invitrogen) per well. Transfection was carried out with FuGeneHD (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions. Cells treated with only the transfection reagents served as mock-treated controls. Cells were harvested at 24 h after transfection, unless otherwise specified and utilized for further analysis.

4.5 Construction of vp13 plasmids.

HEV ORF3 was amplified from pSK-E2 by PCR using Venus N1-H3 For and Venus N1-H3 Rev primers (Table 1) that contain restriction sites of *Eco*RI and *Bam*HI, respectively. The forward primer was designed to begin from the third in-frame initiation codon AUG in ORF3 that was recently found to be an authentic translation initiation codon for this gene (Huang *et al.*, 2007). The PCR-amplified product was digested with the two enzymes, and then ligated to a VenusN1 vector, using T4 DNA ligase (Promega), upstream of the Venus reporter gene. The ligation mixture was transformed into DH5α competent cells and positive clones were selected by PCR. Cloning was further confirmed by restriction enzyme digestion and bidirectional nucleotide sequencing using an ABI 3100 DNA sequencer (Applied Biosystems). The recombinant plasmid,

VenusN1-H3, was used in the following studies. The ORF3 gene was also similarly cloned into VenusC1 vector to confirm the expression pattern.

The ORF3 gene was also cloned into pCMVTag2 vector for expression of FLAG-tagged fusion protein in mammalian cells. PCR primers used for the cloning were designed similarly as above to include restriction enzyme sites and make sure ORF3 gene was in-frame with FLAG sequence in the vector. The presence of ORF3 gene in the recombinant plasmid pCMV-H3 was confirmed by DNA sequencing.

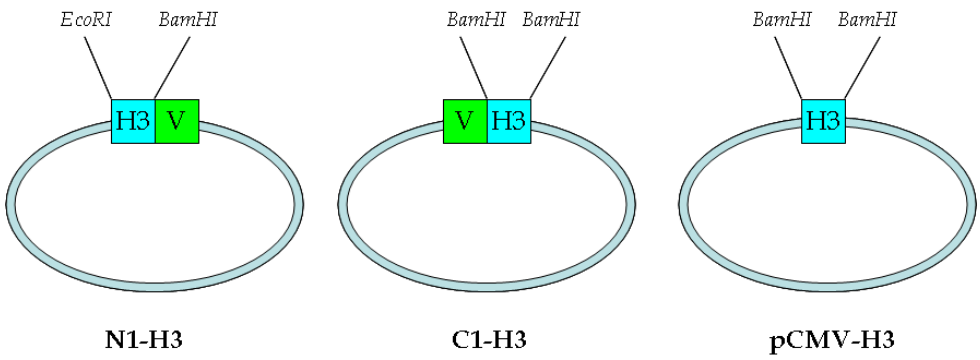

Fig 2. Construction of HEV ORF3 expression plasmids. The ORF3 of HEV (H3) was cloned into Venus N1 vector, upstream of the Venus reporter gene (N1-H3) using the indicated restriction enzymes. Similarly, H3 was also cloned into Venus C1 vector, downstream of the Venus reporter gene (C1-H3). Additionally, H3 was also cloned into pCMV vector, to determine if the Venus fusion could affect H3 expression pattern.

Table 1. Oligonucleotides used in construction of ORF3 expression plasmids

Plasmid	Primer sequence (5′ to 3′)
Venus N1-H3	For – GC*GAATTC*ATGGGTTCGCGACCATGCG Rev – CCGGATCCTTGCGGCGCGGCCCCAGCTGTG
Venus C1-H3	For – GC*GAATTC*AATGGGTTCGCGACCATGC Rev – CCGGATCCTTGCGGCGCGGCCCCAGCTGTG
Venus N1-H3D1	For – GC*GAATTC*ATGGGTTCGCGACCATGCG Rev – CAGGATCCGTTGGTTGGATGAATATAG
Venus N1-H3D2	For – GC*GAATTC*ATGGGTTCGCGACCATGCG Rev – CTGGATCCCTGGTCACGCCAAGCGGA
Venus N1-H3D3	For – GC*GAATTC*ATGCGCCACCGCCCGGTCAG Rev – CCGGATCCTTGCGGCGCGGCCCCAGCTGTG
Venus N1-H3D4	For – GC*GAATTC*ATGATTCATCCAACCAACCC Rev – CCGGATCCTTGCGGCGCGGCCCCAGCTGTG
Venus C1-H3NH	For – GC*GAATTC*AATGGGTTCGCGACCATGC Rev – CTGGATCCTTAGTGGCGCGGGCAGCATAG

All the forward primer sequences contained restriction site for *Eco*RI enzyme (italicized and underlined), while all the reverse primer sequences contained restriction site for *Bam*HI enzyme (underlined).

Various truncation mutants of ORF3 were constructed by cloning fragments of ORF3 into Venus vector (Fig 3). Primers were designed accordingly to amplify the individual fragments of ORF3 by PCR (Table 1). Cloning was performed, in a manner similar to that of full length ORF3, into VenusN1 or Venus C1 vectors.

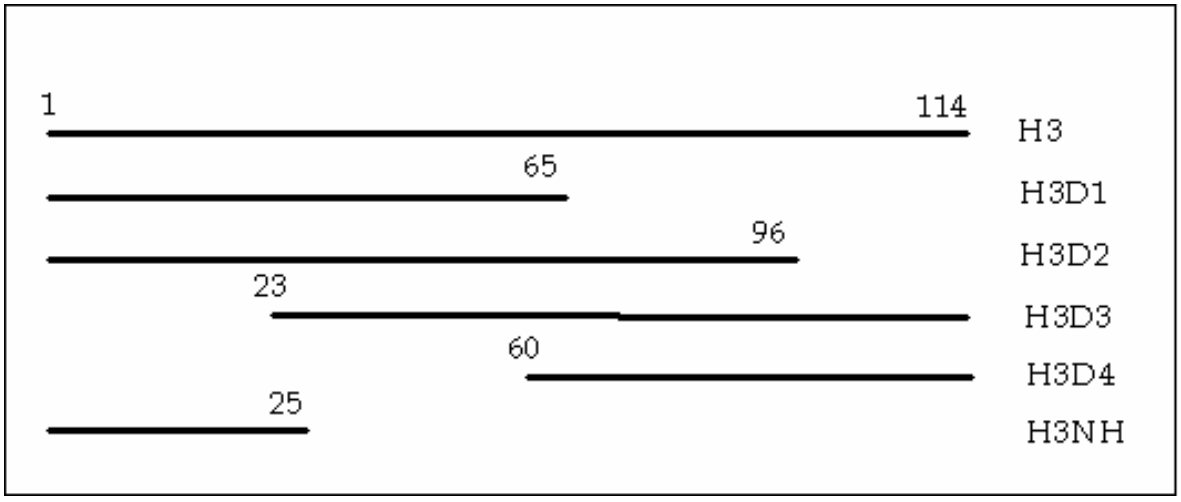

Fig 3. Schematic illustration of ORF3-truncation plasmids. The numbers above each line indicate starting and ending nucleotides of ORF3 or truncations. H3 contains the full-length vp13. H3D1 contains both hydrophobic domains at N-terminal end of vp13, but lacks a stretch of 49 aa residues at its C-terminal end. H3D2 contains both the hydrophobic domains at the N-terminal region, but lacks 15 aa residues at its C-terminal end. H3D3 lacks the first N-terminal hydrophobic domain but contains the second hydrophobic domain of vp13. H3D4 lacks both the hydrophobic domains while the H3NH construct contains only the first hydrophobic domain at N-terminal end of vp13.

4.6 Fluorescence microscopy.

Cells were seeded directly onto cover slips in cell culture plate, incubated overnight, and transfected the next day. Twenty-four hours post transfection, the cells were observed directly under fluorescence microscopy (Venus vector-transfected cells) or fixed with 1% paraformaldehyde and mounted onto slide with anti-fade mounting solution (Invitrogen, Carlsbad, CA) before observation. For the cells treated with NOC, culture medium was replaced with medium containing the drug at 24 h post transfection and the cells were incubated for an additional 2 h. The NOC was then removed and washed twice with PBS, and were observed under fluorescence microscopy. For studying the recovery from NOC-treatment, the treated cells were further incubated for 4 h with culture medium after the removal of NOC-containing medium.

Immunofluorescence assay (IFA) was carried out as reported previously (Zhang *et al.*, 1998) with an rabbit anti-vp13 antibody. A FITC conjugated goat anti-rabbit IgG (Invitrogen) was used to detect vp13 by fluorescent microscopy.

4.7 Western blot analysis.

HeLa cells were transiently transfected with VenusN1-H3 or empty vector. At 24 h post transfection, the cells were lysed with Laemmli sample buffer. SDS-PAGE and Western blot analysis were performed as described previously (Zhang *et al.*, 2007). Briefly, the cell lysates were electrophoresed on 12% SDS-polyacrylamide gels. The separated proteins were transferred onto nitrocellulose membrane and probed with rabbit anti-vp13 antibody. Any specific reaction was detected with goat anti-rabbit IgG

conjugated with horseradish peroxidase (Sigma) and the addition of chemiluminescence substrate. Chemiluminescence signal was collected by a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). Beta-Tubulin was detected on the same blot membranes to normalize protein loading in the analysis. Digital image acquisition and densitometry analyses were conducted using Quantity One software (Bio-Rad). The expression of other proteins, tested in this study, was detected in a similar manner using corresponding antibodies.

4.8 Cell viability assay.

Cell viability was determined with CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI). Briefly, cells were cultured in 96-well plate and CellTiter-Glo reagent was added and incubated for 10 minutes at room temperature. The luminescence signal was measured with VICTOR$^{3™}$ Multilabel Counter (Perkin-Elmer, Waltham, MA). Relative percentages of luminescence intensity were calculated by comparison to mock-treated controls. All experiments were performed at least in duplicates and were repeated at least three times.

4.9 Caspase-3 and -7 activity detection.

Caspase-3 and -7 activities in the transfected cells were detected using Caspase-Glo^{TM} 3/7 Assay kit (Promega), as the manufacturer's instructions. Briefly, 50 µl of Caspase-Glo reagent was added to each well containing the cells in 50 µl of culture media and incubated for 30 minutes at room temperature. The luminescence signal was measured using a VICTOR$^{3™}$ Multilabel Counter (Perkin-Elmer, Waltham, MA) and

relative percentages of luminescence intensity were calculated. All caspase assays were performed at least in duplicates and were repeated at least three times.

4.10 Real-time PCR.

Total RNA was isolated from cells lyzed in TRIzol® Reagent (Invitrogen), according to manufacturer's instructions. For quantitative RT-PCR analysis, RNA was first treated with RNase-free DNase (Promega) to remove carryover DNA from the RNA isolation procedure. Random-hexamer-primed reverse transcription was carried out using AMV reverse transcriptase (Promega). Primers for real-time PCR were designed based on cDNA sequences of target mRNA. Real-time PCR with SYBR Green detection was done as described previously (Patel *et al.*, 2008). The results depicted are averages of three independent experiments.

