# Phosphorylation and membrane association of the Rubella virus capsid protein is important for its anti-apoptotic function

**Steven Willows,[1] Carolina S. Ilkow[1†] and Tom C. Hobman[1,2,3*]**

[1]*Department of Cell Biology, University of Alberta, 5-14 Medical Sciences Building, Edmonton, Canada, T6G 2H7.*
[2]*Department of Medical Microbiology & Immunology, University of Alberta, 5-14 Medical Sciences Building, Edmonton, Canada, T6G 2H7.*
[3]*Li Ka Shing Institute of Virology, University of Alberta, 5-14 Medical Sciences Building, Edmonton, Canada, T6G 2H7.*

## Summary

**Rubella virus (RV), a member of *Togaviridae,* is an important human pathogen that can cause severe defects in the developing fetus. Compared to other togaviruses, RV replicates very slowly suggesting that it must employ effective mechanisms to delay the innate immune response. A recent study by our laboratory revealed that the capsid protein of RV is a potent inhibitor of apoptosis. A primary mechanism by which RV capsid interferes with programmed cell death appears to be through interaction with the pro-apoptotic Bcl-2 family member Bax. In the present study, we report that the capsid protein also blocks IRF3-dependent apoptosis induced by the double-strand RNA mimic polyinosinic-polycytidylic acid. In addition, analyses of cis-acting elements revealed that phosphorylation and membrane association are important for its anti-apoptotic function. Finally, the observation that hypo-phosphorylated capsid binds Bax just as well as wild-type capsid protein suggests that interaction with this pro-apoptotic host protein in and of itself is not sufficient to block programmed cell death. This provides additional evidence that this viral protein inhibits apoptosis through multiple mechanisms.**

## Introduction

Rubella virus (RV) is a member of *Togaviridae*, a family of enveloped positive strand RNA viruses. It is a human pathogen normally associated with the relatively mild, self-limiting disease Rubella, or German measles. Common complications include arthralgia, arthritis and thrombocytopenia; however, severe neurological disorders such as progressive panencephalitis may also occur. Significantly, RV can cause severe birth defects when contracted during the first trimester of pregnancy (reviewed in Hobman, 2013). While little is known about the pathogenic mechanisms of RV infection, it has been suggested that viral persistence is important. For example, long-term shedding of virus has been observed in infants with congenital rubella syndrome (Phillips *et al.*, 1965) and in adults patients suffering from RV-associated arthritis (Chantler *et al.*, 1982; 1985).

An important aspect of viral persistence is escape from apoptosis, a process that can be induced by the immune system or through cellular stress caused by viral replication (reviewed in Griffin and Hardwick, 1997). Unlike alphaviruses, which comprise the majority of togaviruses, RV is a slowly replicating virus that has an unusually long eclipse period. Infection of mammalian cells by RV has been reported to cause apoptosis (Pugachev and Frey, 1998; Hofmann *et al.*, 1999), but generally this does not occur until long after the peak virus production periods. We recently demonstrated that RV-infected cells are in fact resistant to multiple apoptotic stimuli (Ilkow *et al.*, 2011), a property that would be expected to provide a window of opportunity for replication. The resistance of RV-infected cells to apoptosis is thought to be dependent wholly or in part, on the capsid protein, which binds to and inhibits the pro-apoptotic host protein Bax. In the present study we demonstrate that binding to Bax is not sufficient for the apoptotic function of capsid; however, loss of phosphorylation and membrane association leads to decreased anti-apoptotic activity. These data suggest that

specific localization and or the ability to interact with additional factors are critical for its function in blocking apoptosis.

## Results

### *Recombinant capsid protein affects the integrity of isolated mitochondria*

Previous studies from this laboratory established that expression of the RV capsid protein has multiple effects on mitochondria (Beatch and Hobman, 2000; Beatch *et al.*, 2005; Ilkow *et al.*, 2010; 2011). In order to further understand how this viral protein affects mitochondrial physiology, we examined the effects of adding recombinant capsid protein to freshly isolated mitochondria. Because capsid protein blocks apoptosis in part through its interaction with Bax, we anticipated that treatment of isolated mitochondria with purified capsid protein would protect from pro-apoptotic factors such as tBid (Li *et al.*, 1998). Surprisingly, addition of capsid to mitochondria had a similar effect as tBid *in vitro* (Fig. 1A). Specifically, mitochondria treated with recombinant RV capsid or tBid exhibited a dramatic loss of membrane potential compared to buffer alone, an event that often precedes release of cytochrome *c* during the early stages of apoptosis (Liu *et al.*, 1996). Consistent with these results, exposure of mitochondria to recombinant capsid protein resulted in efflux of cytochrome *c* (Fig. 1B). Addition of a control protein, Sec17, did not induce release of cytochrome *c* from the mitochondria. Similar to RV capsid protein Sec17 is a peripheral membrane protein with a molecular mass of 34 kDa (Griff *et al.*, 1992). The effect of RV capsid on cytochrome *c* efflux was not linear but rather appeared to have a threshold limit.

