INTRODUCTION

Classical swine fever virus (CSFV) is a member of the genus Pestivirus in the family Flaviviridae. CSFV is the causative agent of classical swine fever (CSF), an economically important disease of pigs with an almost-worldwide distribution. The clinical symptoms of CSF vary between acute and chronic forms depending on the strain of infection (Le Potier et al., 2006; Lindenbach et al., 2007).

CSFV is an enveloped virus with a single positive-stranded RNA genome that consists of a single large ORF of approximately 12.5 kb in length. Cap-independent translation occurs via an internal ribosome entry site (IRES) to yield a polyprotein, which is processed into 12 known proteins (Lindenbach et al., 2007). Given the small number of proteins encoded by the CSFV genome, it is feasible that most, if not all, have multiple functions within the host cell. The first protein to be translated from the CSFV genome is the N-terminal autoprotease (Npro). Npro helps evade the innate interferon response by targeting interferon regulatory factor-3 for proteasomal degradation and also participates in the evasion of dsRNA-induced apoptosis. To elucidate the mechanisms by which Npro functions, we performed a yeast two-hybrid screen in which the anti-apoptotic protein HAX-1 was identified. The Npro–HAX-1 interaction was confirmed using co-precipitation assays. A dramatic redistribution of both Npro and HAX-1 was observed in co-transfected cells, as well as in transfected cells infected with wild-type CSFV, but not in cells infected with an Npro-deleted CSFV strain.

RESULTS

Identification of HAX-1 as an Npro-interacting protein

To help elucidate the mechanisms by which the Npro protein functions, we carried out a yeast two-hybrid screen in order to identify proteins that physically interact with Npro, and identified the anti-apoptotic protein HAX-1 (HS-1-associated protein X-1) as a binding partner. HAX-1 was initially reported as a binding partner of HS-1, a protein specifically expressed in haemopoietic cells and involved in the transduction of signals for both clonal expansion and deletion of B- and T-cells (Suzuki et al., 1997). However, an increasing number of studies in recent years have indicated that HAX-1 is a multifunctional protein involved in the regulation of various pathways, with a prominent role in the regulation of apoptosis (Fadeel & Grzybowska, 2009). With this in mind, we decided to investigate the interaction of Npro with HAX-1 further. We now report that: (i) Npro physically interacts with HAX-1 in co-precipitation binding assays; (ii) Npro causes a dramatic relocation of HAX-1 in co-transfected cells and in CSFV-infected cells; and (iii) Npro contains a functional consensus HAX-1-binding region.
HAX-1, in addition to 24 aa encoded by the 5' UTR. In order to confirm the interaction, and dismiss the possibility that it was facilitated by the portion of 5' UTR, further yeast two-hybrid analysis was performed. Plasmids encoding Npro, only the ORF of human HAX-1 (hHAX-1) or various controls, were co-transformed into yeast cells. Positive clones were identified by blue growth on selective minimal medium containing the substrate Xα-Gal. Excluding the positive control, only yeast co-transfected with the hHAX-1 and Npro constructs grew, indicating that the interaction was indeed specific (Fig. 1a).

**Npro interacts with HAX-1 in co-transfected cells and in CSFV-infected cells**

To determine whether Npro could interact with hHAX-1 in mammalian cells, Max kidney ('Max') cells were infected with a vaccinia virus strain constitutively expressing the T7 RNA polymerase (MVA–T7) and then co-transfected with hHAX-1 and GST–Npro (glutathione S-transferase–Npro) constructs which were under the control of both the human cytomegalovirus and T7 promoters. Western-blot analysis confirmed GST–Npro, but not GST alone, was indeed able to co-precipitate hHAX-1 in the presence of cellular proteins. In addition, a GST fusion protein containing only the first 70 aa of Npro was unable to co-precipitate hHAX-1, further confirming the specificity of the Npro interaction (Fig. 1b).