Table 2. Oligonucleotides used in real-time PCR

Gene	**Primer**
p53	For - 5' TCAACAAGATGTTTTGCCAACTG 3'
	Rev - 5' ATGTGCTGTGACTGCTTGTAGATG 3'
p21	For - 5' ATGAAATTCACCCCCTTTCC 3'
	Rev - 5' AGGTGAGGGGACTCCAAAGT 3'

4.11 MT isolation and salt-extraction assay.

MT isolation and salt-extraction assays were performed as described previously, with some modifications (Goode & Feinstein, 1994; Pipeleers *et al.*, 1977). Briefly, HeLa cells were transfected with C1-H3 DNA and at 24 h post-transfection lysates were collected with MT stabilization buffer (100 mM PIPES pH6.9, 5 mM $MgCl_2$, 1mM EGTA), both with and without the addition of ATP and GTP. Taxol (40μM) was included to artificially stabilize the MT. Cell lysates were collected with a cell scraper, transferred to a centrifuge tube, and homogenized with a syringe and 25-gauge needle. After 10 min of incubation at 37°C, 2μl of the sample was mixed with trypan blue, and checked for at least 95% cell lysis. The sample was then centrifuged at 100,000g for 30 min at 37°C, using buffers and rotors pre-warmed at 37°C. After centrifugation, the supernatant was transferred to a pre-chilled tube and the pellet was resuspended with salt extraction buffer (80 mM PIPES pH6.9, 1 mM $MgCl_2$, 1mM EGTA, and 500 mM KCl). Taxol was added at 40 μM and the sample was centrifuged again at 100,000 xg for 30 min at 37°C. After centrifugation, the supernatant was transferred to a pre-chilled tube. The pellet was resuspended in MT stabilization (MTS) buffer and then boiled in 1X SDS-PAGE sample buffer and heated at 95°C for 2 min. The supernatants were precipitated with TCA and then analyzed by SDS-PAGE and Western blot using antibodies against vp13, acetylated α-tubulin and β-tubulin. For experiments with addition of detergent in lysis buffer, the cells were lyzed in buffer PEMT (100 mM PIPES pH6.9, 5 mM $MgCl_2$, 1 mM EGTA, 0.1%Triton, 0.1%Tween20, 0.001% Antifoam). Cell homogenization and centrifugation were done as described above.

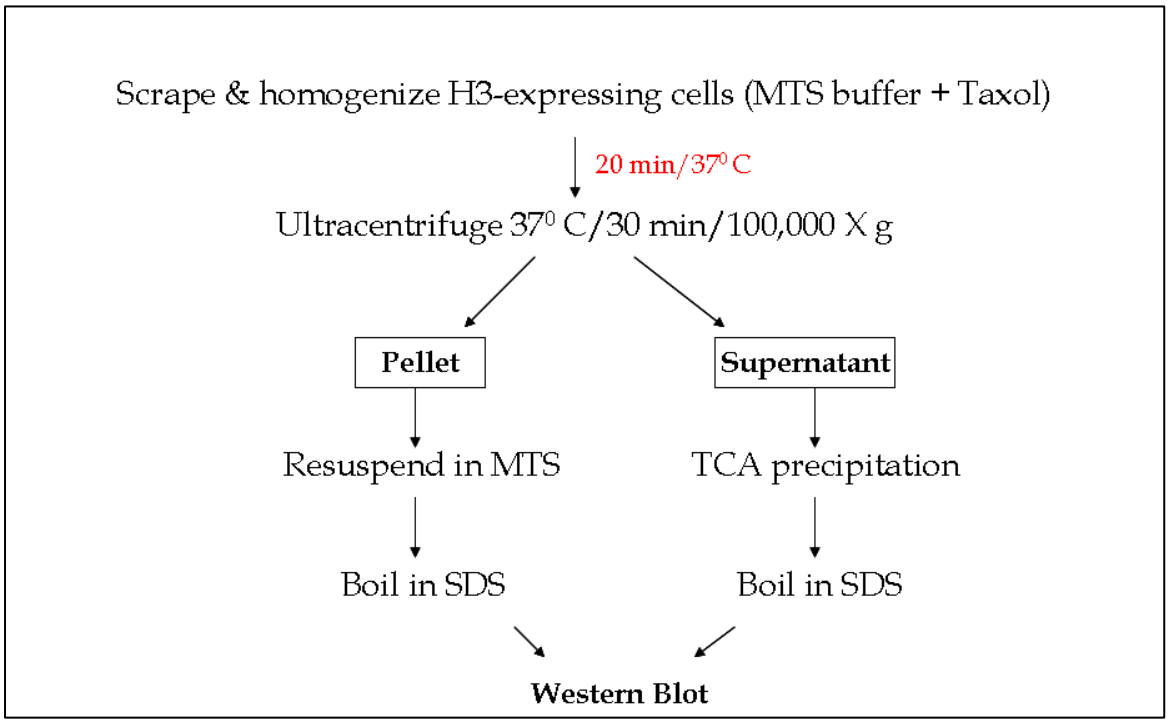

Fig 4. MT isolation and salt extraction assay. MTS buffer – MT stabililizing buffer; SDS – sodium dodecyl sulphate; TCA – tricholoacetic acid

Chapter 5: Results

5.1 HEV vp13 protein co-localizes with the MTs.

HEV ORF3 (H3) was first cloned into VenusN1 vector for expression of vp13-venus fusion protein. HeLa cells were transfected with VenusN1-H3 plasmid. At 24 h after transfection, live fluorescence microscopy was conducted. Under high magnification (40X and 63X) and a FITC filter, green filamentous structures were observed in the HeLa cells (Fig 5), indicating the expression pattern of vp13 fusion protein and its probable co-localization with MTs. When the cells were fixed with paraformaldehyde, the expression pattern of vp13 fusion protein was unaltered. There was also punctate distribution in the cytoplasm. In contrast, homogeneous bright green fluorescence in both cytoplasm and nucleus was observed in cells transfected with empty vector (Fig 5).

To determine if the position of H3 in the fusion protein affected its expression pattern, H3 was cloned into VenusC1 vector that contains the cloning site downstream of the Venus gene. Transfection of HeLa cells with VenusC1-H3 plasmid also showed the same pattern as vp13-venus fusion protein described above, but with much more intense fluorescence (Fig 5b), indicating that the location of the vp13 protein on the Venus vector did not affect or alter the expression pattern. Cells transfected with the empty vector had homogeneous bright green fluorescence in both cytoplasm and nucleus, as observed with VenusN1 construct.

In order to evaluate if vp13 expression pattern differs with the nature of expression vector used, H3 was also cloned into PCMV vector. Following transfection of HeLa cells with this construct, we observed a similar expression pattern by immunofluorescence assay (Fig 5c). These results indicated that the nature of the vector used to express the vp13 gene did not alter the expression pattern of vp13 in transfected cells.

To determine if the expression of HEV vp13 protein was similar in hepatoma cell line since hepatocytes are the primary target cells of HEV, Venus C1-H3 plasmid was transfected into Huh-7 cell line. Green fluorescence of filamentous pattern was also observed in Huh-7 cell line (Fig 6). The expression of vp13 fusion protein in Huh-7 cells appeared in a pattern similar to HeLa cells (Fig 5), though at a lower rate. Therefore, HeLa cells were used in all subsequent experiments, unless otherwise specified, for assessing vp13 co-localization with MTs.

The vp13 fusion protein expression was also detected by Western blot analysis with an antibody against GFP. The size of the vp13-venus fusion protein was ~ 40 kDa, while Venus protein alone was 27 kDa (Fig 7A). To further confirm the expression of the vp13 fusion protein, a rabbit antibody against HEV vp13 was used in Western blot and the band at expected size was detected, while no bands were visible in lysates from vector- or mock-transfected cells (Fig 7B). These results confirmed the expression of vp13-venus fusion protein in transiently transfected cells.

The expression of vp13 in HeLa cells transfected with pCMV-H3 plasmid was confirmed by Western blot analysis with rabbit anti-vp13 antibody. Since ORF3 was cloned into pCMVTag vector for expression of FLAG-tagged vp13 fusion protein, we also performed Western blot with FLAG antibody. However, no specific signal was detected (data not shown), which indicated that the vp13 expression in HeLa cells transfected with pCMV-H3 was not a FLAG-tagged fusion protein. DNA sequencing confirmed that the full-length ORF3 with initiation codon was in frame with FLAG sequence in the plasmid. Thus, it was speculated that the translation starts from the initiation codon of HEV ORF3 gene, instead of AUG upstream of FLAG tag sequence. Therefore, the pCMV-H3 plasmid was used for expression of vp13 without tag in this study.

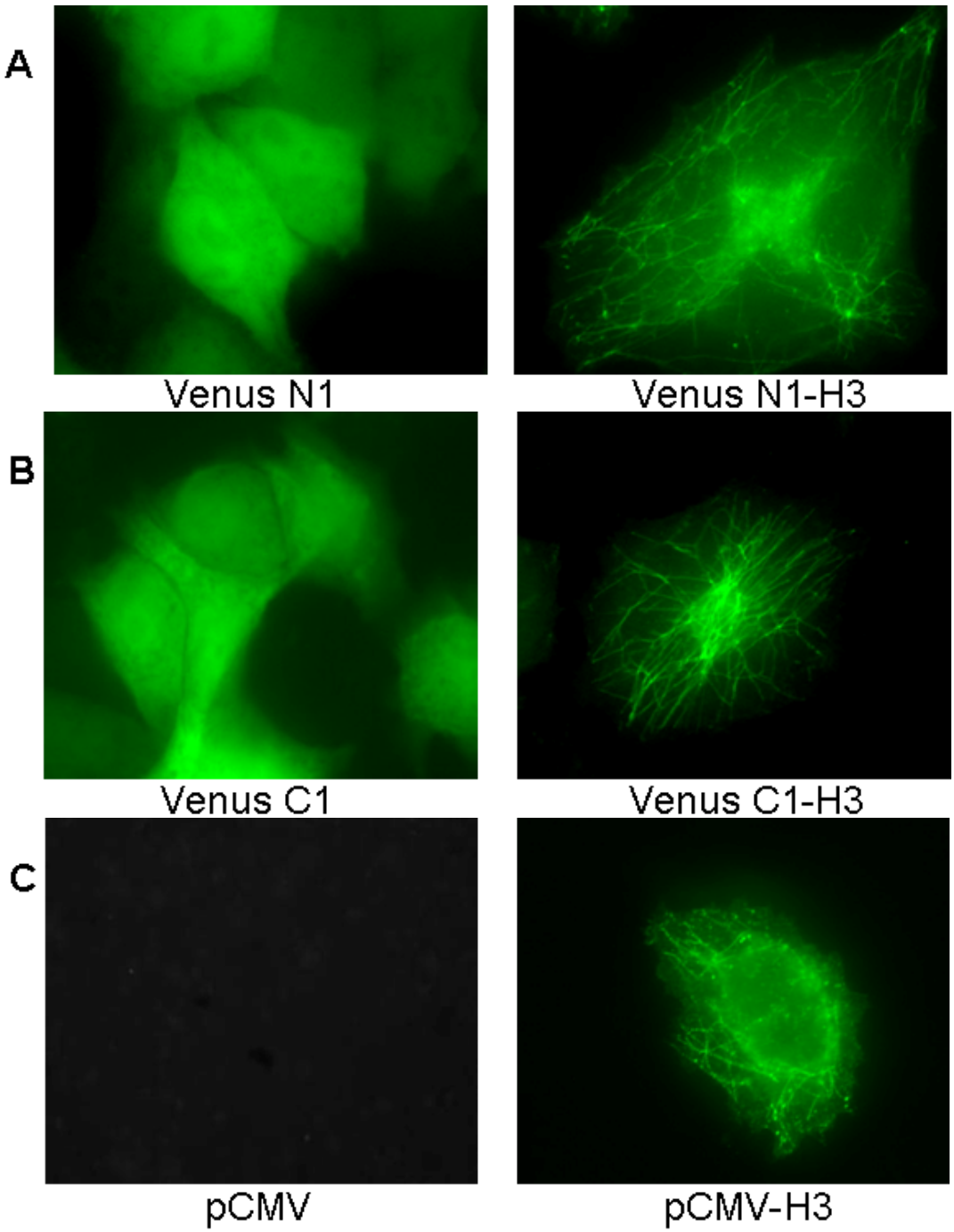

Fig 5. Interaction of vp13 with MT in HeLa cells. Cells were transfected with Venus N1-H3 (A) or Venus C1-H3 (B), pCMV-H3 vectors and, observed by live fluorescence microscopy (A&B) or immunofluorescence (C). Note the filamentous pattern of the vp13 fusion protein (right panels) when compared to empty vectors (left panels).

