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1 Rescue of infectious recombinant Hazara nairovirus from cDNA reveals the nucleocapsid
2 protein DQVD caspase cleavage motif performs an essential role other than cleavage.

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ABSTRACT

The *Nairoviridae* family of the *Bunyavirales* order comprises tick-borne tri-segmented negative strand RNA viruses, with several members associated with serious or fatal disease in humans and animals. A notable member is Crimean-Congo hemorrhagic fever virus (CCHFV), which is the most widely-distributed tick-borne pathogen, and associated with devastating human disease with case/fatality rates averaging 30%. Hazara virus (HAZV) is closely-related to CCHFV, sharing the same serogroup and many structural, biochemical and cellular properties. To improve understanding of HAZV and nairovirus multiplication cycles, we developed for the first time a rescue system permitting efficient recovery of infectious HAZV from cDNA. This system now allows reverse genetics analysis of nairoviruses without the need for high biosafety containment, as is required for CCHFV. We used this system to test the importance of a DQVD caspase cleavage site exposed on the apex of the HAZV nucleocapsid protein arm domain that is cleaved during HAZV infection, and for which the equivalent DEVD sequence was recently shown to be important for CCHFV growth in tick but not mammalian cells. Infectious HAZV bearing an un-cleavable DQVE sequence was rescued and exhibited equivalent growth parameters to wild-type in both mammalian and tick cells, showing this site was dispensable for virus multiplication. In contrast, substitution of the DQVD motif with the similarly un-cleavable AQVA sequence could not be rescued despite repeated efforts. Together, this work highlights the importance of this caspase cleavage site in the HAZV lifecycle, but reveals the DQVD sequence performs a critical role aside from caspase cleavage.

IMPORTANCE

Hazara virus is classified within the *Nairoviridae* family along with Crimean-Congo hemorrhagic fever virus (CCHFV), which is one of the most lethal human pathogens in existence, requiring the highest biosafety level (BSL) containment (BSL-4). In contrast, HAZV is not associated with human disease and thus can be studied using less-restrictive BSL-2 protocols. Here, we report a system able to rescue Hazara virus (HAZV) from cDNAs, thus permitting reverse genetic interrogation of the HAZV replication cycle. We used this system to examine the role of a caspase cleavage site, DQVD, within the HAZV nucleocapsid protein that is also conserved in CCHFV. By engineering mutant viruses, we showed caspase cleavage at this site was not required for productive infection, and furthermore that this sequence performs a critical role in the virus lifecycle aside from caspase cleavage. This system will accelerate nairovirus research due to its efficiency and utility under amenable BSL-2 protocols.

INTRODUCTION

The *Bunyavirales* order comprises over 500 RNA viruses that are the causative agents of infection and disease across a broad range of hosts that encompasses insects, plants, animals and humans. The bunyavirus genome comprises between 2 and 8 segments of negative sense RNA, with some species utilizing an ambisense coding strategy for one or more of their segments [1]. The recent use of metagenomic techniques [2], [3], [4] has led to the discovery of many new diverse bunyaviruses, particularly from within arthropod hosts, and so bunyavirus classification is in a state of flux. Currently, the order is divided into twelve families [1], and viruses associated with human infections are classified within five of these, namely the *Arenaviridae*, *Hantaviridae*, *Nairoviridae*, *Peribunyaviridae* and *Phenuiviridae* families. The *Nairoviridae* family comprises tick-borne tri-segmented RNA viruses, with several members associated with serious and fatal disease in both humans and animals. Nairobi sheep disease virus, after which the family was named, causes acute gastroenteritis in susceptible populations of sheep and goats, with an associated mortality rate of 90%, resulting in significant economic impact [5]. Crimean-Congo hemorrhagic fever virus (CCHFV) is the most widely distributed tick-borne pathogen on earth [6], and is associated with a devastating human disease known as Crimean-Congo hemorrhagic fever (CCHF) with case/fatality rates averaging 30%, but rising as high as 80% in specific outbreaks [7], [8]. CCHF most commonly results from the bite of a CCHFV-infected tick of the *Hyalomma* species, which is widespread within Africa, South-East Europe and Asia, and concerns are growing over the widening habitat of permissive tick vectors in the face of global warming [9]–[12]. Due to the associated risks, CCHFV is one of a select group of pathogens classified within hazard group 4, requiring the highest level of biological containment for its propagation (biosafety level 4; BSL-4), which has restricted research activity and hindered progress in elucidating the molecular and cellular biology of CCHFV, as well as nairoviruses in general.

Hazara virus (HAZV) is a nairovirus that is closely related to CCHFV, such that it is included within the same serogroup [1], however, it is non-pathogenic in humans. As a

consequence, HAZV can be studied in relatively accessible BSL-2 facilities, which permits more rapid and cost-effective research.

The genomes of HAZV and CCHFV are tri-segmented, comprising small (S), medium (M) and large (L) segments. The S segment of both viruses encodes the nucleoprotein (N), which encapsidates the RNA segments through its RNA binding ability. In addition, the CCHFV S segment is thought to express an additional non-structural protein (NSs) using an ambi-sense transcription strategy, with a role in apoptotic activation [13]. The M segment encodes the glycoprotein precursor GPC that is subsequently cleaved into the structural glycoproteins Gn and Gc, and additional non-structural glycoproteins, whereas the L segment encodes the relatively large RNA-dependent RNA polymerase (RdRp) [14], which is involved in replication and transcription of viral RNA, but also encodes an N terminal ovarian tumour (OTU) domain with roles in mitigating host cell innate immunity [15].

We and others recently solved the crystal structure of the N protein of both HAZV and CCHFV, which revealed the location of a conserved caspase-3 cleavage (C3C) site on the exposed apex of the extended arm domain suggestive of an important role at some stage of the nairovirus multiplication cycle [16]–[19]. One possibility was that the C3C site acted as a caspase-3 decoy, diverting caspase-3 away from its native cellular substrates and thus preventing or prolonging apoptosis activation, as has recently been found for the related Junin arenavirus (JUNV) [20]. In support of this, both CCHFV and HAZV-N proteins are extensively cleaved at their C3C sites in mammalian cells, with apoptosis induced relatively late in infection [21],[22]. However, during infection by both HAZV and CCHFV in tick cell lines, the C3C site is subjected to little or no cleavage, revealing a fundamental difference in the tick and mammalian cellular response to nairovirus infection [23],[22]. Recently, a system capable of rescuing infectious CCHFV from cDNA was used to show that the conserved C3C site on the arm apex was dispensable for infection in mammalian cells, but strikingly mutation of this motif resulted in a significant drop in replication ability during infection of cells of tick origin [23]. Taken together, these findings revealed a critical role for the C3C site for CCHFV growth in ticks, although whether the C3C site in HAZV was required remained unknown.