The porcine homologue of the human HAX-1 protein exhibits an identity of 83% and a high similarity of 86% [GenBank accession nos DN109876 (pig) and U68566 (human)]. To confirm Npro could also interact with porcine HAX-1 (pHAX-1) we performed similar co-precipitation assays by co-transfecting Max cells with constructs encoding GST–pHAX-1 and Npro. As expected, GST–pHAX-1 was able to co-precipitate Npro, a result consistent with a physical protein–protein interaction between Npro and HAX-1 (Fig. 1c).

Next we performed co-precipitation assays to investigate the interaction in the context of a CSFV infection. To determine whether CSFV-encoded Npro could physically interact with pHAX-1, Max cells were first infected (m.o.i. of 2) for 24 h with CSFV, superinfected with MVA–T7 and then transfected with a GST–pHAX-1 plasmid. GST–pHAX-1, but not GST alone, was able to co-precipitate Npro from CSFV-infected cells, These results confirm that Npro is capable of binding pHAX-1 during a CSFV infection (Fig. 1d).

**Npro co-localizes with HAX-1 in co-transfected cells**

To date, studies have localized HAX-1 to the mitochondria, endoplasmic reticulum (ER) and the nuclear membrane (Gallagher et al., 2000; Suzuki et al., 1997). Endogenous

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**Fig. 1.** The Npro product of CSFV interacts with hHAX-1; yeast two-hybrid analysis. (a) Yeast cells (AH109) were co-transformed with an hHAX-1 prey construct, and either an empty vector, a p53, a lamin-C (Lam) or a CSFV-Npro, bait construct. Interactions between bait and prey proteins were subsequently judged by growth of the yeast on selective media [–T (tryptophan), –L (leucine), –A (adenine), –H (histidine)]. Negative [simian virus 40 (SV40) large T antigen and Lam] and positive (SV40 large T antigen and p53) controls are indicated. (b) Western blot showing GST–Npro, but not GST–Npro N-terminal (N-ter, aa 1–70) or GST, co-precipitated hHAX-1. Max cells transiently expressing GST, GST–Npro N-ter, or GST–Npro and hHAX-1 were lysed and co-precipitation (CP) assays performed. Cell extracts and co-precipitation samples were analysed for hHAX-1 and GST as indicated. Products of cleavage are indicated by an asterisk. (c) Western blot showing GST–pHAX-1, but not GST, co-precipitated Npro. Max cells transiently expressing GST or pHAX-1 and Npro were lysed and CP assays performed. Cell extracts and CP samples were analysed for Npro and GST as indicated. (d) Western blot showing that GST–pHAX-1, but not GST or beads alone (no GST), co-precipitated CSFV-encoded Npro. CSFV-infected Max cells transiently expressing GST or pHAX-1 were lysed and CP assays performed. Cell extracts and CP samples were analysed for Npro, the structural protein E2 and GST as indicated. All CP assays were performed using glutathione–agarose beads as described in Methods and are representative of at least two independent experiments that gave similar results.
HAX-1 was not detectable by immunostaining. Therefore, to investigate the localization of pHAX-1 in the presence and absence of Npro we used a porcine-kidney cell line (Max) transfected with a construct encoding either pHAX-1 or GFP–Npro, or co-transfected with both constructs. When pHAX-1 was overexpressed it localized to the mitochondria, as confirmed using MitoTracker co-stain (Fig. 2a), which agrees with previous reports for a number of different mammalian cell lines (Suzuki et al., 1997; Vafiadaki et al., 2007; Yedavalli et al., 2005). Although GFP–Npro was present within the cytoplasmic compartment, its localization appeared predominantly nuclear when overexpressed alone (Fig. 2b) (Doceul et al., 2008). However, in 90% of cells co-transfected with Npro and pHAX-1 constructs, a dramatic redistribution of the encoded proteins to a cytoplasmic region adjacent to the nucleus was observed (Fig. 2c). As previously reported, in control cells transfected with only a GFP construct, GFP was distributed evenly throughout the cell (data not shown) (Doceul et al., 2008). Furthermore, no redistribution of pHAX-1 was observed in cells co-transfected with GFP and pHAX-1 constructs (Fig. 2d). It has been reported that phospholamban (PLN) also co-localizes with HAX-1 in a region adjacent to the nucleus which corresponds to the ER (Vafiadaki et al., 2007). In order to investigate whether this is also the case with Npro we used a rabbit antiserum recognizing the C terminus of ER protein 60 (ERP60) to locate the ER (Rouiller et al., 1998). We found that in co-transfected cells, Npro did indeed co-localize with pHAX-1 in the ER (Fig. 2e).