### *Hypo-phosphorylated capsid protein is less effective in blocking apoptosis*

The data from the *in vitro* experiments were at first unexpected; however, it was important to consider that the recombinant capsid protein used in these experiments is different from the *bona fide* capsid that is produced in mammalian cells. First, the latter is phosphorylated on multiple serine and threonine residues, a process that is dependent upon initial phosphorylation of serine 46 (Law *et al.*, 2003; 2006). Therefore, we assayed apoptotic resistance of cells expressing wild-type capsid and point mutants in which serine 46 was changed to alanine (S46A) or a phosphomimetic amino acid aspartic acid (S46D). Cells were transduced with lentiviruses encoding wild-type and mutant capsid proteins and after 2 days, were challenged with apoptotic stimuli that activate death receptor pathways (anti-Fas) or through intracellular path-
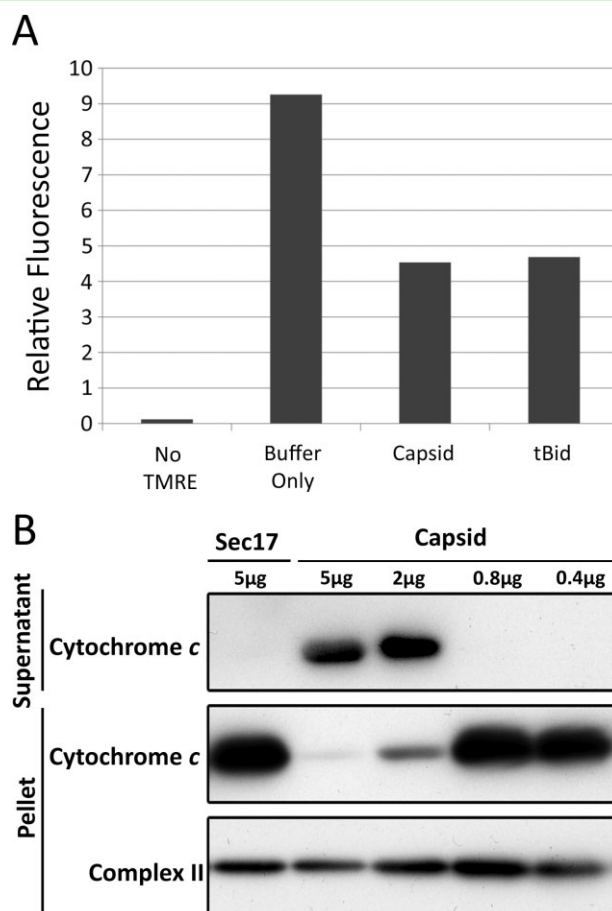


**Fig. 1.** Recombinant capsid has pro-apoptotic-like effects on mitochondria *in vitro*.
A. Crude mitochondria isolated from A549 cells were incubated with recombinant proteins for 15 min at 30°C before addition of 150 nM of TMRE for 30 min at 37°C. The mitochondria were washed and subjected to fluorometric analyses.
B. Mitochondria from rat liver mitochondria were isolated and adjusted to 1 mg ml$^{-1}$ of protein. Isolated mitochondria were then incubated with the indicated amounts of capsid or Sec17 (negative control) for 30 min at 30°C and then subjected to centrifugation after which the membrane pellets and supernatants were analysed by immunoblotting.

ways that detect viral RNA (poly I:C). Apoptosis in the transduced cells was determined by indirect immunofluorescence with antibodies to activated caspase 3. We have previously shown that RV capsid blocks Fas-dependent apoptosis in multiple cell types (Ilkow *et al.*, 2011) however, the effect of poly I:C on capsid-expressing cells has not been investigated previously.

Data in Fig. 2A show that compared to wild-type capsid protein, both S46A and S46D were significantly less effective in protecting cells from Fas-dependent apoptosis. Comparable results were obtained when capsid-expressing cells were challenged with polyI:C (Fig. 2B), a dsRNA mimic that activates apoptosis through
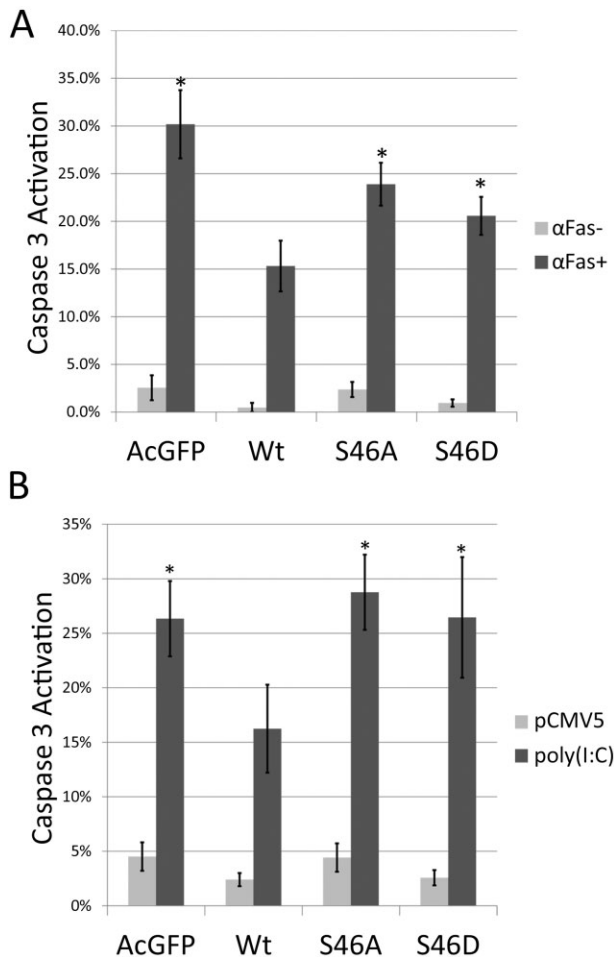
Fig. 2. Phosphorylation of capsid is important for its role in blocking apoptosis. A549 cells were transduced with lentiviruses encoding the indicated capsid constructs and AcGFP (multiplicity of transduction = 2). Two days post transduction, cells were treated with anti-Fas (0.25 µg ml⁻¹) and cycloheximide (1 µg ml⁻¹) for 24 h (A) or 1 µg ml⁻¹ of vector DNA or poly(I:C) for 20 h (B) before processing for indirect immunofluorescence. Apoptotic cells were detected using antibodies against active caspase 3. Results are based on the averages of four (A) and five (B) independent experiments. Bars indicate standard error of the mean.