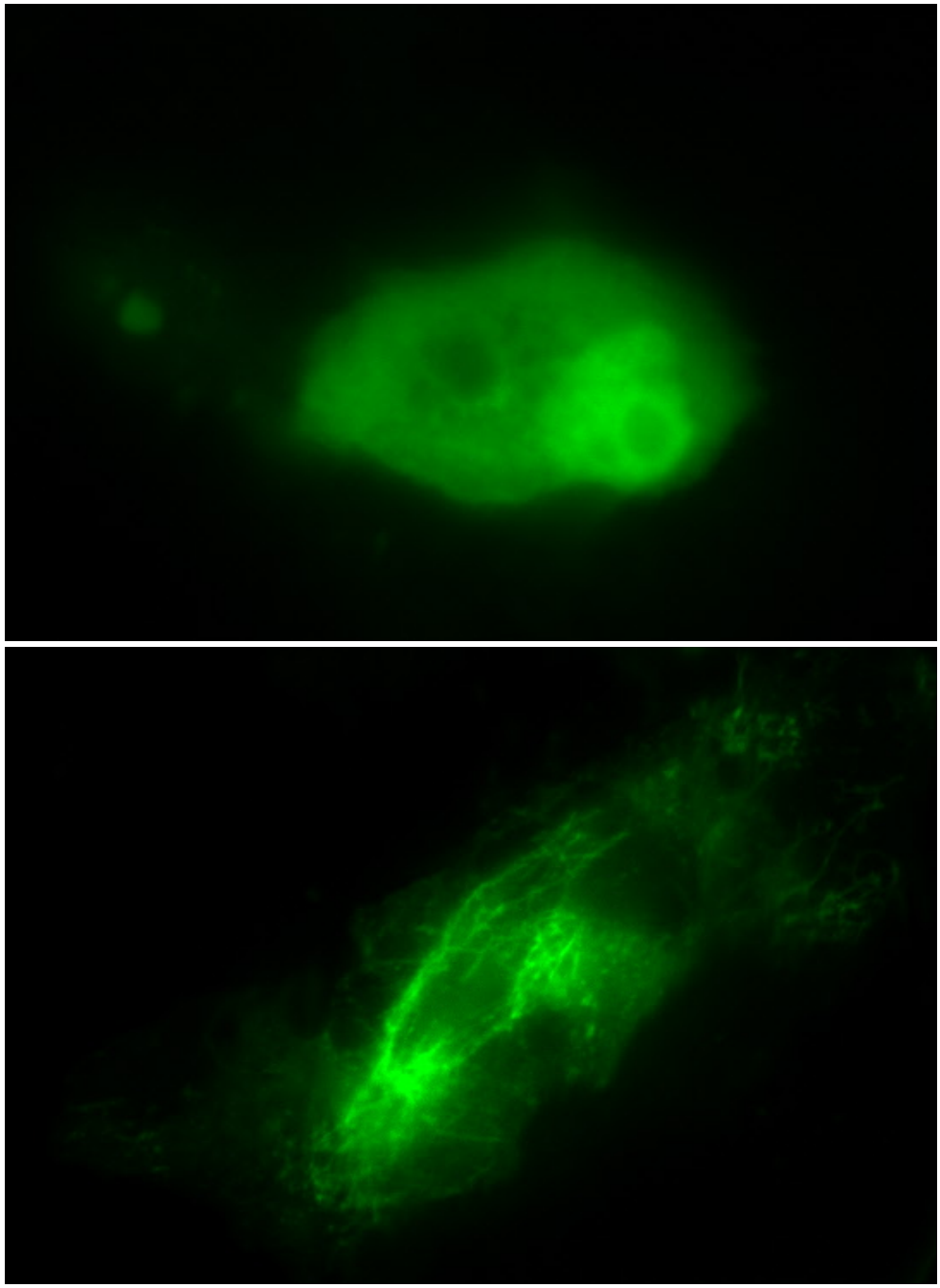

Fig 6. Interaction of vp13 with MT in Huh-7 cells. Huh-7 cells were transfected with Venus N1-H3 and, observed by fluorescence microscopy. Note the filamentous pattern of the vp13 fusion protein (bottom panel) when compared to empty vector (top panel).

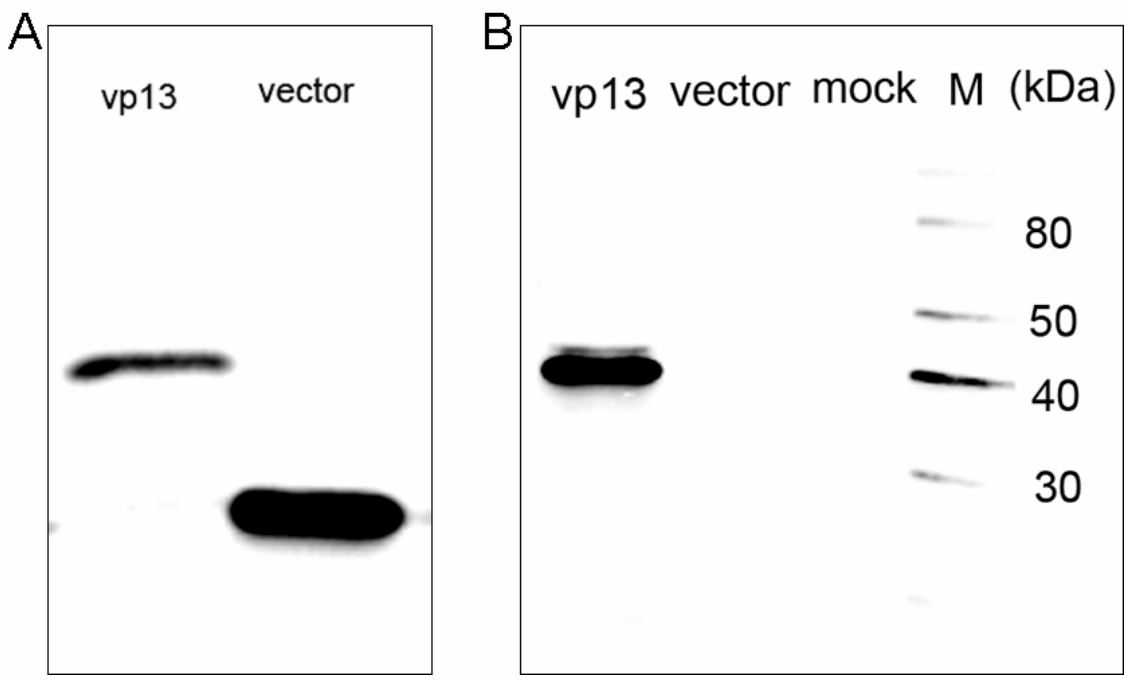

Fig 7. Detection of vp13 fusion protein by Western blot. HeLa cells were transfected with VenusN1-H3, and cell lysates were detected for the expression of vp13 fusion protein by Western blot using mouse anti-GFP antibody (A) or rabbit anti-vp13 antibody (B). Cells transfected with the empty vector or untransfected cells were included as controls. M: MagicMark™ XP Western Protein Standard (Invitrogen).

5.2 Effect of MT-depolymerizing agents on HEV vp13 expression pattern.

The pattern of expression of vp13 in the transfected cells indicated that it co-localizes with MTs. To explore the association of vp13 with the microtubular cytoskeleton, we used a reversible MT-destabilizing drug nocodazole (NOC) on vp13-expressing cells and then observed the expression pattern of vp13. HeLa cells were treated with NOC at a non-toxic concentration of 10 μM for 4 h on day 2 after transfection with VenusN1-H3 plasmid. When observed by fluorescence microscopy, we found that the drug treatment led to disappearance of the filamentous expression pattern of vp13 (Fig 8). Green punctate fluorescence was observed along the cell periphery or subcellular organelles (Fig 8, top panel).

The effects of NOC are reversible. Therefore, to test if the removal of NOC from the cells would restore the vp13 filamentous expression pattern, we washed the cells twice with culture medium and observed the cells after 4 h incubation without the NOC. Upon removal of NOC, the characteristic green filamentous structures were observed in the cells, indicating restored MT nucleation and confirming the co-localization of vp13 with MTs. Together, these results clearly demonstrated that the expression pattern of vp13 fusion protein occurred in a MT-dependent manner.

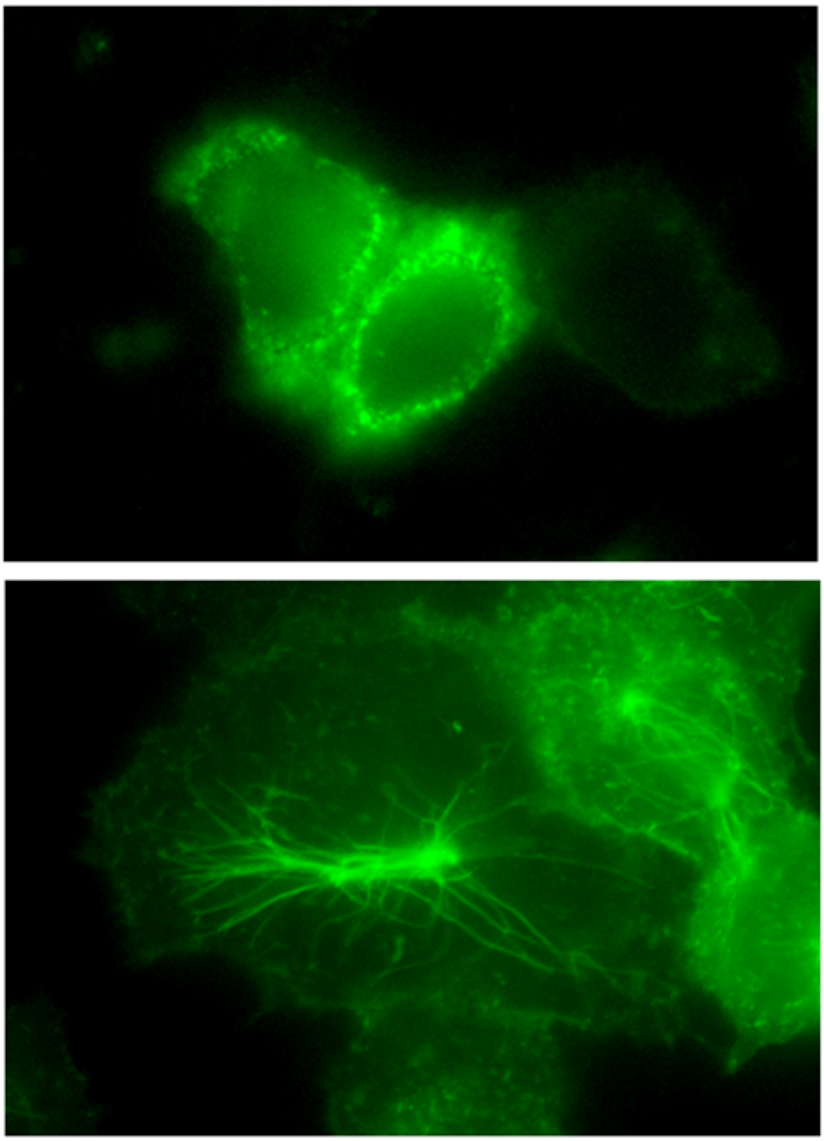

Fig 8. Effect of nocodazole (NOC) on vp13 expression pattern. HeLa cells were transfected with VenusN1-H3 plasmid and treated with (top) or without (bottom) the MT-destabilizing drug NOC for 4 h on day 2 after transfection. The depolymerization of MT abolished the filamentous expression pattern of vp13 fusion protein (top), while the pattern of vp13 expression remained intact in the untreated cells (bottom).