**CSFV-encoded Npro alters the localization of pHAX-1 to a perinuclear region**

Next, we investigated the ability of CSFV (Alfort 187 strain) to alter the localization pattern of pHAX-1. Porcine aortic endothelial cells (AOC) were first infected with CSFV (m.o.i. of 1) and then transfected 48 h later with a plasmid encoding pHAX-1. As expected, in control mock-infected cells pHAX-1 localization exhibited a punctate mitochondrial pattern (Fig. 3a). In contrast, CSFV-infection led to a striking redistribution of pHAX-1 to perinuclear regions.

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**Fig. 2.** Npro causes a redistribution of pHAX-1 from the mitochondria to the ER in co-transfected Max cells. (a) Max cells were transfected with an expression vector for pHAX-1 and protein localization was determined. Mitochondria were stained with MitoTracker red CM-H2XRos prior to fixation. Max cells were transfected with an expression vector for GFP–Npro (b), expression vectors for either GFP–Npro and pHAX-1 (c) or GFP and pHAX-1 (d), and protein localization was determined. (e) Anti-ERP60 polyclonal antibody was used to locate the ER in Max cells transfected with expression vectors for GFP–Npro and pHAX-1. Nuclei were stained with DAPI except when the ER was located.
These results were comparable to the redistribution of pHAX-1 observed in co-transfected cells. In order to confirm the role played by the CSFV N\textsuperscript{pro} product in the redistribution of pHAX-1, we utilized a CSFV strain (EP\#96/2 based on the Alfort Tubingen strain) in which the N\textsuperscript{pro} gene has been deleted [kindly provided by Dr Gregor Meyers, Friedrich Loeffler Institut (FLI), Tubingen, Germany]. The N\textsuperscript{pro}-deleted virus is attenuated in its growth but replicates well in swine-kidney cell line SK6, which lacks a functional type I IFN response (Ruggli et al., 2003). In agreement with the results obtained using AOC cells, transiently expressed pHAX-1 exhibited a punctate mitochondrial pattern of localization in mock-infected SK6 cells (Fig. 3c), whereas the parental CSFV (EP\#98/2) virus was able to relocalize pHAX-1 to a region adjacent to the nucleus (Fig. 3d). However, no such redistribution was seen in SK6 cells infected with the N\textsuperscript{pro}-deleted CSFV strain, thus confirming the requirement for N\textsuperscript{pro} (Fig. 3e). A comparable redistribution of pHAX-1 was observed when similar experiments were performed using AOC cells and either the parental or the N\textsuperscript{pro}-deleted CSFV strain (data not shown).