Here, we developed a system for the rescue of infectious HAZV to authentically-test the importance of caspase cleavage of HAZV-N during infection. By generating a panel of mutant viruses in which the arm domain DQVD was rendered un-cleavable, we showed while this sequence was critical for virus viability, it was not to allow caspase cleavage. Instead, our results show that the DQVD site performs a critical role in the virus life cycle aside from acting as a caspase substrate. To the best of our knowledge, this work represents the first recovery of recombinant HAZV (rHAZV) and also the first nairovirus rescue system that permits virus rescue in BSL-2 facilities, thus facilitating rapid future gains in the understanding of this important group of viruses.

RESULTS

Characterization of the DQVD caspase cleavage site in HAZV-N. In previous work, we identified a number of HAZV-N specific cleavage products during low-MOI infection of mammalian cells [22]. To further characterise these bands, we repeated HAZV infection of human-origin SW13 cells at a higher MOI of 1.0 to increase presence of these cleaved forms of HAZV-N thereby facilitating detection via western blotting. HAZV-N antisera detected prominent N-specific cleavage products with apparent molecular masses of approximately 30 and 22 kDa, and exhibiting a temporal expression pattern, being absent during initial stages of infection, and most abundant at later time points (Fig 1A). Previous work by ourselves and others [16], [19] established that the generation of 30 and 22 kDa products resulted from caspase-3 cleavage of HAZV-N at a consensus DQVD motif (Fig 1B) located at the apex of the HAZV-N arm domain (Fig 1C). The HAZV-N arm domain also possesses the sequence ENKD, which partially-conforms to the caspase-3 cleavage consensus sequence, and is immediately upstream of the DQVD such that these sites overlap forming the sequence ENKDQVD.

Recovery of recombinant HAZV. In order to investigate the role of the ENKD/DQVD caspase cleavage motifs in the authentic context of HAZV infection, we generated a cDNA-based system to allow rescue of infectious HAZV. This system comprised three cDNA plasmids that were designed to transcribe the anti-genomic cRNA strands of the HAZV S, M and L segments under the control of the bacteriophage T7 RNA polymerase promoter, an approach that has been used by others to rescue bunyaviruses of the *Arenaviridae*, *Peribunyaviridae* and *Phenuiviridae* families, as well as CCHFV from the *Nairoviridae* family [24]–[27]. The three corresponding plasmids pMK-RQ-S, pMK-RQ-M and pMK-RQ-L were co-transfected into BSR-T7 cells expressing T7 RNA polymerase, along with plasmid pCAG-T7pol, which allows increased expression of the T7 RNA polymerase, and in our hands further increases the efficiency of virus recovery (Fig 2A). Additional transfections in which HAZV L

segment expression plasmid pMK-RQ-L was omitted were also performed, to act as rescue controls incapable of generating infectious virus.

In cells transfected with all four plasmids ('complete transfection'), HAZV-N protein was abundantly detected by western blotting in the primary transfected cultures at 120 hours post transfection (p.Tr) (Fig 2B; p.Tr , lane 1) suggestive of HAZV rescue. As confirmation, supernatants of primary transfected cultures at 72-, 96- and 120-hours p.Tr were harvested and used to infect further SW13 cells in order to amplify any rescued viruses. At 48-hours post infection (p.Inf) SW13 cell lysates were harvested and tested for presence of HAZV-N by western blot analysis, which revealed abundant N production in 96- and 120-hours p.Inf samples, confirming virus amplification had occurred (Fig 2B; p.Inf, lanes 2-4). Rescue of WT rHAZV was achieved in all attempts using this optimized protocol, indicating the system was robust and efficient.

In contrast, HAZV-N protein was not detected in control transfected cells that received no pMK-RQ-L ('control transfection') in primary transfected cells (Fig 2B; p.Tr , lane 5), and correspondingly, no N was detected in cell lysates harvested at any time points in the p.Inf wells (Fig 2B; p.Inf, lanes 6-8).

To examine the efficiency of HAZV rescue, supernatant samples taken from primary transfected BSR-T7 cells at 72-, 96- and 120-hours p.Tr time points were used to set up plaque assays in SW13 cells. Analysis of the resulting plaques formed showed increasing titres of rHAZV in the p.Tr supernatant increasing through 72-, 96- and 120-hours p.Tr time points (Fig 2C), with titres of rescued virus reaching over 1.2×10^5 pfu/ml in the 120-hours p.Tr supernatants.

Comparison of HAZV and rHAZV growth kinetics. Although the rescued virus was generated from a cDNA source corresponding precisely to HAZV strain JC280, we wanted to verify that rHAZV displayed equivalent growth properties to the parental JC280 isolate, which may have acquired cell culture adaptations through multiple rounds of propagation. To achieve this, we compared multi-step growth kinetics of rHAZV alongside the parental virus isolate and

mock-infected controls. Following infection at an MOI of 0.001, supernatant samples from each infection scenario were harvested every 24 hours for up to 96 hours p.Inf, and used to titre infectious virus by plaque assay (Fig 3A). Similar titres for HAZV and rHAZV were observed at all time points, indicating infectious rHAZV displayed equivalent growth kinetics to the parental virus. Plaque morphology of rHAZV also resembled that of the parental virus (Fig 3B), providing further evidence that recombinant and parental viruses possessed indistinguishable growth properties.

Confirmation of HAZV rescue from a cDNA source by incorporation of a silent mutation. To confirm the recombinant source of rHAZV as cDNA, a single nucleotide change of G723T in the N protein open reading frame (ORF) was engineered into pMK-RQ-S to create pMK-RQ-S(G723T). This change was silent at the amino acid level, but generated a *Hind* III recognition site in the corresponding cDNA sequence (Fig 4A). Plasmid pMK-RQ-S(G723T) replaced the corresponding WT pMK-RQ-S plasmid in a HAZV recovery experiment alongside WT rescue plasmids pMK-RQ-M, pMK-RQ-L, and pCAG-T7pol, described above, and western blotting of post-infection lysates revealed the abundant presence of HAZV-N indicating successful mutant virus recovery (Fig 4B). To verify incorporation of the G723T change, RNA extracted from rHAZV and rHAZV(G723T) infected SW13 cell supernatants was used as a template for RT-PCR amplification, using primers designed to yield an 802 nucleotide-long cDNA fragment encompassing the *Hind* III site. While the WT cDNA fragment was un-cleaved by *Hind* III, the corresponding fragment from rHAZV(G723T) was cleaved to generate two products with lengths of approximately 300 and 500 bp (Fig 4C and 4D), corresponding to the fragments expected following *Hind* III digestion. To confirm that the amplified PCR fragment was templated from a cDNA that originated from an RNA source rather than plasmid carried over from the transfections, we also performed control PCR amplifications without prior RT treatment, and these failed to yield a DNA fragment (Fig 4D). Successful rescue of this rHAZV variant was also confirmed by sequencing of the RT-PCR fragment bearing the introduced

Hind III recognition site. Taken together, these findings confirm the utility of the rescue system to efficiently generate both WT and mutant rHAZV variants.