5.3 Determination of the MT-binding region of vp13.

After finding that vp13 protein interacts with the MT, we attempted to determine the region in vp13 that was required for this interaction. We generated four truncated versions of ORF3 by PCR and cloned them into Venus vectors (Fig 3). Transfection of these plasmids into HeLa cells showed that VenusN1-H3D1 and VenusN1-H3D2 exhibited filamentous expression pattern similar to the full length vp13 fusion protein, though at lower intensity (Fig 9; A, B and F). The rest two plasmids showed homogenous expression throughout the cells similar to patterns produced in cells transfected with empty vector (Fig 9; C and D). These results indicated that N' portion of vp13 was needed for the filamentous expression pattern.

The N' portion of vp13 contains two hydrophobic domains that might be responsible for the MT interaction. Therefore, to further define the binding region, we cloned the fragment containing the first hydrophobic domain into VenusC1 vector. Following transfection, live-cell fluorescence microscopy was conducted and we observed only green punctate fluorescence but no filamentous pattern (Fig 9E), indicating that the first hydrophobic domain of N' region of vp13 is inadequate for MT interaction. Since VenusN1-H3D3 contained sequences of the second hydrophobic domain of N' region of vp13 and had homogeneous expression in transfected cells, the second hydrophobic domain is also inadequate for MT interaction either. Thus, both hydrophobic domains in N' region of vp13 are required for the co-localization of vp13 with MT, as shown in truncation construct VenusN1-H3D1.

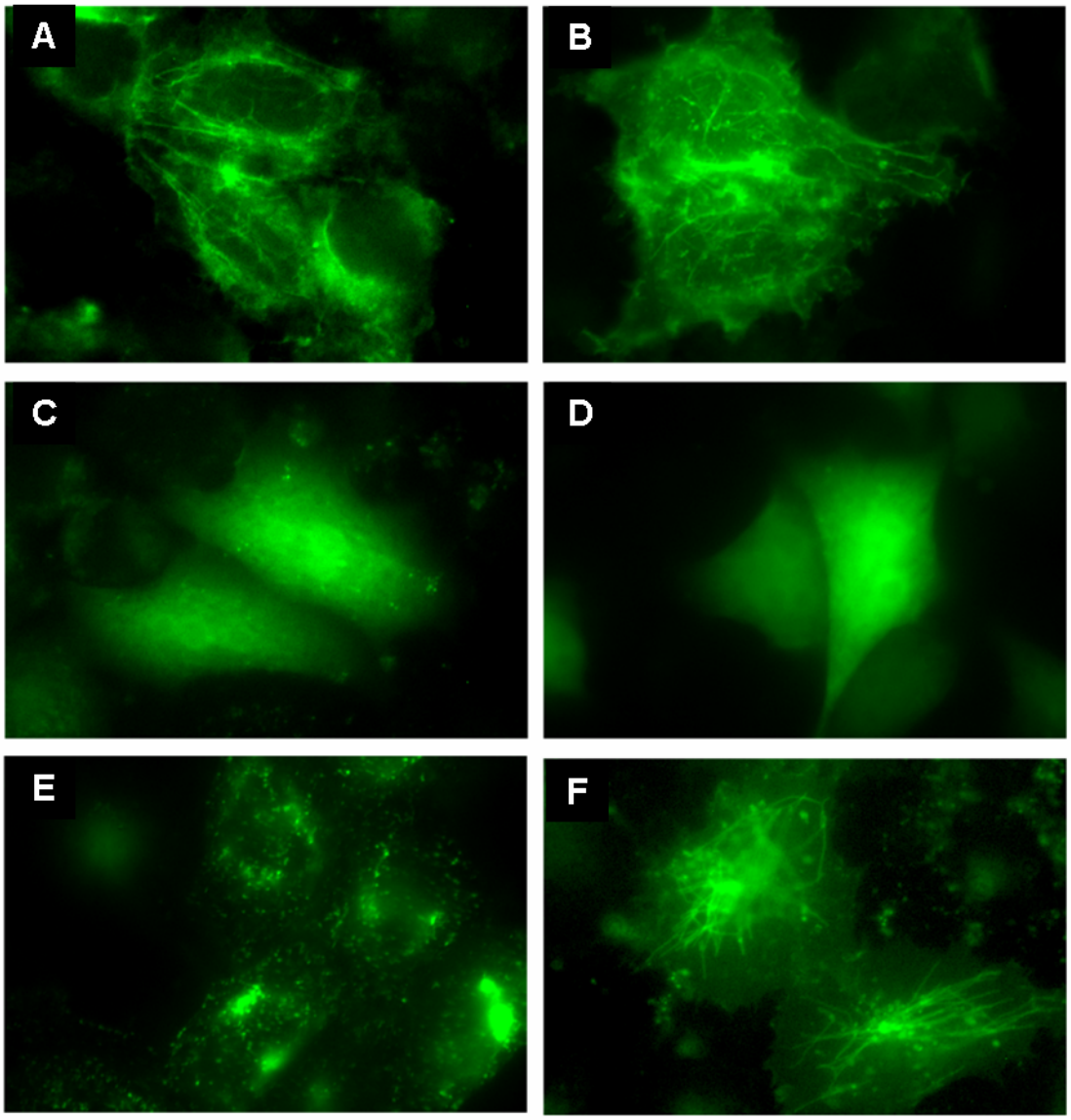

Fig 9. Identification of MT-interacting domain of vp13 protein. Fluorescence images from HeLa cells transfected with the VenusN1-H3D1 (A), VenusN1-H3D2 (B), VenusN1-H3D3 (C), VenusN1-H3D4 (D), VenusC1-H3NH (E), and VenusN1-H3 (F) constructs containing the different ORF3 truncations or full-length ORF3.

5.4 Effect of HEV vp13 on MT stability.

Dynamic instability is a fundamental property of MTs and is critical for diverse cellular functions, and is regulated by many factors. It has been shown that persistent perturbation of MT dynamics with MT-stabilizing drug taxol or MT-destabilizing drug NOC causes significant apoptosis (Sorger *et al.*, 1997). The taxol-perturbation of MT induces apoptosis through a mitochondria-dependent pathway (Wang *et al.*, 2000).

Tubulin acetylation is a well established marker of MT stability. Stable MTs exhibit much higher extent of tubulin acetylation than dynamic, unstable MTs (Westermann & Weber, 2003). Since vp13 interacts with the MTs and hyperacetylation is a quantitative indication of changes in MT stabilization, we examined the level of tubulin acetylation in HeLa cells transfected with VenusN1-H3, by Western blot using a specific antibody against acetylated α–tubulin (Fig 10). Compared to the controls, acetylated α–tubulin was elevated in HeLa cells transfected with vp13 expression plasmid, pCMV-H3 (Fig 10A). Densitometry analysis of the band intensities showed that the level of acetylated α–tubulin in HeLa cells with vp13 expression was 3.2-fold higher than that in mock-treatment control, while the cells transfected with empty vector had a little elevation (Fig 10B). The level of acetylated α–tubulin was also checked in Huh7 cells using C1-H3 vector and similar results were obtained (Fig 10C). These results indicated that expression of vp13 enhanced the stability of MTs, by elevating the levels of acetylated α–tubulin in HeLa cells and Huh7 cells.

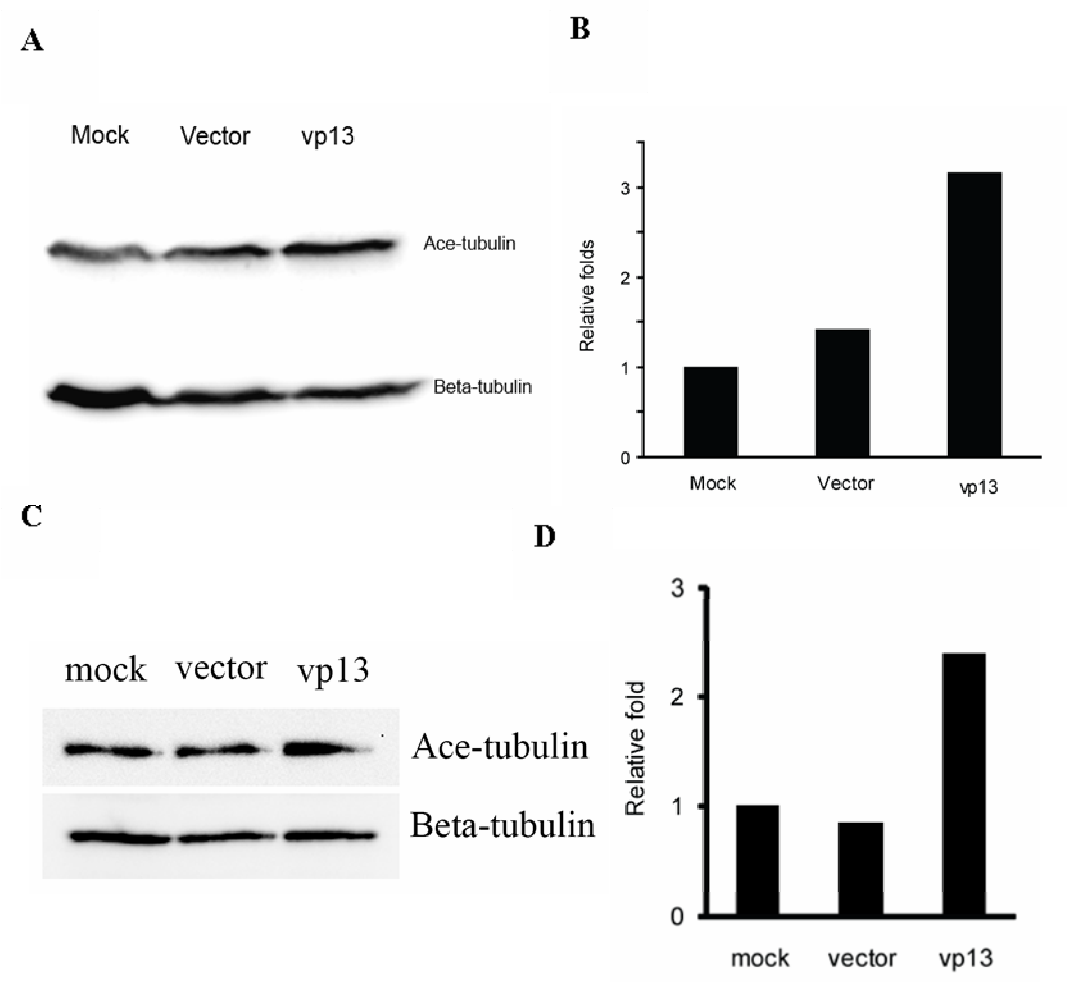

Fig 10. Effect of HEV vp13 on MT stability. A. Western blot detection of acetylated α–tubulin in HeLa cells (A) and Huh-7 cells (C) transfected with vp13-expressing plasmid (vp13 lane), or empty vector or untransfected (mock lane). Detection of β-tubulin in the same blot was conducted for normalization. B. Graphic illustration of densitometry analysis of the digital image of "A". The level of acetylated α–tubulin is presented as relative folds in comparison with mock-treatment control. D. Graphic illustration of densitometry analysis of the digital image of "C" in relative folds.