**pHAX-1 levels are unchanged in CSFV-infected PK-15 cells**

Alternative splicing of the HAX-1 gene yields various splice variants in human, rat and mouse. However, there is a lack of information regarding the expression of different protein isoforms (Hippe et al., 2006; Lees et al., 2008). In short exposures of Western blots, probed with a monoclonal anti-HAX-1 antibody recognizing aa 10–148 of hHAX-1, we routinely detected a single band of approximately 32 kDa in whole-cell extracts of PK-15 cells (Fig. 4a, top). In whole-cell extracts of Max cells a doublet band of similar size was seen (Fig. 4a, top). Doublets of in vivo-synthesized HAX-1 have been reported previously and indicate that the protein may undergo post-translational modification (Al-Maghrebi et al., 2002). Long exposures (5 min) revealed the presence of two putative additional bands in whole-cell extracts from PK-15 and Max cells (Fig. 4a, bottom). These results are comparable to Western blots of human cell-line lysates using the same antibody, in which a central major HAX-1 band of approximately 32 kDa and two minor bands were seen (Lees et al., 2008).
HAX-1 has been reported to be targeted for degradation during apoptosis (Chao et al., 2008; Cilenti et al., 2004; Lee et al., 2008). We hypothesized that HAX-1 would not be cleaved in cells infected with CSFV. Therefore, to determine whether HAX-1 is targeted for cleavage during CSFV infection, we mock-infected or infected PK15 cells (m.o.i. of 2, Alfort 187 strain) for different periods of time and analysed equal quantities of their cell extracts by Western blot. No apparent cleavage or reduction in the level of pHAX-1 during CSFV infection was observed (Fig. 4b). To confirm these results, and to exclude the possibility that Npro orchestrates cleavage of HAX-1 in a similar manner to the high temperature requirement A2 protease (Omi/HtrA2), in vitro-translated 35S-labelled hHAX-1 protein was incubated for different periods of time (up to 5 h) with purified GST–Npro protein (Cilenti et al., 2004). In agreement with the unchanged level of HAX-1 observed during CSFV infection, analysis of the incubated samples revealed no cleavage of HAX-1 (data not shown).

**pHAX-1 levels are unchanged in PK-15 and stable Npro PK-15 cells treated with dsRNA**

Induction of apoptosis with cisplatin, etoposide and H2O2 leads to HAX-1 degradation (Cilenti et al., 2004; Lee et al., 2008). Npro antagonizes dsRNA-mediated apoptosis (Johns et al., 2010; Ruggli et al., 2003, 2005). To determine whether HAX-1 is also degraded during dsRNA-induced apoptosis, we treated parental PK-15 cells and a PK-15 cell line stably expressing Npro with dsRNA (100 μg dsRNA ml−1 added directly to the medium), and then analysed whole-cell extracts by Western blot. Antibodies recognizing cleaved caspase-3 and cleaved poly-ADP-ribose polymerase (PARP) were used as indicators of apoptosis. As Fig. 4(c) clearly shows, in comparison to the parental cells, apoptosis was indeed inhibited in the stable Npro cell line. However, no reduction in HAX-1 levels or HAX-1 cleavage products was observed in either PK-15 cell line treated with dsRNA.

In similar experiments using H2O2 to induce apoptosis, low levels of HAX-1 cleavage were observed but consistent data suggesting Npro protects HAX-1 from cleavage were not obtained. Additionally, H2O2 treatment of both CSFV-infected SK6 cells and a stable Npro PK-15 cell line led to the degradation of Npro, presumably by an activated caspase (data not shown).

**Npro contains a HAX-1 binding consensus**

Yin et al. (2001) analysed the primary structure of a number of proteins that bind HAX-1 and have described a consensus sequence. The consensus in interleukin-1 (IL-1) is capable of physically binding HAX-1. We therefore analysed Npro and discovered that it contains a similar consensus in the C terminus of the protein between aa 110 and 135 (Fig. 5a). Apart from the sequence derived from a pestivirus isolated from giraffe, which contains seven, all the other sequences in the alignment contain eight to ten of the 14 consensus amino acids.