Rescue of infectious HAZV variants with alterations to caspase cleavage sites on the N protein arm apex. Previous work from ourselves and others has identified caspase-3 cleavage motifs on both the CCHFV N (DEVD) and the HAZV-N (DQVD) proteins at the apex of their respective arm domains [16], [17], [19] and mapping these sites onto the corresponding crystal structures show these are in precisely superimposable positions (Fig 5A). Both sites also are preceded by sequences that partially-conform to caspase cleavage consensus sequences suggesting that their cleavage is possible, but nevertheless unlikely. These sequences are KHKD in CCHFV and ENKD in HAZV, and both sites overlap the established DEVD/DQVD cleavage sites at a critical aspartic acid residue (D) (Fig 5A). Both DEVD and DQVD sites are known to be cleaved during infection of mammalian cells by CCHFV and HAZV, respectively [22], [21], and a mutant CCHFV with the DEVD site changed to the non-cleavable AEVA site was shown to be severely growth-attenuated in tick cells, but phenotypically-silent when growth in cells of mammalian origin [23].

To investigate the importance of the DQVD and overlapping ENKD motifs in the context of HAZV infection (Fig 1A and 5A), we created mutant plasmids designed to express HAZV S segments in which the ENKD and DQVD motifs within the N ORF were individually perturbed by mutation. The aspartic acid (D) residues at position 1 within these motifs (underlined) are known to be critical for caspase recognition and cleavage [28], and so in both cases, this residue was changed to glutamic acid (E) to generate the non-cleavable sequences ENKE and DQVE. An additional double mutant plasmid with the sequence AQVA was also generated, concurrently rendering both ENKD and DQVD motifs as un-cleavable, and allowing direct comparison with the AEVA mutant generated for CCHFV, described above [23].

As with the rHAZV recoveries described above, the plasmid expressing the WT HAZV S segment pMK-RQ-S, or its mutant derivatives (named correspondingly DQVE, ENKE or

AQVA) were transfected into BSR-T7 cells along with pMK-RQ-M, pMK-RQ-L, and pCAG-T7pol, and at 72-, 96- and 120-hours p.Tr supernatants were collected and used to re-infect fresh SW13 monolayers for 48 hours. At this time point, lysates were collected and examined for HAZV-N expression via western blotting with anti-HAZV-N antisera (Fig 5A) with HAZV-N detection in virus rescue wells indicating successful recovery of both recombinant WT and mutant viruses. Successful rescue was achieved for HAZV-N mutants rHAZV-DQVE and rHAZV-ENKE, with rescue confirmed by sequencing of an RT-PCR fragment bearing the altered cleavage sites (data not shown) amplified from viral RNA harvested from p.Inf supernatants. In contrast, double mutant rHAZV-AQVA could not be rescued, as evidenced by three failed rescue attempts alongside consistent successful rescue of WT rHAZV.

The growth kinetics of rescued rHAZV-ENKE and rHAZV-DQVE were examined alongside WT rHAZV via plaque assay over a 4-day time course (Fig 5B), and morphology of resulting plaques was assessed (Fig 5C). Viral titres at all time points tested showed no significant differences between WT rHAZV and the rHAZV-ENKE or rHAZV-DQVE mutants (Fig 5D) and taken together these results show that abrogating caspase cleavage at either of these sites has no detectable effect on virus fitness.

The HAZV-N DQVD caspase cleavage motif is required for rescue of infectious virus, but for reasons other than caspase cleavage. The results of the previous section showed HAZV mutants bearing un-cleavable DQVE and ENKE sequences in N could be rescued, whereas an AQVA mutant could not. As expected, abrogation of cleavage at the DQVD site coincided with loss of the customary 30 kDa HAZV-N cleavage product, as measured by western blot analysis (Fig 5E). Interestingly, this band was replaced by a band of increased apparent mass, corresponding to approximately 32 kDa. This 32 kDa HAZV-N fragment is too large to correspond to alternative cleavage at the adjacent and overlapping ENKD, so our results show that during infection with the rHAZV-DQVE mutant, N cleavage at neither the overlapping ENKD site, nor the altered DQVE site, occurs. The fact that infection with the DQVE mutant does not result in generation of a 30 kDa fragment also shows the

ENKD site is not a substrate for caspase cleavage even when the DQVD site can no longer be cleaved, consistent with its only partial similarity to the caspase cleavage consensus. Taken together, these results show that HAZV-N lacking a functional caspase cleavage site at the arm apex can be rescued as infectious virus, and thus a caspase cleavable site at the arm apex is not a prerequisite for HAZV viability.

Thus, our results show that individually, the DQVD and ENKD motifs are dispensable for the HAZV life-cycle in cultured mammalian cells. However, our observation that the double mutant AQVA could not be rescued despite repeated attempts suggests that simultaneous alteration of both sites critically-prevents virus multiplication at some stage of the life cycle. As we show above that cleavage within the arm domain is not required for virus viability, we suggest the fatal deficiency of the AQVA mutant relates to a function other than caspase cleavability.

This observation is interesting in comparison to the previous findings for CCHFV that the corresponding AEVA mutant, which also acts as a double mutant knocking out both KHKD and DEVD sites, could be rescued as infectious virus, and furthermore replicated to wild-type (WT) titres in mammalian cells [23]. Taken together, these findings suggest that sequences within the arm apex of CCHFV and HAZV perform different roles, with those of HAZV being highly sensitive to change, with those of CCHFV being more tolerant.