a pathogen-associated molecular pattern receptor-based pathway (Peng *et al.*, 2009; Kubler *et al.*, 2010).

We next investigated whether phosphorylation of capsid was important for blocking apoptosis in the context of infection. Cells were infected with viruses encoding wild-type, S46A or S46D capsids followed by treatment with anti-Fas. In Fig. 3, it can be seen that compared to the mock population treated with anti-Fas, the proportion of apoptotic cells infected with wild-type RV was approximately half. Conversely, infection with S46A virus or S46D virus conferred virtually no protection compared to mock-infected cells. Together, these data are consistent with a scenario where phosphorylation of the RV capsid protein, particularly at Serine 46, is important for its role in blocking

apoptosis during virus replication. Interestingly, earlier data from our laboratory showed that infectious titres of recombinant RV strains harbouring S46A mutations are only 10-fold lower than wild-type RV (Law *et al.*, 2003). Similarly, an S46D mutant RV strain did not exhibit a dramatic replication defect *in vitro* (data not shown). However, it should be noted that other recombinant RV strains with multiple mutations that impair the anti-apoptotic ability of capsid (Ilkow *et al.*, 2011) are extremely unstable and revert to wild-type in less than two passages. As such, it is possible that any significant replication defects associated with the S46A and D mutants are masked by rapid reversion.

*Interaction with Bax is not sufficient for RV capsid to inhibit apoptosis*

We observed that when higher lentiviral transduction levels were employed, cells expressing S46A and S46D capsids were protected from anti-Fas to a significant degree (Fig. 4A). This was not due to differences in stabilities of the capsid mutants as all of the constructs were expressed at comparable levels to wild-type capsid (data not shown). This suggests that even hypo-phosphorylated capsid protein harbours residual anti-apoptotic activity. One mechanism by which RV capsid may block programmed cell death is through sequestration of the pro-apoptotic host protein, Bax (Ilkow *et al.*, 2011). Accordingly, we questioned whether the mutant capsids, particularly S46A, had a lower affinity for Bax. Surpris-
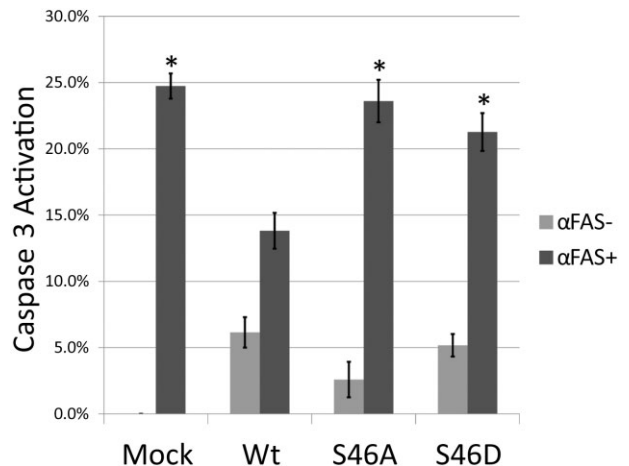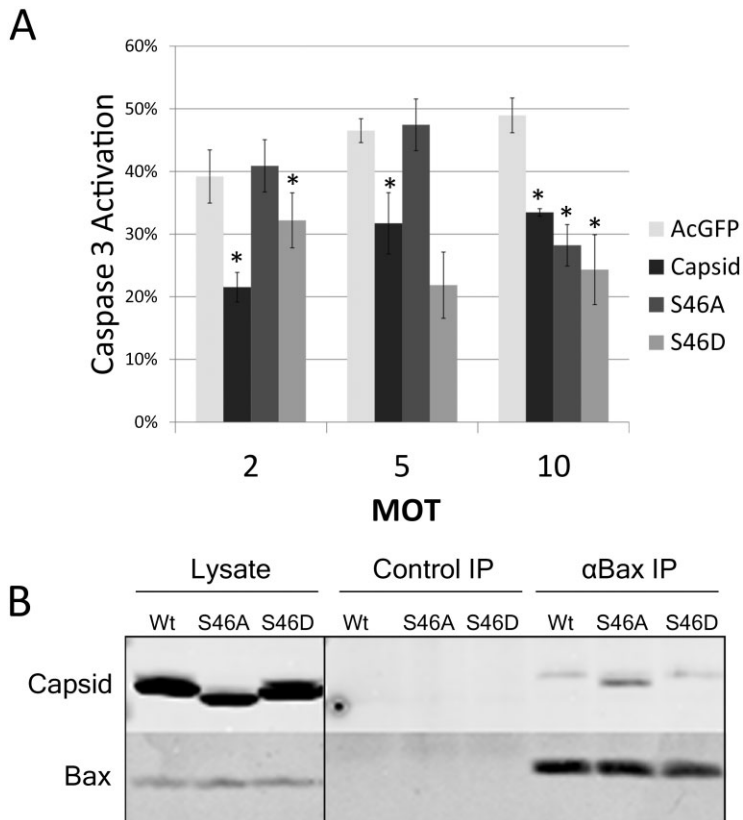