To investigate the putative consensus sequence, GST co-precipitation assays were performed, as previously described (Doceul et al., 2008), using a GST-fusion construct encoding aa 106–143 of Npro ('Npro-consensus'). In agreement with the consensus sequence being a functional binding site, pHAX-1 was successfully co-precipitated. In contrast, there was no such interaction with the GST control (Fig. 5c). To verify these results, a construct encoding GFP-tagged Npro-consensus (GFP-consensus) was transfected, or co-transfected with a pHAX-1 construct, into PK-15 cells. When overexpressed alone GFP-consensus localized to the nucleus...
in all cells, resembling the distribution of full-length Npro. However, two additional patterns of localization were observed. In approximately half of the cells, GFP-consensus was also localized to several perinuclear areas (Fig. 6a). In the remaining cells GFP-consensus exhibited a punctate cytoplasmic pattern (Fig. 6b). Co-transfection of GFP-consensus and pHAX-1 constructs also yielded two patterns of localization. In all cells GFP-consensus was localized to cytoplasmic regions adjacent to the nucleus (Fig. 6c, d). In addition, half of the cells exhibited a punctate cytoplasmic pattern similar to that observed in single transfectants and suggestive of a mitochondrial distribution (Fig. 6d). Importantly, GFP-consensus co-localized with pHAX-1 in both the perinuclear and punctate staining patterns. Taken together, these results suggest that Npro-consensus can interact with endogenous as well as overexpressed pHAX-1 in both mitochondrial and perinuclear regions.

DISCUSSION

In summary, using a yeast two-hybrid screen we identified the anti-apoptotic protein HAX-1 as a binding partner of the Npro protein of the pestivirus CSFV. This interaction was confirmed by co-precipitation assays and confocal microscopy.

In this study we identified a conserved motif within the C terminus of Npro that binds HAX-1. However, it is possible that several other regions are also involved in the interaction. For example, peptide array analysis showed that three different domains interspersed in pre-IL-1α were capable of complexing with HAX-1 (Yin et al., 2001). An alignment of the regions of HAX-1 that interact with cellular and viral proteins indicated that the C-terminal part of HAX-1 is responsible for protein–protein interactions (Fadel & Grzybowska, 2009). In agreement with this, HAX-1 lacking aa 1–91 was able to co-precipitate with GST–Npro, indicating that the C-terminal part of HAX-1 is also responsible for the observed interaction (data not shown).

A growing body of evidence suggests that HAX-1 is a pleiotropic protein with various functions that are probably mediated through a combination of factors such as splice variants, subsequent protein isoforms and subcellular localization (Fadel & Grzybowska, 2009). One prominent function of HAX-1 appears to be the promotion of cell survival. Overexpression of HAX-1 in a T-lymphoma cell line conferred resistance to apoptosis following various stimuli including Fas treatment, serum deprivation, and γ-irradiation (Suzuki et al., 1997). A homozygous deletion of HAX1 in mice resulted in excessive apoptosis of neurons and post-natal lethality (Chao et al., 2008). HAX-1 not only contains weak homology to the anti-apoptotic protein Bcl-2 but also in vitro studies show that HAX-1 and Bcl-2 can physically interact (Matsuda et al., 2003; Sharp et al., 2002). HAX-1 also interacts with the pro-apoptotic protein caspase-9, preventing its cleavage and subsequent activation (Han et al., 2006; Shaw & Kirshenbaum, 2006).

HAX-1 has been reported to interact with a number of viral proteins including Epstein–Barr virus nuclear antigen leader protein (EBNA-LP, also designated EBNA5), the K15 protein of Kaposi’s sarcoma-associated herpesvirus (KSHV), the Vpr and Rev proteins of human immunodeficiency virus (HIV) type 1, and hepatitis C virus (HCV).
core protein (Banerjee et al., 2009; Dufva et al., 2001; Forsman et al., 2008; Kawaguchi et al., 2000; Matsuda et al., 2003; Modem & Reddy, 2008; Sharp et al., 2002; Yedavalli et al., 2005). These studies point to virus-mediated antagonism of host-cell apoptotic response.

Pestiviruses cause persistent infections, which in the case of bovine viral diarrhea virus (BVDV) can be lifelong (Nettleton et al., 1998; Peterhans & Schweizer, 2010). CSFV is a non-cytopathic virus that has the ability to persistently infect porcine cell lines. Transplacental infection, during the second trimester of pregnancy, with viral strains of low virulence often results in persistently infected piglets. These piglets are immunotolerant and may survive for a long time, persistently shedding the virus until a late onset of disease occurs and the animals die (Ribbens et al., 2004). We suggest that it is feasible that, to ensure efficient viral replication, the CSFV N pro product targets the apoptotic response through its interaction with HAX-1.