The DQVD caspase-3 cleavage motif is also dispensable for HAZV replication in cells of tick origin. As described above, previous work by others has shown that a CCHFV variant with an un-cleavable AEVA substitution at the N protein arm apex DEVD site replicates to WT titres in SW13 cells, but cannot replicate in tick cells, suggesting a critical role for this motif in the CCHFV life cycle [23]. Having shown that mutations at caspase cleavage sites ENKD or DQVD at this location on HAZV-N pose no significant reduction in growth in mammalian cells (Fig 5C), we next wanted to examine the role of these motifs for replication in tick cells.

290 To achieve this aim, we examined the ability of the rHAZV-DQVE and rHAZV-ENKE
291 variants to multiply in CTVM/HAE9 tick cells, as determined by their ability to express
292 abundant HAZV-N after 48 hours (Fig 6A). Infection with rHAZV carrying individual DQVE and
293 ENKE substitutions resulted in the synthesis of equivalent quantities of N as WT rHAZV, as
294 measured by western blot analysis with HAZV-N antisera, categorically showing that the
295 corresponding DQVD and ENKD motifs are non-essential for rHAZV replication in cells of tick
296 origin, when individually altered. The equivalent abundance of N proteins expressed by these
297 viruses compared to WT also suggested these viruses were not growth attenuated in these
298 cells.

299 A more direct comparison between the role of the arm apex cleavage sites of CCHFV
300 and HAZV would be achieved using equivalent substituting sequences; AEVA for CCHFV and
301 AQVA for HAZV. However, this comparison was not possible, as the AQVA mutant for HAZV
302 could not be rescued, despite repeated efforts. Nevertheless, these results show the ability of
303 the DQVE virus to replicate in tick cells does not require a cleavable caspase site on the apex
304 of the N arm domain.

DISCUSSION

Here, we report a rescue system allowing the production of infectious HAZV from cDNA that can be utilized in widespread and amenable BSL-2 facilities, and which represents an important advance in the study of nairovirus molecular and cellular biology. Previously, a rescue system for the highly-pathogenic CCHFV was established, permitting insight into the role of multiple nairovirus specific features such as the OTU domain within the L protein ORF, as well as characterization of the processing pathway of GPC, expressed from the M segment ORF [15], [27]. However, due to the extreme pathogenicity of CCHFV, utilization of this rescue system has been restricted to a small number of laboratories that can comply with the highest BSL-4 containment protocols. The development of a HAZV rescue system that can be utilized using less-restrictive BSL-2 protocols will greatly facilitate and accelerate future nairovirus research. A HAZV mini-genome system has also been reported recently [29], which allowed the delineation and characterization of the viral mRNA transcription promoters, and while utility of this system is restricted to analysis of *cis*-acting signals involved in RNA synthesis, it will also allow a rapid accumulation of information regarding nairovirus multiplication.

The HAZV rescue system we describe here was capable of generating a titre of WT HAZV of over 1.2×10^5 pfu/ml in primary transfection supernatants. Correspondingly, rescue of WT rHAZV was successful in every attempt, and allowed rescue of each of the mutants described in this manuscript with minimal experimental replicates. Taken together these findings suggest the HAZV rescue system is highly efficient, a property that will facilitate the rescue of mutant viruses that possess other attenuating lesions.

The N proteins of both HAZV and CCHFV possess caspase-3 cleavage motifs with the sequences DQVD and DEVD, respectively [16], [30], prominently located at equivalent positions on the apex of their arm domains, suggesting their conservation is driven by functional importance. In line with this suggestion, previous work has shown both HAZV and CCHFV N proteins are cleaved *in vitro* at these motifs by purified active caspase-3 [16], [17], [19], as well as by caspase-3 in cultured human cells [22].

To test the importance of cleavage within the HAZV-N arm domain during HAZV infection, we generated mutant viruses for which the overlapping ENKD and DQVD consensus cleavage sites were individually disrupted with glutamic acid substitutions at position 1 (underlined) to render them un-cleavable. The fact that both the resulting viruses rHAZV-ENKE and rHAZV-DQVE were viable and replicated with growth kinetics that were indistinguishable from WT rHAZV is a critical observation. Because the DQVE mutant is cleaved at neither this DQVE site, nor the adjacent ENKD site (Fig 5C), these findings show cleavage within the arm apex is not a requirement for virus viability.

Our failure to rescue the rHAZV-AQVA mutant despite repeated efforts suggests it is deficient in a critical function. We show that caspase cleavage within the arm apex is not a requirement for virus viability, and indeed viruses that cannot be cleaved at this location show equivalent fitness to WT HAZV. Thus, the lack of rescue of rHAZV-AQVD cannot be due to loss of cleavability of the arm apex. This finding is intriguing in light of recent work with CCHFV for which the equivalent AEVA mutant was found to be phenotypically-silent in mammalian cells but significantly reduced in fitness for replication in tick cells. These findings suggest the requirements for HAZV and CCHFV are different, and the molecular basis for these differences likely lie in the subtle differences of arm domain sequences (Figure 5A). Our results here show the DQVD site is important for rescue, but not for caspase cleavage and this provides a starting point for studies to elucidate the critical role of this DQVD motif. So what might this possible role be? One possibility is in N-N multimerization. Previous work has shown the crystallographic N-N interface between adjacent monomers involves the formation of a hydrophobic pocket comprising six residues from the arm domain of one monomer that interacts with a single proline residue located on the base of the globular domain of the adjacent monomer [16]. Interestingly, neither aspartate (D) residues within the DQVD motif that are altered in either DQVE or AQVA mutants are involved in this interaction, which would thus appear to rule out N-N interactions as its critical role. However, we cannot rule out the possibility that the HAZV N-N interaction that occurs in the assembly of an authentic nairovirus ribonucleoprotein (RNP) is different to that revealed in the HAZV N crystal structure, and that

these critical aspartate residues do indeed play important roles in RNP formation. This matter will be resolved by solving the structure of nairovirus RNPs in their native state. An alternative possibility is that the DQVD site is involved in an interaction with a host factor, and the availability of such a component may also explain the differential outcome of infection with CCHFV in mammalian and tick cells.

Previous work by ourselves and others has suggested that caspase cleavage of the nairovirus N protein may act as a caspase decoy, as has been shown for JUNV. Our results presented here showing the essential role of the HAZV DQVD motif is essential for a role other than cleavage does not rule this out. In fact, the finding that the rHAZV-DQVE mutant no longer generates the customary 30 kDa band, but instead is cleaved to yield a 32 kDa product shows that alternative cleavage sites exist that are amenable to cleavage once the dominant sites are removed. Deciphering the caspase cleavage profile of nairovirus N proteins is a complex task, as for HAZV there are a total of 28 aspartate residues that could potentially be cleaved, however we are struck by the high accessibility of the HAZV N protein to caspase digestion. Further work on this and other projects are currently underway, using the rescue system described here, and we hope that its use will accelerate further understanding of nairovirus molecular and cellular biology.