Fig. 3. The serine residue at position 46 of capsid is important for inhibiting apoptosis in RV-infected cells. A549 cells were infected with wild-type and mutant RV strains (moi = 0.5) or mock treated. Two days post infection, cells were treated with anti-Fas (1 µg ml⁻¹) and cycloheximide (1 µg ml⁻¹) for 8 h before processing for indirect immunofluorescence. Apoptotic cells were detected using antibodies against active caspase 3. Results are based on the averages of five independent experiments. Bars indicate standard error of the mean.

**A**



**B**



Fig. 4. Capsid phosphomutants bind Bax and their elevated expression confers resistance to apoptosis.
A. A549 cells were transduced with capsid-encoding lentiviruses using three different multiplicities of transduction. After anti-Fas challenge, apoptotic cells were detected using antibodies to active caspase 3 as described above. Results are based on the averages of three experiments. Bars indicate standard error of the mean.
B. A549 cells were transfected with constructs expressing wild-type, S46A or S46D capsids. Lysates were prepared and co-immunoprecipitations were conducted with rabbit anti-Bax or pre-immune serum. Immunocomplexes were subjected to SDS-PAGE and immunoblotting with antibodies to Bax and capsid.

ingly, co-immunoprecipitation with anti-Bax, consistently resulted in recovery of more S46A capsid compared to wild-type or S46D capsid (Fig. 4B). These data suggest that sequestration of Bax may not be sufficient for capsid to block apoptosis.

*Membrane-association is important for the anti-apoptotic function of RV capsid*

Another major difference between recombinant capsid protein from *Escherichia coli* (Fig. 1) and *bona fide* capsid produced in infected (or transfected cells), is that a significant proportion of the latter is membrane-associated via a hydrophobic peptide that serves as the signal peptide (SP) for the E2 glycoprotein (Suomalainen *et al.*, 1990; Law *et al.*, 2001). Therefore, we analysed the potential importance of the SP domain in the anti-apoptotic role of capsid protein. Cells that were transduced with lentivirus encoding wild-type, S46A and S46D versions of capsid that lack the E2 SP (ΔSP) were challenged with anti-Fas and activation of caspase 3 was determined using indirect immunofluorescence (Fig. 5). The levels of apoptosis among transduced cells expressing capsid proteins that lack the SP domain were significantly higher with S46A ΔSP being the least protective.

One potential explanation for the reduced anti-apoptotic activity of some capsid mutants is that they are localized

away from mitochondria and/or ER membranes. For example, most of the ΔSP mutants localized to cytoplasmic granules, nuclei and/or nucleoli (Fig. 6). It is interesting to note that the least protective capsid mutant S46AΔSP was localized predominantly in the nucleolus
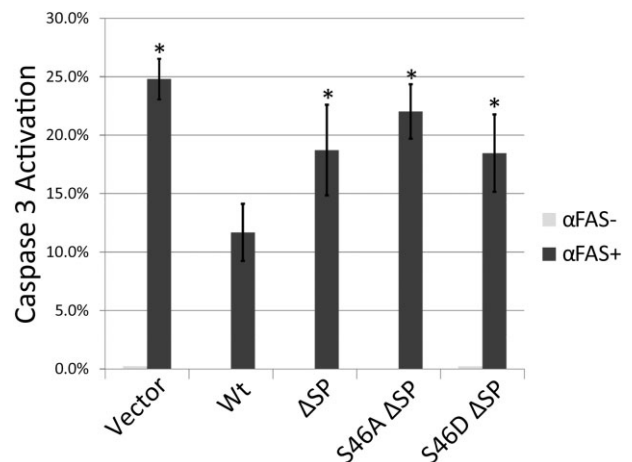