CSFV infection inhibits dsRNA-induced apoptosis at a number of apoptotic checkpoints, preventing caspase-8 activation and loss of mitochondrial membrane potential (Johns et al., 2010). N pro contributes to the antagonism of dsRNA-mediated apoptosis; however the underlying mechanism has not been determined (Johns et al., 2003; Ruggli et al., 2005). In this study, no decrease in HAX-1 levels was observed in PK-15 cells following treatment with dsRNA, indicating that N pro does not inhibit dsRNA-mediated apoptosis by preventing HAX-1 degradation. In addition to antagonism of dsRNA-mediated apoptosis, CSFV has been shown to partially protect cells from overexpression of human truncated Bid, which directly targets mitochondria to initiate apoptosis (Johns et al., 2010).

Interestingly, the core protein of HCV, a member of the family Flaviviridae related to CSFV but lacking the N pro gene, has recently been shown to interact with HAX-1. Banerjee et al. (2009) showed that HepG2 cells expressing HCV core protein were sensitized to undergo apoptosis when treated with the chemotherapeutic agent 5-fluorouracil. They suggested that sensitization is probably linked with the association of HCV core protein and HAX-1, and showed that cell death occurred via a mechanism involving p53 and activation of caspase-2 and -7, in association with HAX-1.

HAX-1 has been shown to localize to the mitochondria, ER and the nuclear membrane (Gallagher et al., 2000; Suzuki et al., 1997). More recently, Jeyaraju et al. (2009) showed that the association of HAX-1 with mitochondria is peripheral, and that HAX-1 is neither resident within the mitochondria nor anchored to membranes of the organelle. It is therefore feasible that HAX-1 is accessible to cytoplasmic N pro. We observed no obvious change in the expression level of HAX-1 during CSFV infection, confirming that N pro does not target HAX-1 for degradation or initiate new protein synthesis. However, in this study a dramatic redistribution of HAX-1 and N pro to the ER compartment was observed in co-transfected cells. HAX-1 was also redistributed to a perinuclear region in the presence of CSFV-encoded N pro. N pro may orchestrate this redistribution of HAX-1 to the ER during infection to increase cellular resistance to apoptosis. A similar pattern of co-localization has been reported for other proteins which bind HAX-1 including the Vpr protein of HIV type 1, the K15 protein of KSHV, HCV core protein and PLN (Banerjee et al., 2009; Sharp et al., 2002; Vafiadaki et al., 2007; Yedavalli et al., 2005). Importantly, the redistribution of HAX-1 to the ER in the presence of PLN correlated with stronger resistance to apoptosis (Vafiadaki et al., 2007). Anti-apoptotic properties have also been reported for ER-localized Bcl-2, a protein that, like HAX-1, is usually localized to mitochondria (Hacki et al., 2000; Rudner et al., 2001).
Also of interest are several reports indicating that HAX-1 plays a role in protein localization. The N-terminal peptide of pre-IL-1α, which contains a bipartite nuclear localization signal (NLS) and is liberated during intracellular processing, interacts with HAX-1 (Kawaguchi et al., 2006; Yin et al., 2001). This association is required for nuclear localization of pre-IL-1α (Kawaguchi et al., 2006). HAX-1 also interacts with EBNA-LP, a protein expressed as variable isoforms with different numbers of W1W2 repeats. EBNA-LP is primarily localized in the nucleus; however, EBNA-LP containing a single W1W2 repeat is predominantly localized in the cytoplasm where it co-localizes with HAX-1 (Dufva et al., 2001; Kawaguchi et al., 2000). It has been suggested that HAX-1 may bind to the NLS within the W1W2 repeat of EBNA-LP and implement cytoplasmic retention (Yin et al., 2001). Relocation of the nucleus to the cytoplasm of HIV type 1 Rev and Vpr proteins was also observed in cells overexpressing HAX-1 (Modem & Reddy, 2008; Yedavalli et al., 2005). Interestingly, Npro is localized in the cytoplasm and nucleus, but when co-transfected with a HAX-1 construct the amount of Npro seen within the nucleus is substantially reduced (see Fig. 2b, c, respectively). This raises the possibility that HAX-1 may regulate Npro localization between cellular compartments.