MATERIALS AND METHODS

Cells and viruses. BSR-T7 cells (derived from baby hamster kidney, containing a T7 polymerase expression gene) were maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma Aldrich) containing 2.5 % foetal bovine serum (FBS) (Invitrogen). SW13 cells (derived from human adrenal cortex) were maintained in DMEM containing 10 % FBS. All cell culture media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and grown at 37 °C in a 5 % CO₂ atmosphere. Tick-derived HAE/CTVM9 (*Hyalomma anatolicum*) cells [31] were maintained in L15 / MEM containing 20 % FBS, 10 % tryptose phosphate broth, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 30 °C.

389

390 **Plasmids.** Full-length cDNAs representing the S, M and L segments were synthesized
391 (Genewiz) using the HAZV strain JC280 (Genbank accession numbers: M86624.1,
392 NC_038710 and DQ076419.1, respectively) as reference and incorporated into the pMK-RQ
393 plasmid, resulting in the generation of pMK-RQ-S, pMK-RQ-M and pMK-RQ-L, able to express
394 S, M and L segment-specific RNAs, respectively. Viral gene segments were flanked by the
395 bacteriophage T7 polymerase promoter, and hepatitis delta virus ribozyme to ensure correct
396 formation of 3' and 5' terminal sequences, with the cDNAs orientated such that primary T7
397 polymerase transcripts were of positive sense. pCAG-T7pol was a gift from Ian Wickersham
398 (Addgene plasmid #59926). Generation of mutant plasmids was achieved using the Q5 Site
399 Directed Mutagenesis (SDM) kit (New England Biolabs) according to the manufacturer's
400 instructions, with all mutant plasmid sequences confirmed via sequencing (Genewiz)

401

402 **Virus rescue.** Six-well plates were seeded with 2×10^5 BSR-T7 cells/well 1 day prior to
403 transfection in 2 ml DMEM supplemented with 2.5 % FBS. 16-24 hours later, cells were
404 transfected with 1.2 μ g of pMK-RQ-S, pMK-RQ-M, pMK-RQ-L and 0.6 μ g pCAG-T7pol,
405 combined with 2.5 μ l Mirus TransIT-LT1 transfection reagent (Mirus Bio) per μ g of DNA in 200
406 μ l OPTI-MEM (Life Technologies). For mutant recovery, the WT plasmid was replaced with
407 the corresponding mutant plasmid. A control sample was set up alongside each experiment
408 in which transfection of pMK-RQ-L was omitted. Cell supernatants were collected 72-, 96- and
409 120-hours post transfection and 300 μ l supernatant was passaged in a 6-well plate of SW13
410 cells grown in DMEM supplemented with 10 % FBS for 48 hours. 100 μ l supernatant was also
411 used to titre virus following transfection using a standard plaque assay protocol.

412

413 **Virus infections.** SW13 monolayers were infected with HAZV at the specified
414 multiplicity of infection (MOI) in serum-free DMEM (SFM) at 37 °C. After 1 hour, the inoculum
415 was removed and cells washed in phosphate buffered saline (PBS), fresh DMEM containing

2.5 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin was then applied for the duration of the infection. Infections of HAE/CTVM9 cells of tick origin were carried out at an MOI of 0.01 in a similar manner to mammalian cell infections, but with an incubation temperature of 30 °C.

Inhibition of caspases. SW13 monolayers were pre-treated with 20 µM Z-FA-FMK for 45 minutes in SFM at 37 °C prior to infection with HAZV at an MOI of 0.1. Following infection, virus and inhibitor were removed, cells were washed three times in PBS and 2.5% FBS DMEM was reapplied containing 20 µM Z-FA-FMK. At 24-, 32- and 48-hours timepoints total cell lysates were harvested and prepared for western blot.

Cell viability assays. SW13 monolayers were pre-treated with a range of Z-FA-FMK concentrations for 1 hour 45 minutes in SFM, matching the duration of the pre-treatment and infection stage. At this point, media was changed to 2.5% FBS DMEM containing the same concentration of drug, again matching the infection procedure. Following a 48-hours infection period, cell viability was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions.

Western blotting. For preparation of cell lysates, monolayers were washed in ice cold PBS followed by incubation in ice cold RIPA buffer (150 mM sodium chloride, 1.0% NP-40 alternative, 0.1% SDS, 50 mM Tris, pH 8.0) and agitated for 120 seconds. Cells were then harvested via cell scraping and transferred to pre-chilled Eppendorf tubes, after which lysates were centrifuged at 20,000 x g for 15 minutes to pellet insoluble material. SDS-gel loading buffer containing DTT was added to the supernatant prior to storage at -20°C. Proteins were separated on 12 % SDS polyacrylamide gels by electrophoresis and transferred to fluorescence compatible PVDF (FL-PVDF) membranes. Sheep anti-HAZV-N antiserum generated as previously described [32] was used to detect HAZV-N, and was subsequently

visualised using fluorescently labelled anti-sheep secondary antibodies. Membranes were visualised on the LiCor Odyssey Sa Infrared imaging system.

Virus titration. Determination of virus titre for construction of growth curves was achieved through plaque assay. Briefly, SW13 cells were seeded (2×10^6) into 75 cm² flasks 24 hours prior to infection with HAZV, rHAZV or mutant rHAZV at an MOI of 0.001. Supernatant was collected at time points of 24-, 48-, 72-, 96- and 120-hours post infection, and serially-diluted to infect fresh monolayers of SW13 cells in a 6-well plate. Following infection, media containing virus was removed and replaced with 1:1 2.5 % FBS DMEM and 1.6 % methylcellulose and returned to incubate for a further 6 days prior to fixing and staining with crystal violet. Plaques were then counted and virus titre determined.

Extraction of viral RNA. Viral RNA was first extracted from cell-free supernatant using the Qlamp Viral RNA kit (Qiagen) and treated with DNase to remove any contaminating DNA before a cDNA copy was generated using ProtoScript II Reverse Transcriptase (New England Biolabs) according to manufacturer's instructions. PCR amplification of an ≈ 800 bp fragment using primers specific to the HAZV S segment was achieved using the Q5 High Fidelity Polymerase (New England Biolabs). Restriction digest analysis was performed by incubating *Hind* III (New England Biolabs) with 500 ng PCR product at 37 °C for 1 hour prior to resolving DNA bands on a 1 % agarose gel containing 0.01 % SYBR Safe (ThermoFisher).

