Investigating the role of the different NS3 functional domains in the AHSV infection cycle in mammalian cells.

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African horse sickness (AHS) is a notifiable, often fatal disease of equids endemic to sub-Saharan Africa. It's caused by African horse sickness virus (AHSV), an arbovirus transmitted by *Culicoides* biting midges (Coetzer *et al.* 2004). As the disease is of great economic importance with no specific treatment available (Mellor *et al.* 2004), research that improves our understanding of the virus infection cycle is of paramount importance. This study focusses on the AHSV non-structural protein NS3. It's an integral membrane protein that mediates virus trafficking and release (Celma *et al.* 2009), and contributes to cellular pathogenesis by acting as a viroporin, increasing cell membrane permeability (Han *et al.* 2004). While several functional domains have been described for NS3, limitations of previous work included the inability to correlate the activity of each of these to specific aspects of the infection cycle.

The aim of this study was to gain insight into the contribution of six different NS3 domains to the protein's function. To do this, seven mutant AHS viruses expressing modified versions of NS3, with one or more of these functional domains disrupted, were generated using a reverse genetics approach. NS3-mutant strains were then compared to the wildtype virus in mammalian cells. We employed transmission electron microscopy (TEM), TEM with immunogold labelling, confocal laser scanning microscopy (CLSM) and phase contrast light microscopy to describe the localisation of the modified NS3 proteins and the behaviour of the mutant viruses.

Four of the mutant NS3 proteins (containing cytoplasmic domain modifications) exhibited an intracellular distribution pattern indistinguishable from that of the wildtype protein, localising to clusters of cytoplasmic perinuclear vesicles and to the plasma membrane (Fig. 1A). The other three mutant NS3 proteins (with transmembrane domain modifications) showed an unexpected intranuclear localisation, either as round foci or as bundles of thin fibres (Fig. 1B). These fibres formed due to the co-localisation of the mutant NS3 proteins with that of another viral protein, NS1 (Fig. 1C). The significance of this association is unknown, but hints at an as yet undescribed function of NS1 in managing misfolded proteins. Cells infected with any of the seven mutant viruses showed varying degrees of delayed cell death compared to the wildtype control. The mutants also exhibited a reduced efficiency in virus release and spread, with small or no plaque formation, and significantly higher viral titres in all cell-associated fractions. TEM analysis revealed, that in contrast to the wildtype virus, most of the mutants showed an aggregation of virus particles in the cell cytoplasm (Fig. 1D and E), and in some cases tethering to the plasma membrane (Fig. 1F).

This study allowed us to map four cytoplasmic domains of NS3 that interact with host trafficking proteins and virus particles, as their disruption prevented virus release and resulted in an accumulation of newly formed virions within the cytoplasm. It also allowed us to identify the two transmembrane domains of NS3 as the main determinants required for optimal functioning of the protein, mediating its proper processing and shuttling within the cell following synthesis.

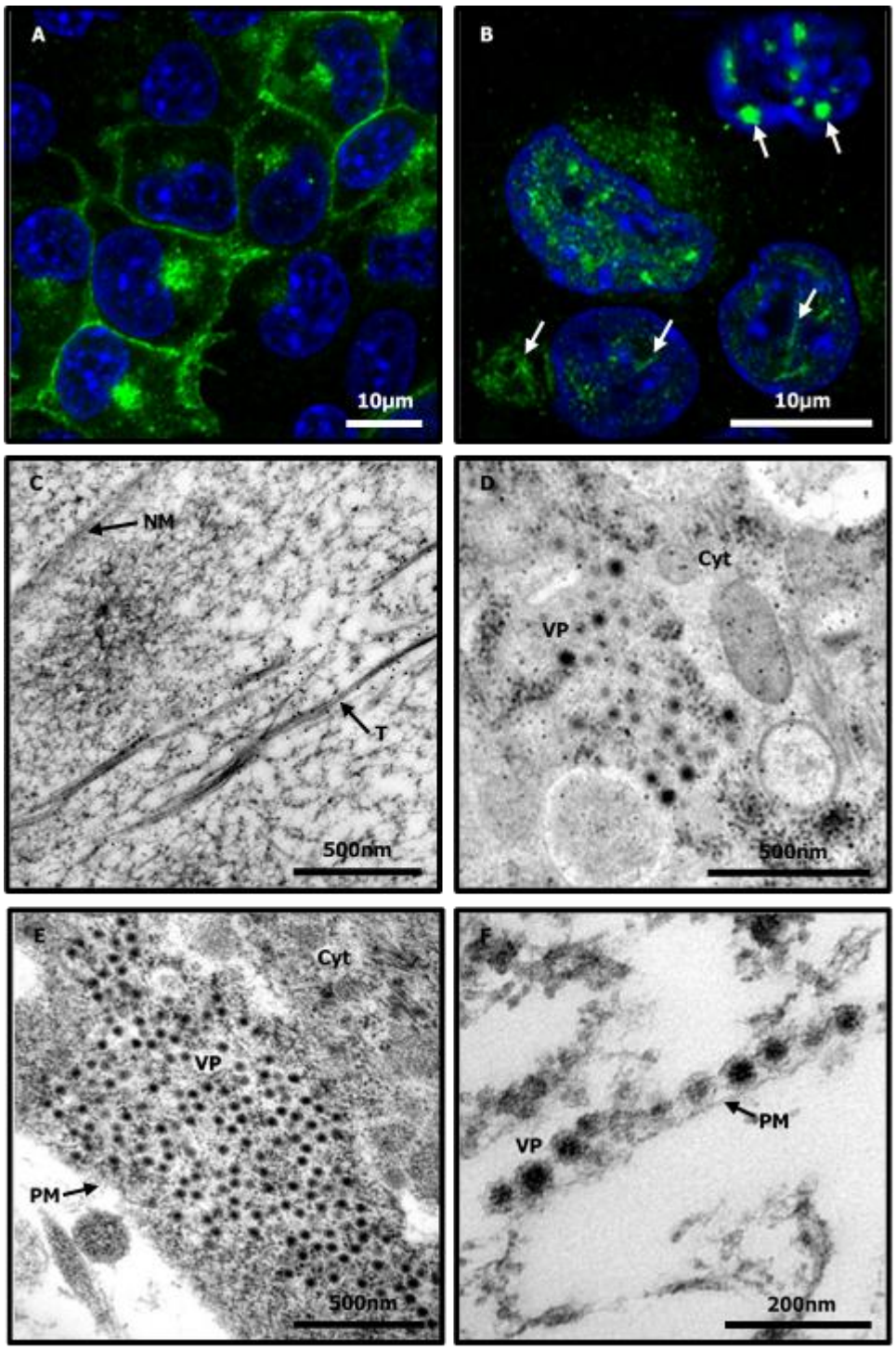

Figure 1: CLSM and TEM micrographs of mammalian cells infected with respective AHS viruses. A and B) Intracellular localisation of wildtype (A) and transmembrane mutant (B) NS3 proteins BSR cells; nuclei stained with DAPI and cells labelled with anti-NS3 and Alexa Fluor 488; arrows indicate NS3 foci and fibres. C) Transmembrane mutant NS3 localising to NS1 tubules within the nucleus; cells immunolabelled with anti-NS3 and gold conjugated secondary antibodies. D and E) Virus particle localisation in BSR cells infected with wildtype (D) and mutant (E) virus strains. F) Mutant virus particles tethered to the plasma membrane. (NM - nuclear membrane, T - tubules, Cyt - cytoplasm; PM - plasma membrane; VP - virus particles).