5.5 Effect of HEV vp13 on cellular p53 levels.

Because MT dynamics is critical for diverse cellular functions, modulation of MTs may result in various biological consequences. p53 tumor suppressor protein has been shown to associate with MTs and use the MT-dependent motor complex dynein/dynactin for nuclear targeting under conditions of DNA damage (Giannakakou *et al.*, 2000). p53 is a key transcription factor that can induce growth-arrest, apoptosis and cell senescence. It is a tumor suppressor phosphoprotein that is usually inactive in normal cells, but becomes active after being activated by variety of stress types and oncogenes . So, we determined the levels of p53 in COS-7 cells expressing vp13. HeLa cells naturally contain an inactive p53 (Liang *et al.*, 1995) and, therefore, were not suitable for this study.

COS-7 cells were transfected with vp13-expressing VenusN1-H3 plasmid, or with empty vector or left untransfected. Lysates were harvested at 24 h post-transfection and subjected to SDS-PAGE and immunoblotting using a p53-specific antibody. Our results showed that in COS-7 cells expressing vp13, p53 level was elevated compared to those transfected with empty vector or untransfected cells (Fig 11A). Densitometry analysis of the bands showed that p53 level in the cells with vp13 expression was 2.6-fold higher than the vector-transfected or untransfected cells (Fig 11B). These results indicated that vp13 expression induced p53 elevation in COS-7 cells, while the empty vector alone did not have any effect.

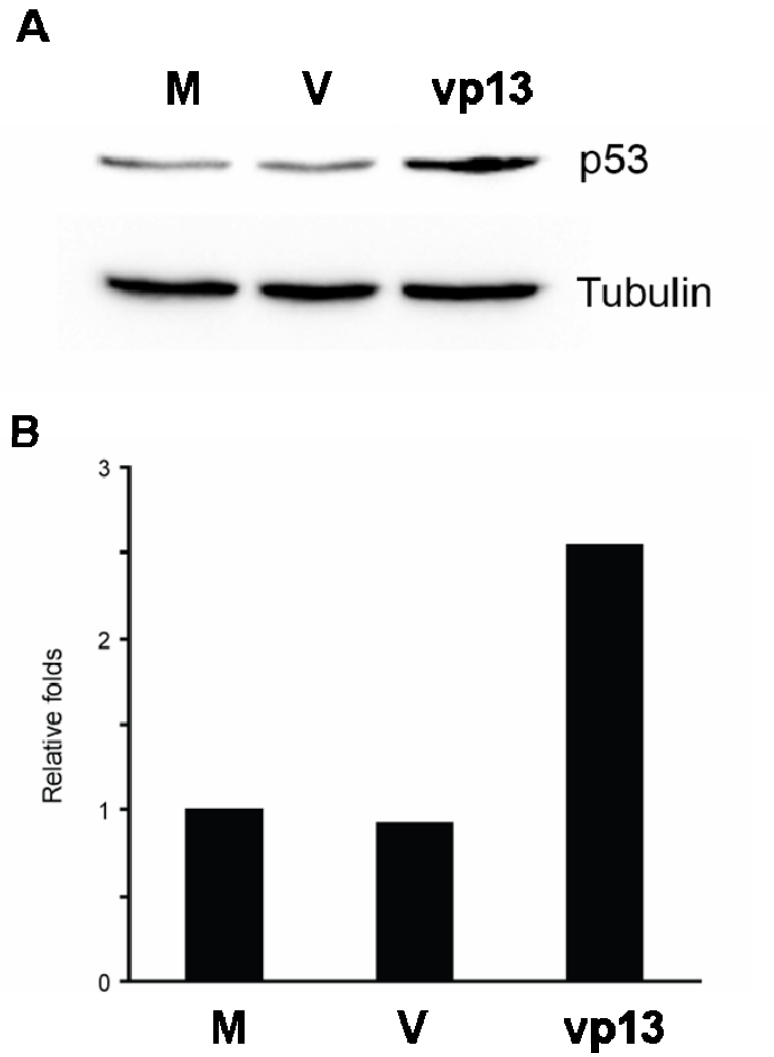

Fig 11. Effect of HEV vp13 on levels of cellular p53. **A**. Western blot with p53 antibody. COS-7 cells were transfected with VenusN1-H3 plasmid (vp13), or empty vector (V) or left untransfected (M). Lysates were collected and subjected to SDS-PAGE and immunoblotting using p53-specific antibody. Tubulin was detected in the same blot for normalization. **B**. Graphic illustration of densitometric analysis of the digital image of p53 blot. The p53 protein level is presented as relative folds in comparison with untransfected control.

To determine if the p53 elevation was due to increased p53 mRNA transcripts or protein accumulation, we conducted real time RT-PCR to assess p53 mRNA level in the COS-7 cells. In comparison with mock-treated control, cells with vp13 expression had more than 2.5 folds higher level of p53 transcripts (Fig 12), while empty vector did not cause any significant change.

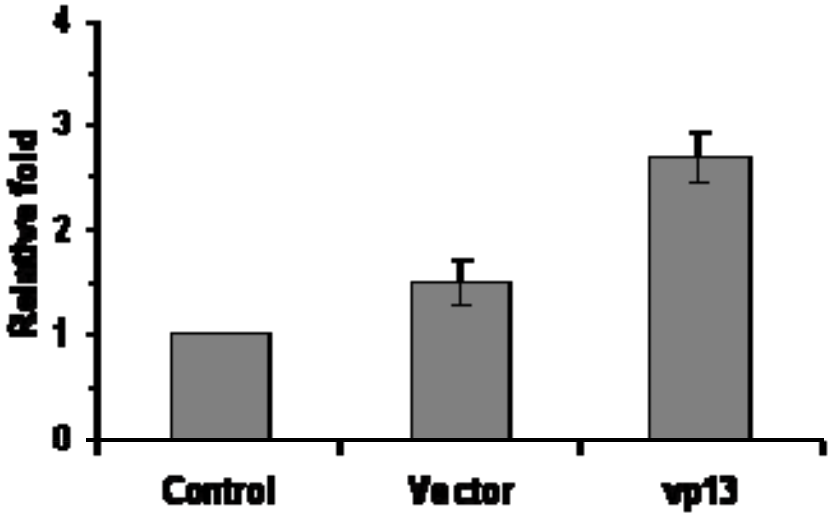

Fig 12. Expression levels of p53 mRNA in vp13-expressing cells. Quantitative RT-PCR to assess the transcript levels of p53 was performed and the results are shown as relative fold compared with mock-treatment control.

5.6 Effect of HEV vp13 on cellular levels of p21$^{Cip1/WAF1}$.

Cyclin-dependent kinase inhibitor p21$^{Cip1/WAF1}$ (p21) is transcriptionally activated by tumor suppressor p53. Induction of p21 is essential for p53-mediated arrest of cell cycle in G1 phase in response to DNA damage (el-Deiry *et al.*, 1994). Since p53 was elevated in cells expressing vp13 (section 4.5), we evaluated the cellular levels of p21, the protein which is activated upstream by p53, in vp13-expressing cells (Fig 13). By immunoblotting, we found that the p21 levels in COS-7 cells expressing vp13 were higher than those in cells transfected with empty vector (Fig. 13A). Densitometric analysis showed that p21 levels in vp13-expressing cells were 2.9-fold higher than those in untransfected or vector-transfected cells (Fig.13B).

To determine if the p21 protein elevation was due to higher transcription of p21 mRNA, real time RT-PCR was conducted to assess p21 mRNA level in the COS-7 cells transfected with vp13-expressing plasmids. In comparison with mock-treatment control, the cells that express vp13 had 2.5-fold higher p21 mRNA level (Fig. 13C), while the empty vector did not lead to any change. These results indicated that the mRNA levels of p21 were elevated in vp13-expressing COS-7 cells, which correlated with the increased p21 protein levels as observed by Western blot. Taken together, these results demonstrated that vp13 expression led to p21 elevation, which in turn may inhibit cell cycle progression.

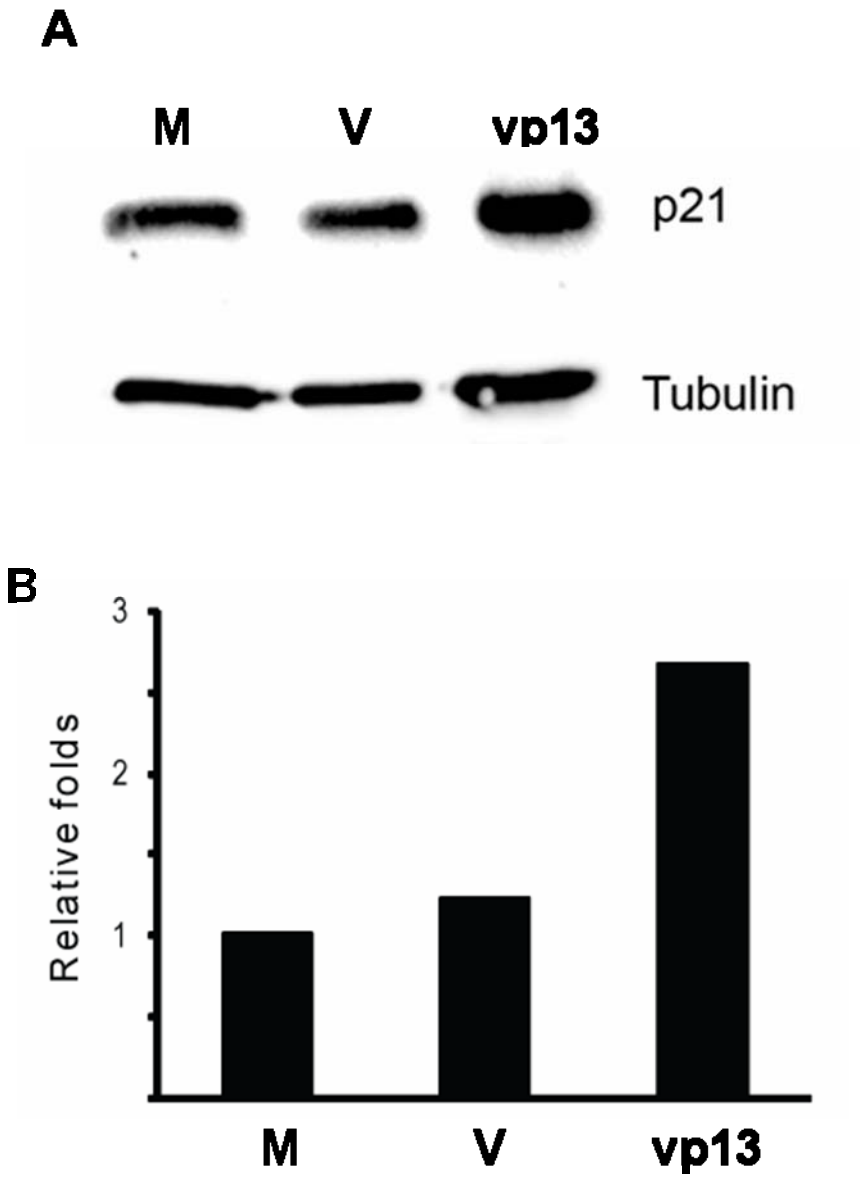

Fig 13. Effect of HEV vp13 on p21 expression. **A**. Western blot using p21 antibody. COS-7 cells were transfected with VenusN1-H3 plasmid (vp13), or empty vector (V) or left untransfected (M). Lysates were collected and subjected to SDS-PAGE and immunoblotting using p21-specific antibody. Tubulin was detected in the same blot for normalization. **B**. Graphic illustration of densitometric analysis of the digital image of p53 blot. The p53 protein level is presented as relative folds in comparison with untransfected control.