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AUTHOR CONTRIBUTIONS

Author contributions are as follows: JF, RAS, GS, RH, JM and JNB conceptualized the study; JF, RAS, GS performed the experimental investigation; JF and JNB wrote the original draft manuscript; RAS and GS reviewed and edited the manuscript; JM, RH and JNB supervised the core team; JNB provided management and coordination of the research activities and acquired the financial support for the project.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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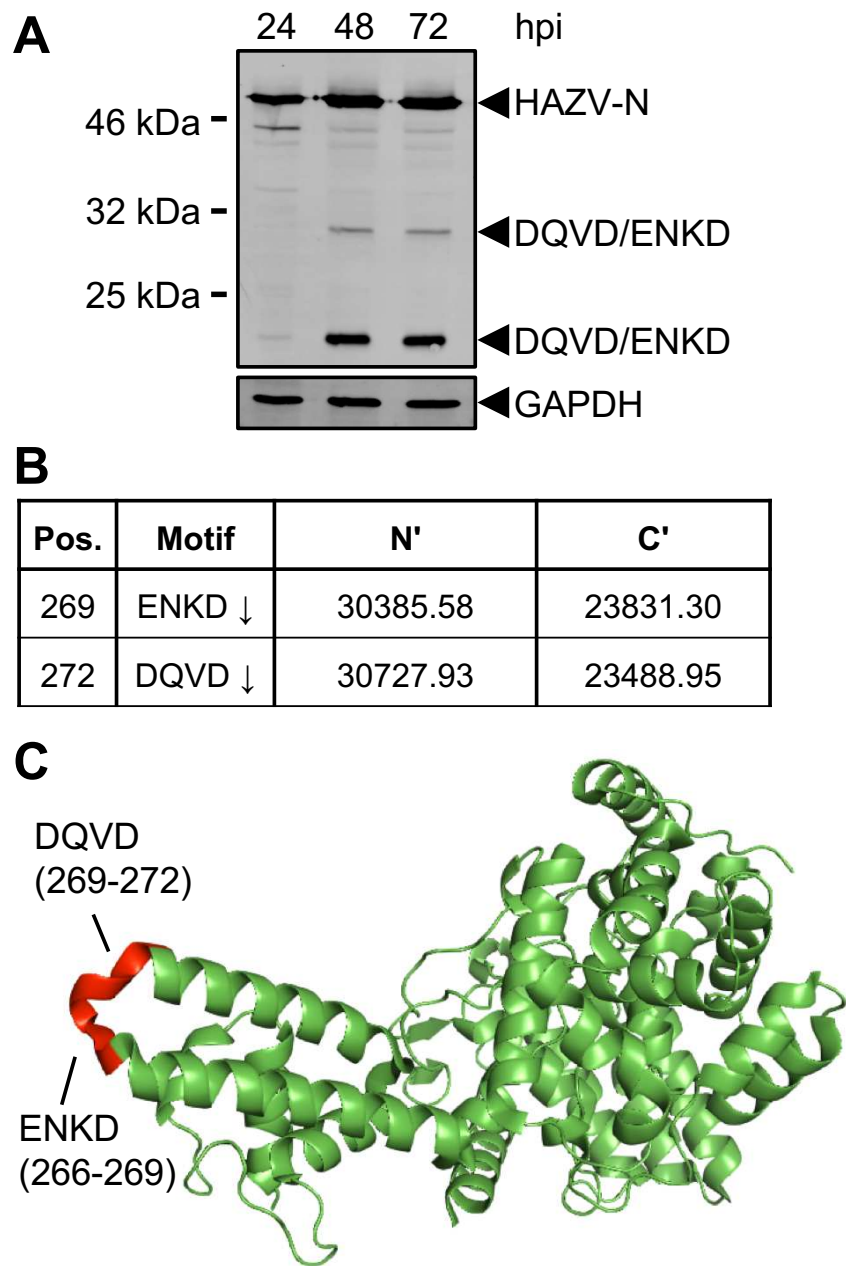


Figure 1. Detection of multiple HAZV-N cleavage products. A) Monolayers of SW13 cells were infected with HAZV at an MOI of 0.01. At the indicated hour time points post infection (hpi), total cell lysates were collected and analysed for HAZV-N expression by western blotting with HAZV-N antiserum. B) Predicted cleavage motifs of HAZV-N and associated predicted molecular weights of N' and C' fragments resulting from cleavage in Daltons. C) Schematic showing solvent-accessible location of motifs described in part B.