Future experiments will investigate in more detail the functional significance of the HAX-1–Npro interaction, using infectious copy virus with targeted mutation of Npro to specifically ablate binding to HAX-1.

**METHODS**

**Plasmids.** The bait construct, CSFV Npro–pGBK7T, as well as GFP–Npro and GST–Npro have been described by Doceul et al. (2008). GST- and GFP-fusion constructs used for cell transfections were generated from existing DNA templates by PCR and ligation into pcDNA3.1/V5–His–TOPO or pcDNA3.1/NT–GFP–TOPO (Invitrogen), respectively. The prey construct containing the ORF of hHAX-1 was created from existing DNA templates by PCR and ligation into pcDNA3.1/His–TOPO. Thirty hours after transfection with expression constructs, cells were lysed in PD buffer (50 mM Tris/HCl pH 7.6, 1% Triton X-100, 200 mM NaCl, 0.5 mM DTT, 1 mM PMSF and 1 μg protease inhibitors ml⁻¹) with sonication. Clarified lysates were incubated with 30 μl of 50% glutathione–Sepharose bead slurry (Amersham Biosciences) overnight at 4 °C. Protein–bead complexes were washed seven times with PD buffer and reducing buffer was added.

**Western blots.** Western blots were performed as described by Doceul et al. (2008). Rabbit anti-Npro serum was generated for use as a primary antibody by inoculating rabbits with the peptide, KTNKQKPMGVEEPYPDTAGKPLFGDPS, which corresponds to N-terminal aa 11–37 of Npro (DS14 serum; La Rocca et al., 2005). Mouse anti-γ-tubulin (T6557; Sigma), goat anti-GST (27-4577-01; Amersham Biosciences), mouse anti-HAX-1 (610824; BD Transduction Laboratories), rabbit anti-cleaved PARP (ab4830; Abcam) and rabbit anti-cleaved caspase-3 (9664; Cell Signaling Technology) were used as primary antibodies where indicated. Bound primary antibodies were detected by horseradish peroxidase-conjugated anti-mouse (Bio-Rad), anti-rabbit (Bio-Rad) or anti-goat (Promega) antibodies.

**Immunohistochemistry and fluorescence microscopy.** Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and blocked in 0.5 % BSA in PBS or 30% normal goat serum. Monoclonal anti-CSFV E2 antibody (WH303), anti-HAX-1 (610824; BD Transduction Laboratories) and anti-C terminus of ERP60 (produced in house) were used where indicated. Secondary antibodies were goat anti-mouse Alexa Fluor 488- or Alexa Fluor 568-conjugated (Molecular Probes). Nuclei were stained with DAPI (Sigma). For mitochondrial staining, 250 nM Mitotracker red CM-H2XRos (Molecular Probes) diluted in complete growth medium was added to the cells 45 min prior to washing and fixation.

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**Transfection and vaccinia infection.** Lipofectamine (Invitrogen) was used for all transfections. MVA–T7 was used to enhance the expression of transfected constructs. When superinfection was performed, Max cells were first infected with CSFV for 24 h, infected with MVA–T7, and finally transfected.

**GST co-precipitation binding assays.** Thirty hours after transfection with expression constructs, cells were lysed in PD buffer (50 mM Tris/HCl pH 7.6, 1% Triton X-100, 200 mM NaCl, 0.5 mM DTT, 1 mM PMSF and 1 μg protease inhibitors ml⁻¹) with sonication. Clarified lysates were incubated with 30 μl of 50% glutathione–Sepharose bead slurry (Amersham Biosciences) overnight at 4 °C. Protein–bead complexes were washed seven times with PD buffer and reducing buffer was added.

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