C

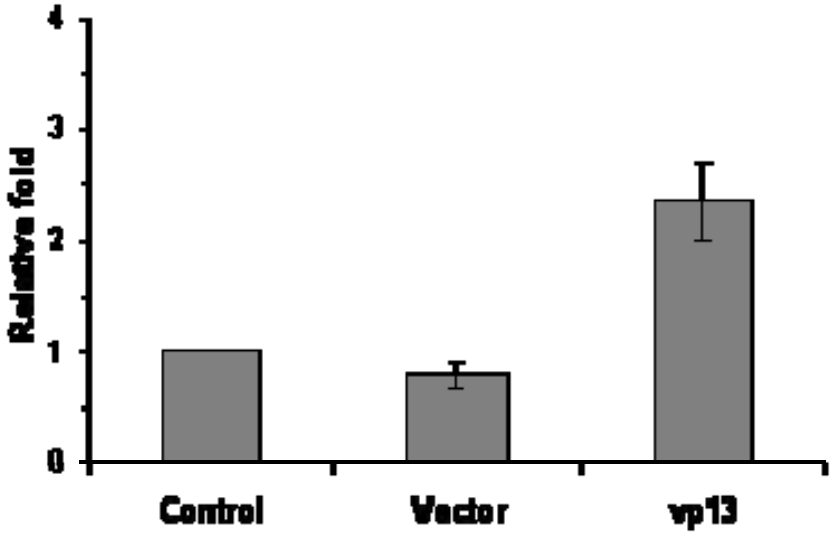

Fig 13 (Contd.). **C**. Quantitative RT-PCR to assess the transcript levels of p21 was performed and the results are shown as relative fold compared with mock-treatment control.

5.7 Effect of HEV vp13 on negative regulator of p53: MDM2.

As described in section 5.5, our results indicated that the levels of p53 are elevated in vp13-expressing cells. The p53 elevation could be either due to a direct effect of vp13 on p53 or, due to the interference of vp13 with p53 degradation that indirectly leads to elevated p53 levels. MDM2 is an important negative regulator of p53 since it acts as an E3 ubiquitin ligase, targeting p53 for proteosome-mediated degradation (Kubbutat *et al.*, 1997). Moreover, increase in MDM2 level also acts as a surrogate marker of nuclear localization of p53 (Giannakakou *et al.*, 2000). Therefore, we evaluated the levels of MDM2 expression in the cells expressing vp13 by Western blot

(Fig 14). We could not detect any detectable increase in MDM2 protein levels in vp13-expressing cells (Fig 14).

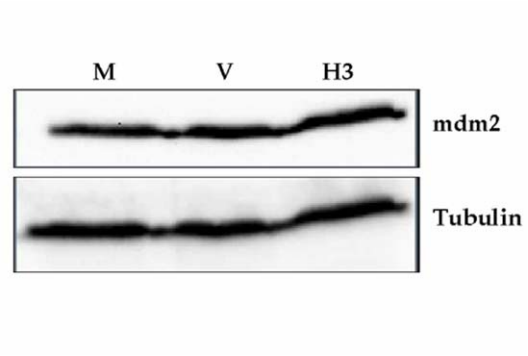

Fig 14. Effect of HEV vp13 on MDM2 expression. Western blot for MDM2 was performed on lysates collected from COS-7 cells that were transfected with vp13-expressing plasmid (H3), or empty vector (V) or left untransfected (M). Tubulin was detected in the same blot for normalization.

5.8 Activation of apoptotic pathway by HEV vp13.

One of the consequences of interference of MT dynamics is apoptosis, and activation of p53 in turn leads to activation of apoptotic responses (Vousden & Woude, 2000). Given that vp13 interacts with MT and possibly interferes with MT dynamics by altering the level of acetylated tubulin, and that vp13 also increases the levels of p53, it is possible that vp13 expression could result in apoptosis. Therefore, we analyzed for various events, in vp13-expressing cells, that are characteristic of the apoptotic pathway. Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear DNA-binding zinc finger protein that influences DNA repair and apoptosis (Kaufmann *et al.*, 1993). Proteolytic cleavage of PARP-1 (p116) yields a cleavage product (p85) and considered as a classical hallmark for apoptosis. Caspases are proteolytic enzymes that are the central mediators of apoptosis. Therefore, we tested for the cleavage of PARP-1 and for the expression of caspases in cells that transiently or stably express vp13, to determine if HEV vp13 activated the apoptotic pathway.

Western blot analysis was performed to evaluate the cleavage of PARP-1 indicated that in vp13-expressing HeLa cells. Our results indicated that cleavage of PARP-1 occurred at an increased level when compared to that in vector transfected or untransfected cells (Fig 15A). Densitometric analysis performed to determine the level of the cleavage product (p85), indicated that the p85 levels were about 4-fold higher in vp13-expressing HeLa cells compared to those in the vector-transfected cells (Fig 15B). To further determine the effect of vp13 on apoptosis, we generated HeLa cells that stably-expressed vp13, as described in materials and methods (section 3.1.1). We

observed PARP-1 cleavage in vp13-positive cells, but not in vp13-negative cells (Fig 15C). The level of PARP p85 in vp13-positive stable cells was over 60-fold higher than vp13-negative cells (Fig 15D).

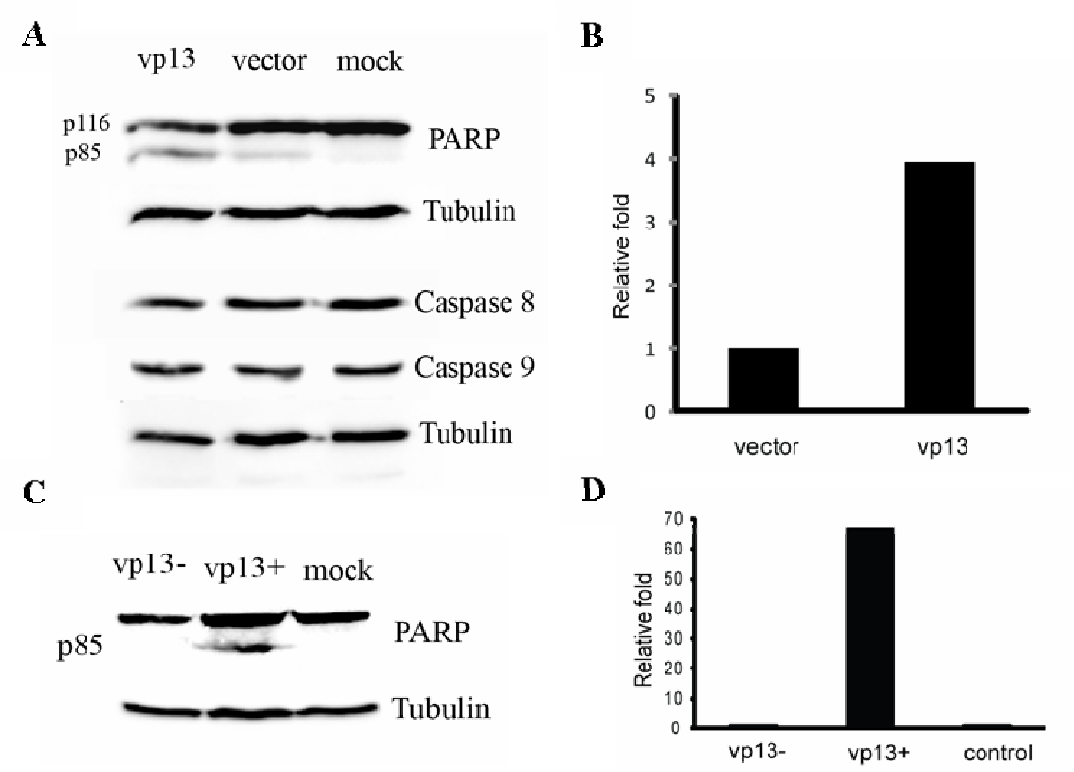

Fig 15. Activation of apoptotic pathway by vp13. **A**. Western blot detection of PARP-1 cleavage in HeLa cells transfected with pCMV-H3 construct (vp13) or empty vector or left untransfected (mock). Cleavage of caspases 8 and 9 was also tested in the same blot. Detection of β-tubulin in the same blots was conducted for normalization. **B**. Densitometric analysis of the digital image of PARP-1 blot. The PARP-1 p85 level is presented as relative folds in comparison with empty vector control. **C**. Western blot detection of PARP-1 in stable vp13-positive (vp13+) or -negative (vp13-) and normal HeLa cells (mock). **D**. Densitometric analysis of the digital image in 'C'.

Surprisingly, in HeLa cells that transiently express vp13, Western blot analysis indicated that there was no significant change in the levels of upstream initiator caspases, caspase-8 and -9, in vp13-expressing cells compared to cells transfected with empty vector or untransfected cells (Fig 15A). The caspase-8 subunit p18 or caspase-9 subunit p10 was not detected, indicating no cleavage or activation of these two initiator caspases.

For clones of the HeLa cells that stably express vp13, we found that the levels of caspase 8 and 9 were similar between the stable cells and control, but the activity levels of caspase-3 and -7 in vp13-positive cells was 40% higher than that in vp13-negative cells (Fig 16A). We believed that this increased activity of caspases 3 and 7 accounted for the increased levels of PARP-1 cleavage observed in these cells. In order to determine if the increased caspase activities resulted in any alteration in cell survival and growth, we performed a cell viability assay. Our results from the cell growth assay did not detect any change in the growth rate of stable HeLa cells of both clones compared to normal HeLa cells (Fig 16B). These results indicate that the apoptosis pathway activation by HEV vp13 was probably abortive or incomplete.

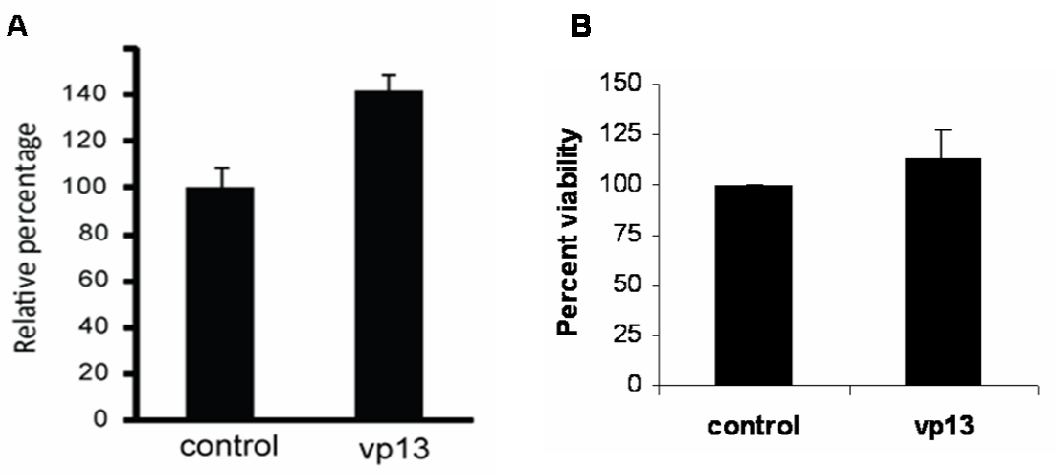

Fig 16. Caspase -3 and -7 activities (A) and cell viability (B) in vp13-positive stable HeLa cells. Relative percentage is shown in comparison with normal HeLa cells.