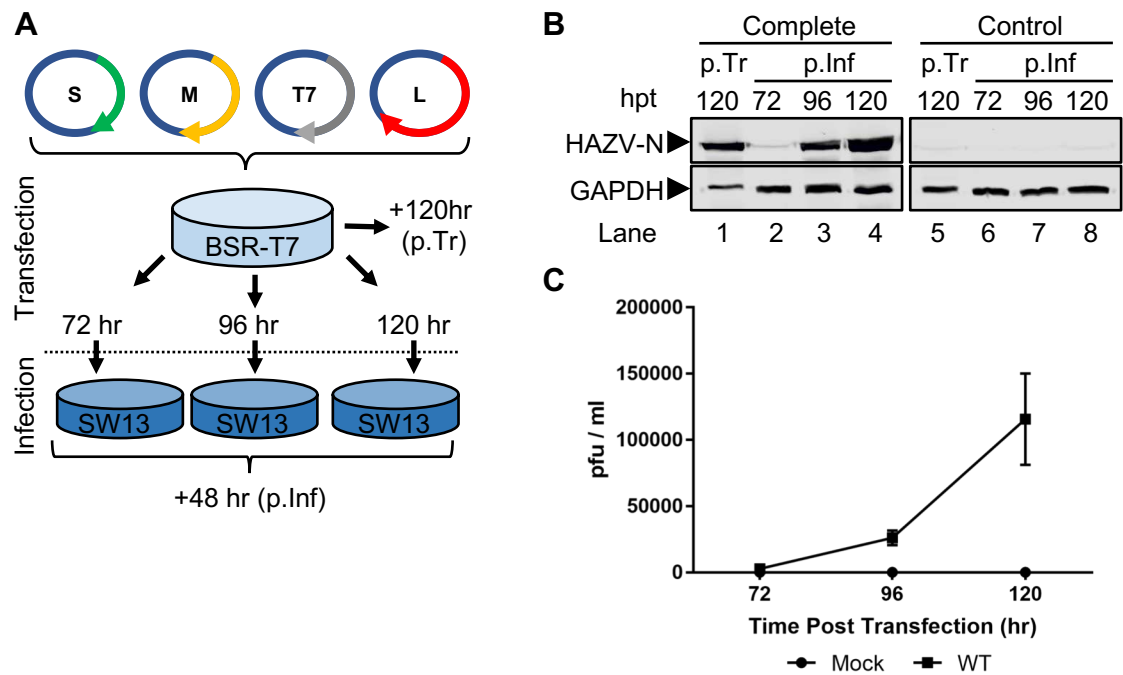


Figure 2. Recovery of recombinant HAZV. A) Schematic showing work-flow for recovery of rHAZV. Plasmids were transfected into BSR-T7 cells for the indicated duration, prior to harvest of supernatant and subsequent 48-hour infection of SW13 monolayers. B) Detection of HAZV-N protein post transfection (p.Tr) of BSR-T7 cells and subsequent 48 hour post infections (p.Inf). Supernatant samples collected from transfected BSR-T7 cells at 72-, 96- and 120-hours post transfection (hpt) were used to infect monolayers of SW13 cells. Following a 48-hour infection, lysates were collected (p.Inf, lanes 2-4) and analysed for N expression by western blotting alongside lysates collected from the initial transfected BSR-T7 cells (p.Tr, lane 1). Recovery of wild-type (WT) rHAZV (Complete) was carried out alongside a control recovery omitting transfection of the essential pMK-RQ-L plasmid (p.Tr and p.Inf, lanes 5-8). Detection of GAPDH abundance was included as a loading control. C) Titre of infectious rHAZV released into supernatant at 72-, 96-, and 120-hour time points p.Tr was assessed via plaque assay, error bars represent average of two repeats.

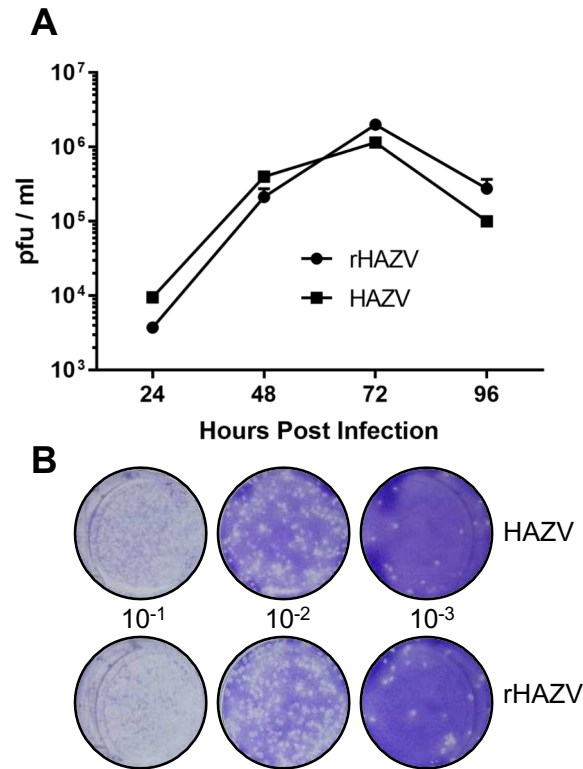


Figure 3. Growth kinetics of rHAZV versus parental isolate, A) Titre of infectious rHAZV (circle) and its parental strain of HAZV (square) harvested at 24-hour intervals for 4 days following infection of SW13 cells at an MOI of 0.001. B) Representative plaque assays used to determine titre of infectious HAZV and rHAZV in (A) showing plaque morphology to be similar in both instances. Serial dilutions were generated from virus harvested 24 hours post infection and used to infect SW13 monolayers for 1 hour prior to addition of a methyl cellulose overlay. Cells were then fixed and stained 6 days post infection and viral plaques were counted.

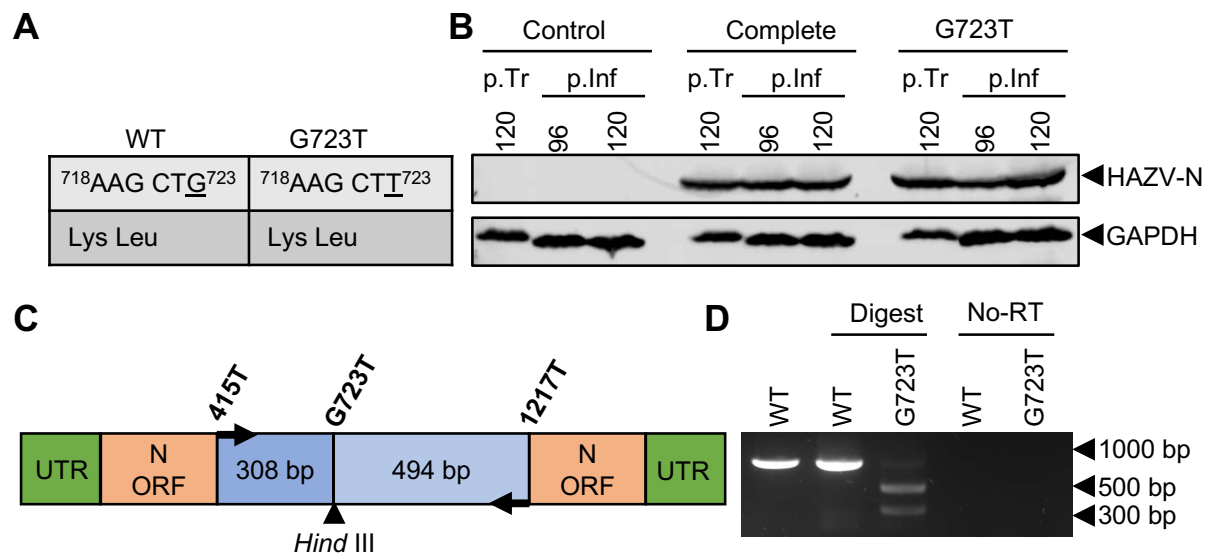


Figure 4. Confirmation of cDNA origin via recovery of mutant rHAZV. A) Table outlining the change (underlined) to both cDNA sequence of the HAZV-N ORF and the resulting amino acid sequence. B) Detection of HAZV-N by western blotting following transfection (p.Tr) of BSR-T7 cells and subsequent 48 hour infections (p.Inf). Supernatant samples collected from transfected BSR-T7 cells at 96 hours p.Tr were used to infect monolayers of SW13 cells. Following a 48-hour infection, lysates were collected and analysed by western blotting for N expression. Recovery of rHAZV containing a *Hind* III restriction site (rHAZV-G723T) was carried out alongside a complete and control recovery of rHAZV. Detection of GAPDH abundance was included as a loading control. C) Schematic showing the location of the inserted *Hind* III restriction site into the S-segment ORF cDNA. D) Restriction digest of dsDNA fragment following RNA extraction of rHAZV and rHAZV-G723T containing supernatants, first strand synthesis and PCR amplification of viral genetic material.