Fig. 5. The membrane-anchoring (SP) domain of capsid is important for its anti-apoptotic function. A549 cells were transduced with lentivirus expressing the indicated capsid proteins and apoptosis was induced. Apoptotic cells were quantified based on activated caspase 3 detection as described above. Results are based on the averages of four independent experiments. Bars indicate standard error of the mean.
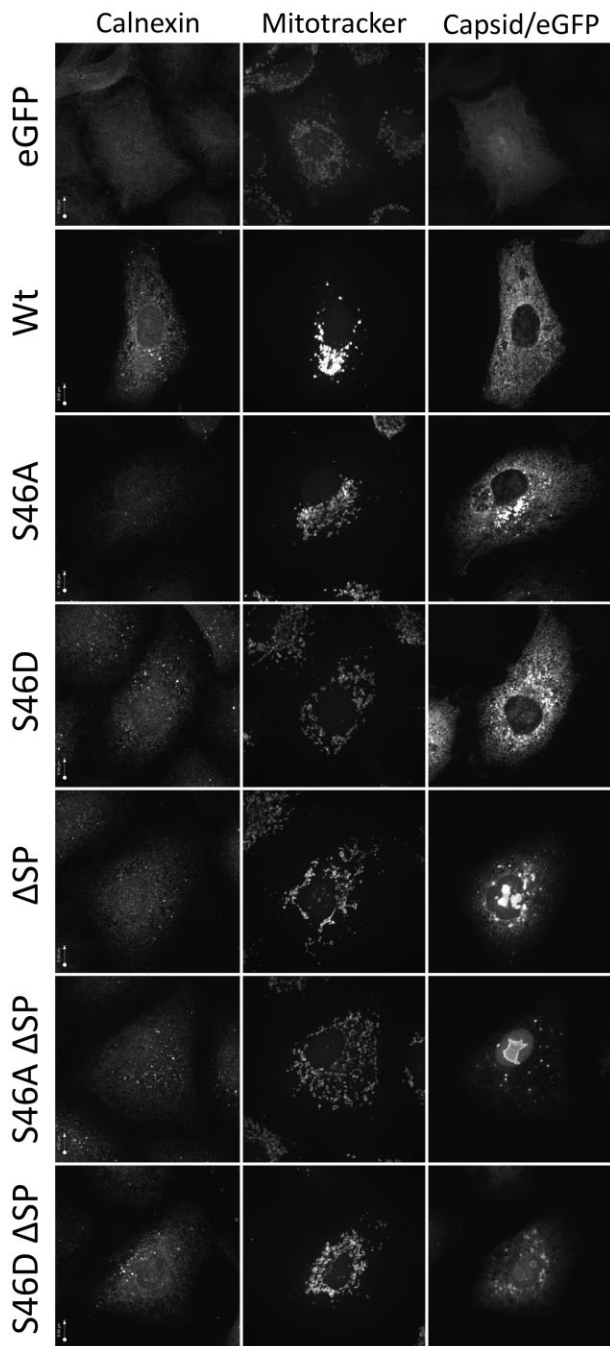
**Fig. 6.** Subcellular localization of capsid mutants. Cells were transfected with indicated constructs, incubated for 2 days before being labelled with Mitotracker and processed for indirect immunofluorescence. Capsid proteins were detected using a mouse monoclonal antibody and rabbit anti-calnexin was used to detect the endoplasmic reticulum.

whereas a larger proportion of ΔSP and S46DΔSP was detected in the cytoplasm. The punctate cytoplasmic structures to which ΔSP mutants localized resembled processing bodies (P-bodies), which are RNA-rich granules that function in mRNA metabolism (Anderson and Kedersha, 2009). Based on colocalization of the anchorless capsid mutants with the P-body marker DCP1 (Fig. 7, arrowheads), it is tempting to speculate that membrane-association and possibly phosphorylation, are important to prevent capsid protein from accumulating in these structures, which similar to the nucleolus, have high concentrations of RNA. One major consequence of capsid accumulating in these RNA-rich structures is its loss from the cytoplasm and consequently, ability to interact with host proteins or membranes that function in apoptosis. Together, these data suggest that localization of capsid to cytoplasmic membranes or the E2 SP itself is important for the anti-apoptotic role of RV capsid.

## Discussion

The teratogenic effects of RV on the fetus likely require the virus to persist *in utero*, a process that in turn may involve interfering with the innate immune response. To this end, we showed that the capsid protein affects several mitochondria-dependent pathways including apoptosis (Ilkow *et al.*, 2008; 2010; 2011). The ability of the RV capsid protein to interact with multiple host cell proteins may be related to the fact that its structure is flexible (Mangala Prasad *et al.*, 2013). Data from the present study indicate that the ability of RV capsid to inhibit apoptosis is a complex process that does not simply involve sequestering the host protein Bax. Indeed, some capsid constructs that do not bind Bax can still protect cells from apoptotic stimuli (Ilkow *et al.*, 2011) suggesting that this viral protein interferes with programmed cell death pathways through more than one mechanism. Certainly, some large DNA viruses employ a multi-pronged strategy to limit apoptosis. Human cytomegalovirus for example, utilizes two different proteins vMIA and vIBO to inhibit Bax and Bak respectively (Goldmacher *et al.*, 1999; Cam *et al.*, 2010). While the RV capsid does not protect cells from Bak overexpression (Ilkow *et al.*, 2011), it cannot be ruled out at this point that it affects steps that lead to activation of Bak or Bax for that matter. Recent findings indicate that these proteins can be activated through multiple mechanisms, some of which are rather unconventional. For instance, in addition to promoting Bax and Bak activity through transcription-dependent mechanisms following detection of viral dsRNA, the transcription factor IRF3 can also activate Bax through direct binding (Chattopadhyay *et al.*, 2010).