5.9 MT isolation and salt extraction assay.

Since vp13 colocalized with MTs, we speculated that there could be a physical interaction between vp13 and MTs and, therefore, isolated MTs would carry vp13 protein along with them. HeLa cells, transiently transfected with vp13-expression plasmid, were homogenized in MT-stabilization buffer PEM. The cell lysate was centrifuged to separate MTs from free tubulin and other soluble cytoplasmic fraction. Any proteins associated with MTs would pellet with them during the centrifugation. SDS-PAGE analysis showed that vp13 was in the pellet fraction, but not in the supernatant (Fig 17A), indicating that vp13 physically interacted with the MTs. Acetylated α-tubulin was only detected in pellet, as expected. Resuspension of the MT pellet in high salt (500 mM KCl) PEMS buffer extracts MT-associated proteins (MAP) or MAP-like proteins as the binding of these proteins to the MTs is charge-dependent [see review (Maccioni & Cambiazo, 1995)]. A new round of centrifugation with high salt PEMS buffer was carried out to separate MTs from MAP and MAP-like proteins. The vp13 was detected in both the pellet and supernatant fractions (Fig 17A). The salt extraction of vp13 from MT pellet indicates that vp13 behaves as MAP-like protein and the MT association appeared to involve an electrostatic interaction. The presence of vp13 in the pellet after the salt extraction indicates that vp13 also associates with subcellular organelles, which is consistent with the observation of punctate distribution of vp13 in addition to the linear array pattern under fluorescence microscopy (Fig 5). Acetylated α-tubulin was found only in pellet fraction. The absence of acetylated α-tubulin in the supernatant after salt extraction confirmed the stable polymerization of tubulin

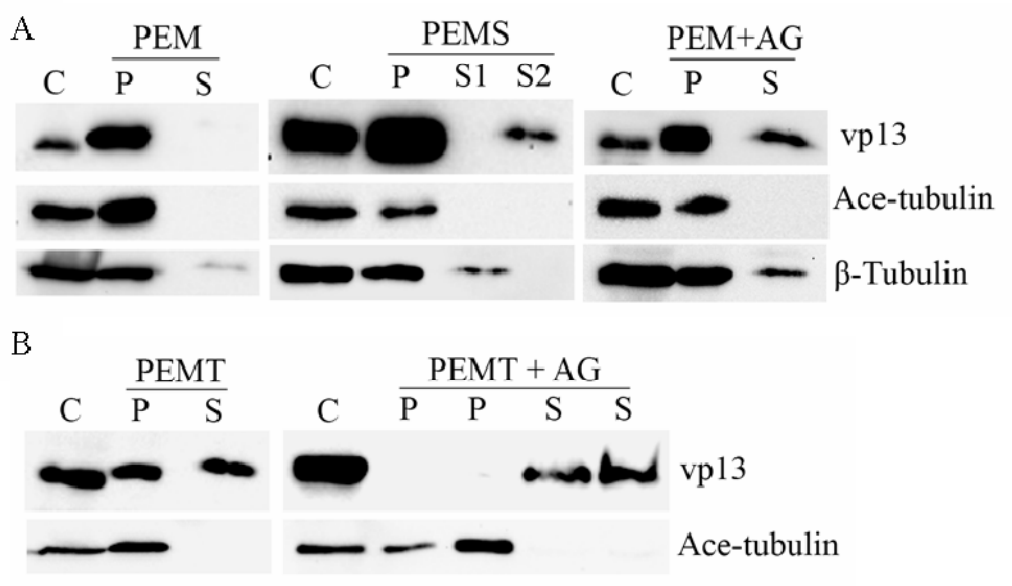

Fig 17. Microtubule isolation and salt extraction assay. **A**. MT isolation with PEM buffer and salt extraction. The vp13 protein was detected in the pellet fraction only when the cells were homogenized in PEM buffer and MTs were pelleted. A portion of vp13 protein was shifted to supernatant fraction after extraction of MT pellet with high salt PEMS buffer. A portion of vp13 protein was also detected in supernatant fraction when the cells were homogenized in PEM buffer supplemented with ATP and GTP (AG) and MTs were pelleted. Acetylated α-tubulin (Ace-tubulin) was detected only in the pellet fraction in all of the experiments. β-tubulin served as loading control. The supernatant fraction (S2) after extraction of MT pellet with PEMS buffer does not contain free tubulin. **B**. MT isolation with PEMT buffer and release of vp13 in the presence of the nucleotides. The vp13 protein was detected in both the pellet and supernatant fractions when the cells were homogenized in PEMT buffer, but in supernatant fraction only if ATP and GTP were included in PEMT lysis buffer. Acetylated α-tubulin was detected only in the pellet fraction in the experiments. C – Cell lysate; P – Pellet; S – Supernatant.

As the supernatant fraction after salt extraction with PEMS buffer contains motor proteins, MAP and MAP-like proteins, we speculated that vp13 might associate with motor proteins. The cytosolic motor proteins, dynein and kinesin, can be eluted from MTs by nucleotides, ATP and GTP (Paschal *et al.*, 1987). To investigate a possible association of vp13 with the motor proteins, MT isolation assay was done in PEM buffer supplemented with ATP and GTP. Western blot showed that vp13 was present in both the pellet and supernatant fraction (Fig 17A). Dynein was also found in the supernatant fraction. The presence of exogenous nucleotides reduced ability of motor proteins to interact with MTs, which led to shift of vp13 to supernatant.

From our results, we observed vp13 in the pellet after PEM lysis or salt extraction. We speculated that vp13 was also present in subcellular organelles besides interacting with MTs and that the lysis buffer PEM was unable to lyze the organelles. Thus, we used the PEMT lysis buffer that included Triton X-100, to determine if the detergent would release vp13 from the subcellular organelles and, to see if the pellet after this lysis with PEMT still contains vp13 or not. Both the pellet and supernatant contained vp13 after lysis with PEMT buffer (Fig 17B), as expected. Addition of ATP and GTP in this lysis buffer shifted vp13 from pellet to supernatant (Fig 17B), indicating that the association of vp13 with the MTs is sensitive to the presence of nucleotides, a property similar to other motor proteins. Acetylated α-tubulin was found only in pellet fraction. These results collectively indicate that vp13 physically associates with the MTs and this association is nucleotide-sensitive. They also indicate that vp13 probably acts as a MAP-like protein, suggesting a potential association of vp13 with other cellular motor proteins.

5.10 Involvement of the dynein motor protein in vp13-MT interaction

Dynein is a large protein complex and functions as a molecular motor that transports various cellular cargo towards the minus end of microtubules (King, 2000). Many viruses sequester the dynein machinery of infected cells to move along the MTs to reach their replication destinations (Alonso *et al.*, 2001; Mabit *et al.*, 2002; Ploubidou *et al.*, 2000; Suomalainen *et al.*, 1999). Furthermore, dynein binding to membranous organelles occurs through a second large protein complex, dynactin (King, 2000). Over expression of a dynactin subunit, p50/dynamitin, disrupts the dynactin complex and thereby dynein function (Echeverri *et al.*, 1996).

Since vp13 colocalizes with MTs and potentially associates with motor proteins, we examined the role of dynein motor protein in the vp13 distribution pattern. For this purpose, we co-transfected HeLa cells with p50/dynamitin expression plasmid and VenusC1-H3 and observed the cells under live fluorescent microscopy at 24 h post-transfection. We observed that the linear array pattern of vp13 expression was abolished in the cells co-transfected with p50/dynamitin plasmid while no such observation was made in the cells transfected with vp-13 plasmid only (Fig 18, top panels). Many green punctates in cytoplasm were also observed. Sodium vanadate (Na_3VO_4) is a well-described inhibitor of dynein activity (King, 2000). Incubation of HeLa cells with a non-toxic concentration of 100 μM Na_3VO_4 for 2 h abolished the linear array pattern of vp13 in the cells expressing vp13 (Fig 18, bottom panels), validating the specificity of the

above observations with p50/dynamitin expression. Therefore, these results demonstrate an important role of dynein motor protein in the vp13-MT interaction process.

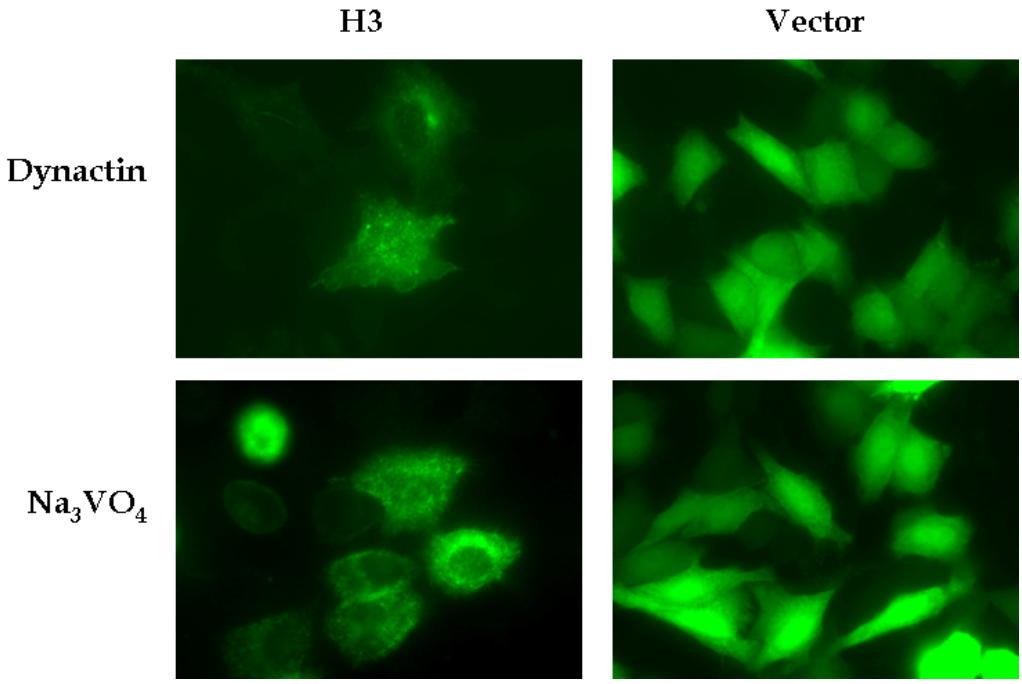

Fig 18. Involvement of dynein in vp13-MT interaction. HeLa cells were co-transfected with VenusN1-H3 and p50/dynamitin constructs (left panels). Fluorescence microscopy was conducted 24 h after transfection. Treatment of these cells with sodium orthovanadate (Na_3VO_4) for 2 h abolished vp13 filamentous expression pattern (bottom left). HeLa cells transfected with the vector alone are shown as controls (right panel).