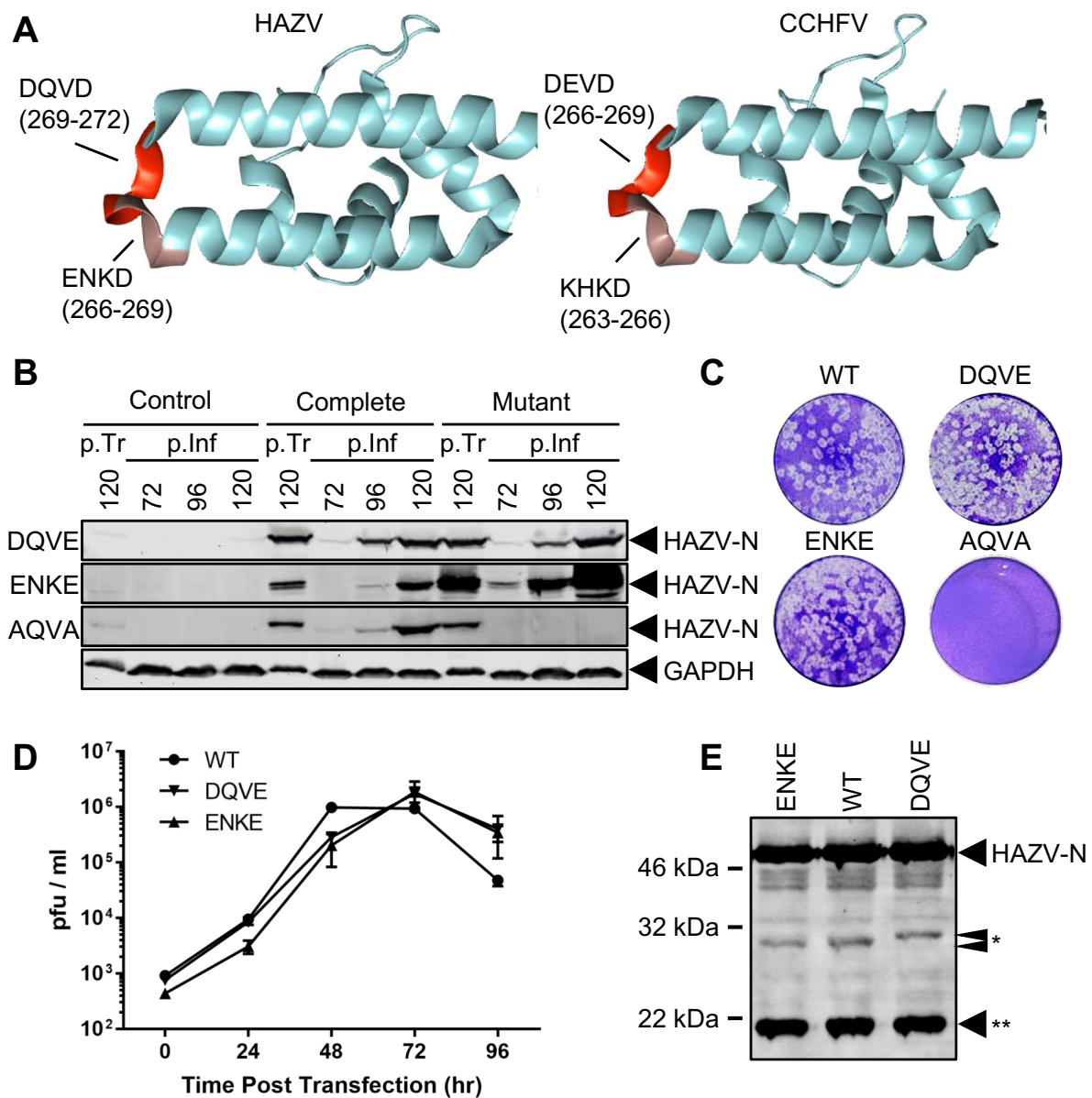


Figure 5. Recovery of mutant rHAZV targeting multiple caspase motifs. A) Schematic showing location of caspase cleavage motifs on the apex of the arm domains for HAZV and CCHFV, with amino acid positions indicated numerically. B) Western blot detection of HAZV-N protein for rHAZV-DQVE, ENKE and AQVA mutants following transfection (p.Tr) of BSR-T7 cells and subsequent 48 hour infections (p.Inf) of SW13 cells using supernatant harvested 72-, 96- and 120-hours p.Tr. Recovery of all mutants was carried out alongside independent complete and control recoveries of WT rHAZV. Detection of GAPDH abundance was included as a loading control. C) Representative plaque assays from supernatant taken 120 hours p.Tr

displaying plaque morphology for recovered viruses. D) Titre of infectious WT rHAZV (circle) versus DQVE (triangle↓) and ENKE (triangle↑) at 24-hour intervals following infection of SW13 cells at an MOI of 0.001. E) Detection of HAZV-N and associated cleavage products at 30 and 32 kDa (*) and 20 kDa (**) following a 48-hour infection at an MOI of 0.01.

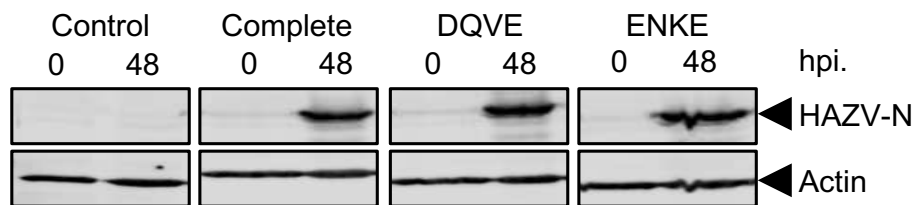


Figure 6. The ability of rHAZV, rHAZV-DQVE and rHAZV ENKE to replicate in the tick cell line HAE/CTVM9 was examined via western blot detection of HAZV-N in lysates taken 48 hours post infection (hpi) at an MOI of 0.01. Actin was included as a loading control.