The unexpected findings from *in vitro* assays in which recombinant capsid was found to have pro-apoptotic effects on isolated mitochondria led us to examine *cis*-acting elements that are important for the anti-apoptotic function of capsid. Our findings indicate that phosphorylation and the membrane-binding region of capsid, the E2 SP, are both important for its ability to inhibit
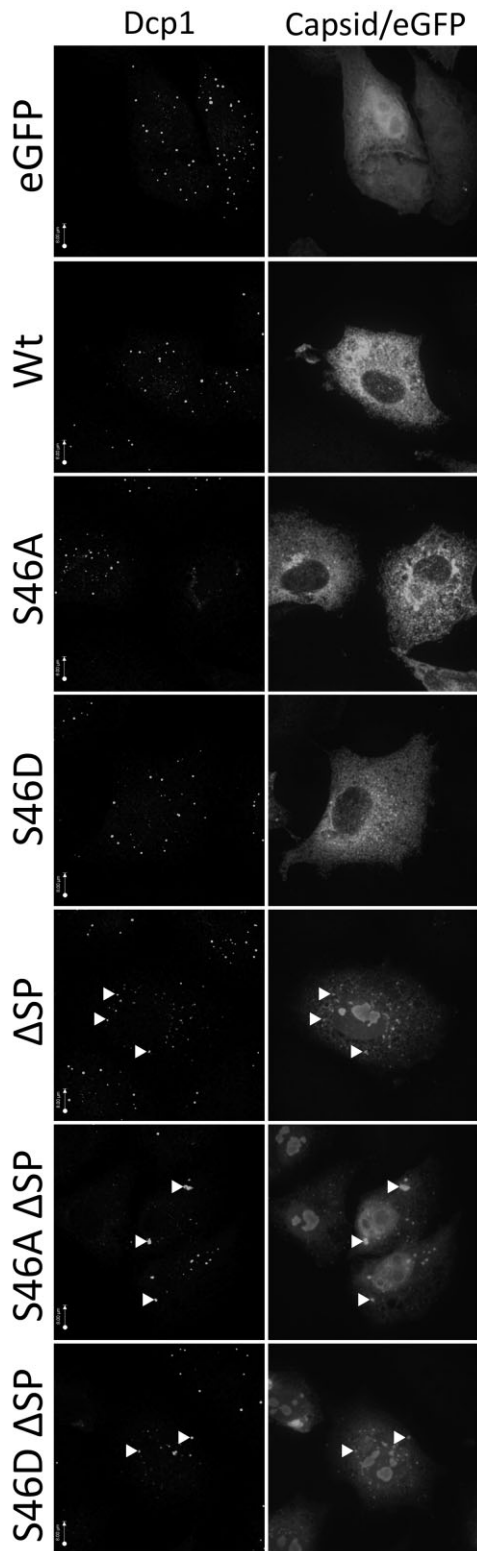
**Fig. 7.** Anchorless capsid mutants localize to areas of high RNA content. A549 cells were transfected with indicated capsid constructs and incubated for 2 days before processing for indirect immunofluorescence. Capsid proteins were detected using a mouse monoclonal antibody and rabbit anti-DCP1 was used to detect the P-bodies. Arrowheads indicate colocalization between anchorless capsid constructs and DCP1.

programmed cell death. Membrane-association *per se* may not be critical for blocking apoptosis, but rather, localization of capsid to specific cytoplasmic compartments such as the surface of the ER and/or mitochondria. The RV capsid does not possess catalytic activity and therefore changes in its phosphorylation state likely affects intermolecular interactions. For example, the S46A mutant and other hypo-phosphorylated mutants bind RNA with higher affinity than wild-type capsid (Law *et al.*, 2003; 2006). Given that the S46D phosphomimic mutant is significantly less protective than wild-type capsid, we are left to conclude that serine 46 *per se*, or the phosphorylated version of this amino acid, plays a critical role in its anti-apoptotic function. While substitution of an aspartate at this position is sufficient to induce downstream phosphorylation of other residues in capsid, it may not mimic phosphoserine well enough to facilitate interaction with host cell proteins involved in apoptosis. Indeed, a hypo-phosphorylated capsid mutant (P3D), in which three phospho-serine/threonine residues in the RNA binding region were changed to aspartates, behaved similarly to S46A in an *in vitro* RNA-binding assay. Specifically, the aspartates were expected to decrease RNA-binding of capsid but surprisingly, the P3D mutant exhibited significantly higher affinity for viral genomic RNA than wild-type capsid protein (Law *et al.*, 2006).

None of the capsid mutants when expressed in mammalian cells, including S46AΔSP, recapitulated the 'pro-apoptotic' effects of capsid protein *in vitro*. One potential explanation for these seemingly discordant data is that unlike recombinant capsid protein, which is completely devoid of phosphorylation, S46A mutants are not completely devoid of phosphorylation but instead are hypophosphorylated (Law *et al.*, 2003). High concentrations of non-phosphorylated capsid protein may be toxic for mitochondria and certainly, the ratio of capsid to mitochondria is higher in the *in vitro* system than in transfected or transduced cells. Indeed, there appears to be a threshold after which recombinant capsid protein behaves in this manner. Specifically, whereas addition of 0.8 μg of protein to mitochondria had no effect, addition of 2 (or more) μg resulted in near complete efflux of cytochrome *c*. Finally, it cannot be ruled out at this point that the 'pro-apoptotic' activity of recombinant capsid is simply an artefact of the *in vitro* system. Nevertheless, data from the *in vitro* experiments prompted further investigation, which showed that

both phosphorylation and membrane association are important for the anti-apoptotic function of RV capsid *in situ*.