Chapter 6: Discussion and Conclusion

HEV is an important virus of public health concern. HEV infections lead to several outbreaks in developing countries worldwide. Several aspects of HEV biology and pathogenesis are not clearly known due to two main reasons – one, the lack of an efficient cell culture system for virus propagation, and two, lack of an animal model system. Most of the knowledge currently available is based on studies conducted with in-vitro synthesized replicons or individual viral proteins expressed in cells. Scientists around the world are involved in studying and understanding the various aspects of HEV molecular biology, replication and pathogenesis in spite of these limitations.

HEV vp13 is a small phosphoprotein encoded by ORF3. Though it is believed to play a regulatory role in viral pathogenesis, its exact functions are not clearly known. Several researchers have found that vp13 plays a role in various cellular signaling pathways and that it is essential for infectivity *in vivo*, at least in macaques and pigs. In this study, we have attempted to understand and characterize the functional role of the vp13 protein. In particular, our work has focused on the interaction of vp13 with the cell structural component, mainly the microtubules and the consequences of this interaction. We have also studied the effect of vp13 expression on several cellular proteins that are involved in cell survival and growth. Our findings indicate that vp13 interacts with the MTs, which probably might facilitate HEV trafficking inside cells and enable efficient viral infection in vivo.

MTs are a major component of the cell structural framework. They are polarized filaments that have important roles in various cell functions such as cell shape maintenance, cell migration and intracellular transport. Several viruses use the cellular cytoskeletal machinery, such as actin, MTs, and their associated molecular motors, to traffic to the site of replication, assembly, and release (Dohner *et al.*, 2005; Dohner & Sodeik, 2005; Smith & Enquist, 2002). Many viruses such as herpesvirus and adenoviruses traffic within infected cells specifically using the MTs (Dales & Chardonnet, 1973; Kristensson *et al.*, 1986). The Tat protein of HIV, which is a powerful activator of viral gene expression, was also found to target MTs by interacting with tubulin and induce apoptosis (Chen *et al.*, 2002). Our findings clearly indicate that vp13 protein of HEV co-localizes with the MTs. The expression pattern of vp13 was abolished when transfected cells were treated with nocodazole, an agent that specifically targets and destabilizes the MTs, confirming that vp13 interacts with MTs.

Previous reports have shown that vp13 is present in the cytoskeletal fraction and that in vp13 expressing cells, the protein is often seen as "speckles" in the cytoplasm (Tyagi *et al.*, 2004; Zafrullah *et al.*, 1997). A recent report shows that vp13 localizes in early and recycling endosomes and causes delay in the postinternalization trafficking of epidermal growth factor to late endosomes/lysosomes (Chandra *et al.*, 2008). A major difference in our experiments was the use of Venus reporter vectors to express vp13 as a fusion protein. Venus vectors contain an improved version of YFP (yellow fluorescent protein), named "Venus" (Nagai *et al.*, 2002). YFP is a variant of GFP that has been used in various applications in biological studies. Antibody against GFP reacts with YFP and

other related variant fluorescent proteins. One major advantage of venus vector is monomeric-expression of the reporter protein, thereby avoiding over-expression and thus aggregation. Therefore, our results indicate a filamentous pattern of vp13 expression though a speckled appearance was also visible. However, when a difference vector was used, we also observed aggregated proteins in big speckles in HeLa cells. Our results also suggest that the Venus fusion protein is a better method to be used for localization studies. The expression of vp13 fusion protein in COS-7 and Huh-7 cells also appeared in filamentous pattern, although at a lower rate, but the filament lengths were shorter than those observed in HeLa cells. The difference in filament length may be due to the difference in cell type and MT organization. Since the observation of the filamentous pattern was better in HeLa cells, these cells might serve as a better system to study vp13 distribution and MT interaction.

After our findings demonstrated that vp13 interacts with the MT in a specific manner, we determined the specific domains of vp13 that are responsible for this interaction. The MT interaction domain of vp13 was located in the two N-terminal hydrophobic domains. Both the domains were found to be essential for the interaction, since constructs with one of the domains did not show the filamentous expression pattern in cells after transient transfection. Interestingly, punctate expression pattern was observed in cells transfected with the construct containing the first hydrophobic domain of vp13, which indicates that the fusion protein could be expressed in subcellular organelles such as endoplasmic reticulum or Golgi. The truncation constructs containing H3D3 and H3D4 had homogenous expression throughout the cells (Fig. 4), which

indicates that the fusion proteins were soluble in cytoplasm. We also noticed that expression of the reporter with full-length vp13 had stronger fluorescence than that of truncation constructs containing the two N-terminal hydrophobic domains. It is speculated that the full-length vp13 might have a better interaction with MTs. The functional significance of vp13-MT interaction, in general, and the importance of the individual domains of vp13 for the MT interaction, in specific, need to be studied in further detail and could serve as future focus areas of this research.

Acetylation and deacetylation under normal physiological conditions act as powerful and dynamic means of controlling MT dynamics. Cross-linking of MTs by cellular structures occur along their length or cap on their ends are known to cause acetylation and stability on MTs (Westermann & Weber, 2003). MT dynamics, a fundamental property of MTs, is critical for diverse cellular functions and regulated by many factors. Interference of the dynamics can lead to adverse consequences, including apoptosis (Sorger *et al.*, 1997). Having observed that vp13 and MT interact, we continued to study the effect of vp13 interaction on MT stability. Therefore, we observed changes in MT stability, using the levels of acetylated α-tubulin as an indicator. In vp13-expressing cells, MT stability was enhanced, as shown by the elevation of acetylated α-tubulin. Thus, our results demonstrate the effect of vp13 on MT dynamics. It would be interesting to know if MT stability or levels of acetylated α-tubulin are also elevated in natural viral infections. However, our results provide a strong speculation that vp13 could serve as an important virulent factor in HEV infections.

We detected elevation in PARP-1 cleavage in cells expressing vp13. PARP-1 cleavage is regarded as a hallmark for apoptosis. It is known that severe DNA damage triggers a PARP-mediated apoptosis and PARP-1 cleavage is mediated by caspase-3 during the late phase of apoptosis (Jin & El-Deiry, 2005). Increased levels of p53 were demonstrated during PARP-1 cleavage following DNA damage. In the cells expressing vp13, elevation of p53 and caspase-3 and -7 activities were detected and may account for the PARP-1 cleavage. However, we did not detect any change in caspase 8 and 9 protein level or their cleavage in Western blotting analysis. The result indicates that elevation of caspase-3 and -7 was either by other unknown factors instead of caspase 8 and 9, or by low level of caspase-8 and -9 activities that was below detection level by Western blot. It has also been observed that PARP-1 activation can cause the translocation of apoptosis inducing factor from the mitochondria to the nucleus, leading to a caspase-independent apoptotic pathway (Yu *et al.*, 2002). Whether such events also occur in vp13-expressing cells needs to be investigated. Though the vp13 cells have elevated PARP-1 cleavage, we did not observe apoptosis or change in cell viability. The reason could be that vp13 blocks cytochrome C release (Moin *et al.*, 2007) and vp13 has other functions that compromised the vp13 activation of the apoptosis pathway and protected the cells from death.

Another important aspect of this work was to determine the effects of vp13-MT interaction on several cellular proteins involved in cell cycle. When we analyzed the levels of the tumor suppressor protein p53 and cyclin-dependent kinase inhibitor p21 expression, we observed that the levels of both these proteins were elevated in vp13-

expressing cells when compared to those that do not have vp13. The vp13 interaction with MTs could be the stress factor that led to the p53 elevation. However, our work does not exclusively demonstrate the relationship of MT interaction and p53 elevation, since vp13 may bind to other cellular factors to activate the p53. As downstream players of p53, the levels of p21 and MDM2 were elevated accordingly. Because of its inhibitory function in cell cycle progression, p21 elevation was expected to result in slowdown of cell growth. However, we did not observe any significant cell growth change in cells with vp13 expression. It is known that p21 has dual roles in apoptosis (Pavelic *et al.*, 2008) and can inhibit p53-mediated apoptosis (Sohn *et al.*, 2006). Thus, in cells with vp13 expression, the p21 elevation might inhibit apoptosis. However, other possible reasons for the absence of apoptosis in the presence of vp13 should also be considered.

Molecular motors such as kinesin and dynein play important roles in cellular trafficking, including trafficking of viruses (Dohner *et al.*, 2005). Cytoplasmic dynein, together with its activator dynactin, is a multisubunit macromolecular complex necessary for cargo transport. Further, dynamitin (p50) is a component of the dynein complex whose overexpression results in disruption of dynein-dependent transport by a dominant-negative effect (Dohner *et al.*, 2002). In our study, dynein motor function was found to be critical for the vp13 colocalization with MTs since over expression of p50/dynamitin abolished the vp13 expression pattern. In addition, treatment of cells with sodium vanadate, a well-described inhibitor of dynein activity, altered the filamentous pattern of vp13 expression. These results indicate that vp13 might bind the dynein motor complex

and the binding lead to the colocalization with MTs. However, we do not have direct evidence to demonstrate interaction between vp13 and dynein complex.

To further address the interaction of vp13 with MT, we resorted to a MT isolation assay that used detergent and salt treatments. Our results indicated that vp13 interaction with MTs occurs through the dynein motor protein complex since vp13 was found in MT pellet and extracted from the pellet with high salt buffer. This result was consistent with the observation of fluorescence microscopy that showed the vp13 colocalization with MTs. In this assay, the vp13 transiently transfected cells were used for MT isolation. The presence of vp13 in MT pellet indicated direct interaction of vp13 with MTs. Salt extraction of the MT pellet removed vp13, which indicated that vp13 behaved like an MAP or MAP-like protein, since high salt buffer did not favor their binding to MTs. The presence of vp13 in the supernatant fraction in the presence of ATP and GTP showed that vp13 may associate with motor proteins complex. It has been proposed that HEV ORF3 protein may have a role in virion release (Takahashi *et al.*, 2008). Furthermore, recent studies have shown that ORF3 protein was detected on the surface of virions released from infected cells, but not those from feces (Takahashi *et al.*, 2008). Our findings that vp13 protein interacted with motor proteins indicated that it may utilize the motor protein for virion trafficking. We speculate that vp13 interaction with MTs is due to vp13 binding with other MAP-like proteins because ionic interaction was responsible for the interaction and the two N-terminal domains of vp13 were needed for the colocalization with MTs. It may be the reason why vp13 was found in cytoskeleton fraction, but

immunoprecipitation with tubulin antibody failed to precipitate vp13 (Zafrullah *et al.*, 1997).

In conclusion, our data demonstrate that vp13 interacts with MTs and interferes with their dynamics, which could create a conducive intracellular-environment for the establishment of successful HEV infection. The vp13 association with MTs led to modulation of MT dynamics by elevating their stability. The association also led to activation of apoptosis pathway, shown by elevation of PARP-1 cleavage and caspase-3 and -7 activities. However, no adverse on cell growth was detected, which might be vp13's other functions aborting the apoptosis induction. Furthermore, we have identified the MT-binding regions of vp13 in both the N-terminal hydrophobic domains. Our results also showed that vp13 expression in HeLa cells increased the levels of cellular proteins p53 and p21. An important finding in this study was the involvement of dynein in vp13-MT interaction and that vp13 behaves as a MAP-like protein. These results advance our understanding of HEV vp13 function. Further studies are needed to elucidate the mechanism and the biological effects of the MT modulation. Studying the vp13 interaction with the MTs and the role of molecular motors dynein and kinesin in the interaction will assist our understanding of HEV pathogenesis. Moreover, delineating the strategy for viral protein binding to the MTs provides clue to the development of novel antiviral drugs that can disrupt intracellular viral trafficking.

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