We hypothesize that phosphorylation and membrane-association prevents the RV capsid from becoming sequestered in other areas of the cell with high RNA content; a situation that would allow capsid to remain concentrated at sites where it fulfils both structural (nucleocapsid assembly) and non-structural functions (blocking apoptosis). The fact that S46A∆SP, which has the lowest anti-apoptotic activity, localizes to organelles with high RNA content (P-bodies and the nucleus/nucleolus) is consistent with this idea. Indeed, even a pool of anchor-less but still phosphorylated capsid (∆SP) localized to these RNA-rich structures. Whether this has any bearing on the inherent differences between RV capsid and the closely related alphavirus capsid proteins is debatable. However, given that cells infected with alphaviruses generally produce much higher levels of antigen than RV-infected cells, it is possible that alphaviruses can better tolerate the mis-localization of a pool of their capsid proteins away from replication and/or assembly sites. With the exception of hantavirus nucleocapsid, a pool of which localizes to P-bodies in order to fulfil a role in 5′ cap-snatching (Mir *et al.*, 2008), RNA virus capsid proteins have not been reported to associate with cytoplasmic RNA granules. In fact, it is well documented that other togaviruses and flaviviruses inhibit formation of these RNA-rich granules which are thought to function in anti-viral defence (McInerney *et al.*, 2005; Emara and Brinton, 2007; Fros *et al.*, 2012; Panas *et al.*, 2012).

Phosphorylation appears to be common mechanism in which to limit interaction of viral capsid proteins with cellular RNAs, but interestingly, it is not always the capsid proteins that are phosphorylated. For example, in vesicular stomatitis virus-infected cells, phosphorylation of P protein, which binds to the capsid protein (N), prevents N protein from binding cellular RNAs (Chen *et al.*, 2013). Trafficking of several other viral capsid proteins between the cytoplasm and nucleus including those of severe acute respiratory syndrome coronavirus nucleocapsid and West Nile virus is affected by phosphorylation (Surjit *et al.*, 2005; You *et al.*, 2005; Bhuvanakantham *et al.*, 2010). As with RV capsid, phosphorylation of these proteins also affects RNA binding (Chen *et al.*, 2005; Cheong and Ng, 2011).

In summary, this is the first study demonstrating that post-translational modification of an RNA virus protein is important for interfering with apoptosis. In general, very little is known about how capsids or other proteins encoded by RNA viruses, function to inhibit apoptosis. Because there are so many ways to activate programmed cell death, it is likely that other viral proteins that function

analogously to RV capsid act through multiple pathways. Indeed, as well as blocking death receptor-dependent apoptosis (Ilkow *et al.*, 2011), in the present study, we show that RV capsid interferes IRF3-dependent apoptosis [induced by poly(I:C)]. Through identification of cellular kinases and membrane-associated host proteins that interact with virus capsids and/or other proteins, we will gain a better understanding of how these multifunctional proteins act at various stages of the virus life cycle.

## Experimental procedures

### Reagents and cells

A549, Vero, BHK-21 and HEK 293T cells were obtained from the American Type Culture Collection (Manassas, VA). Protein A and G Sepharose beads were obtained from GE Healthcare Bio-Sciences Corp (Princeton, NJ). Media and fetal bovine serum (FBS) were obtained from Life Technologies-Invitrogen (Carlsbad, CA).

### Plasmid construction and virus production

Several plasmids used in this study including pBRM33, pBRM33-S46A, pCMV5-Capsid, pCMV5-S46A, pCMV5-S46D and pCMV5-Cap∆SP have been previously described (Yao and Gillam, 1999; Law *et al.*, 2001; 2003; 2006). S46A∆SP and S46D∆SP cDNAs were constructed by PCR amplification of the template plasmid pCMV5-Capsid using a forward primer that begins at an internal SalI site (5′-CGC CGC CAT AGT CGA CGC GGT AGA AGA CC-3′) and a reverse primer that introduces a stop codon and BamHI site just before the E2 signal peptide (5′-TTG GAT CCT TAG CGG ATG CGC AAA GGA TG-3′). These fragments were then used to replace the 400 bp BamHI–SalI fragments in pCMV5-S46A or pCMV5-S46D respectively. The plasmid pBRM33-S46D was constructed by replacing the NotI–BsrGI fragment in pBRM33 with the analogous fragment from pCMV5-S46D followed by reinsertion of the NotI–NotI fragment. Platinum Taq® DNA Polymerase High Fidelity (Life Technologies) was used for all PCR amplification reactions.

For production of recombinant lentiviruses encoding capsid proteins, the vector pTRIP-AcGFP (Urbanowski and Hobman, 2013) was employed. To introduce capsid cDNAs into this plasmid, pCMV5-Capsid, pCMV5-Capsid S46A, pCMV5-S46D, pCMV5-Cap∆SP, pCMV5 S46A ∆SP and pCMV5 S46D ∆SP were digested with EcoRI and BamHI. The capsid cDNAs were then purified and ligated together with EcoRI–SpeI adaptors (5′-pCTA GTG AGC TCA GCG-3′ and 5′-pAAT TCG CTG AGC TCA-3′) into pTRIP-AcGFP that had been digested with BamHI and SpeI.

To produce lentiviral stocks, HEK293T cells ($2.5 \times 10^6$ per 100 mm dish) seeded the previous day, were co-transfected with pTRIP AcGFP constructs and HIV gag-pol and vesicular stomatitis virus envelope protein plasmids in a ratio of 7:7:2 (Urbanowski and Hobman, 2013) using TransIT-LT1 (Mirus, Madison, WI) transfection reagent. Lentivirus-containing media were collected 3 days post transfection and then passed through 0.44 μm filters before aliquoting and storage at −80°C until use.

RV infectious RNA was transcribed from plasmids pBRM33, pBRM33-S46A and pBRM33-S46D using mMESSAGE

mMACHINE® SP6 Kit (Ambion) as described (Yao and Gillam, 1999; Law *et al.*, 2003). Virus was reconstituted by electroporating BHK-21 cells with infectious RNA and collecting the media 3 days post infection. Media were cleared by centrifugation at 900 *g* for 5 min before aliquoting.

### Cell culture and virus infection

A549 and HEK293T cells were cultured in Dulbecco's minimal essential medium (high glucose) containing 10% FBS, 2 mM glutamine, 1 mM Hepes, penicillin and streptomycin in a humidified incubator at 37°C with 5% $CO_2$. Where indicated, cells were infected with RV by adding appropriate amounts of viral stock on top of cells in half the normal volume of media (1 ml/35 mm dish). Cells were incubated for 6 h with virus inoculum after which media were replaced. RV-infected cells were maintained at 35°C in a humidified atmosphere containing 5% CO.

Cells were transduced with lentivirus by diluting an appropriate amount of viral stock in 4 ml/35 mm well of DMEM containing 3% FBS, 20 mM Hepes and 4 $\mu$g ml$^{-1}$ polybrene. Cells were then spinoculated by centrifuging at 1200 *g* for 45 min at 37°C. After spinoculation, lentivirus-containing media were removed and replaced with normal growth media containing 10% FBS.

### In vitro *mitochondria assays*

Mitochondria from rat liver were prepared as described (Ilkow *et al.*, 2010). For cytochrome *c* release assays, freshly isolated mitochondria were adjusted to 1 mg ml$^{-1}$ of total protein by diluting with 250 mM sucrose, 10 mM Hepes-KOH, 1 mM ATP, 5 mM Na succinate, 0.08 mM ADP, 1 mM DTT, 2 mM $K_2HPO_4$ (pH 7.5) and were then incubated with the indicated amounts of recombinant capsid or control protein (Sec17) for 30 min at 30°C. Mitochondria were then pelleted by centrifugation for 10 min at 8000 *g* and then pellets and supernatant were analysed by immunoblotting with a monoclonal rabbit antibody to cytochrome *c* (from Dr L. Berthiaume, University of Alberta, Canada).

To determine effect of RV capsid protein on mitochondrial membrane potential, crude mitochondria were prepared from A549 cells. Cells were homogenized in ice-cold mitochondria isolation buffer [0.2% (w/v) BSA, 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes-KOH, pH 7.5] supplemented with Complete™ EDTA-free protease inhibitors using a dounce homogenizer with a loose fitting pestle. Unbroken cells and nuclei were pelleted by centrifugation at 500 *g* for 10 min at 4°C. The supernatants were then centrifuged at 10 000 *g* for 20 min at 4°C to obtain crude mitochondrial pellets. Mitochondrial pellets were resuspended in mitochondria resuspension buffer (250 mM sucrose, 10 mM Hepes-KOH, 1 mM ATP, 5 mM Na succinate, 0.08 mM ADP, 1 mM DTT, 2 mM $K_2HPO_4$, pH 7.5). Mitochondria were incubated with recombinant proteins for 15 min at 30°C, then with 150 nM of tetramethylrhodamine, ethyl ester (TMRE) for 30 min at 37°C. Mitochondria were then washed twice with 0.25 M sucrose 2 mM Hepes buffer and analysed by fluorometry.

### Apoptosis assays

Caspase 3 activation in capsid-encoding lentivirus-transduced or RV-infected cells was detected by indirect immunofluorescence as described (Ilkow *et al.*, 2011). Unless otherwise indicated, A549 cells were transduced with lentiviruses at a multiplicity of transduction of two and then apoptosis was induced 2 days later by adding $\alpha$FAS (0.25 $\mu$g ml$^{-1}$) and cycloheximide (1 $\mu$g ml$^{-1}$) for 24 h. Alternatively, apoptosis was induced in transduced cells by transfection with polyinosinic-polycytidylic acid sodium salt poly(I:C) (Sigma-Aldrich, St. Louis, MO). Two days post transduction, cells were transfected with poly(I:C) (1 $\mu$g ml$^{-1}$) for 20 h prior to apoptosis assays. Transfection with an equal concentration of plasmid DNA (pCMV5) was used as a negative control. Finally, cells were also infected with wild-type (M33) and capsid mutant strains of RV at an moi of 0.5. Two days post infection, apoptosis was induced with $\alpha$FAS (1 $\mu$g ml$^{-1}$) and cycloheximide (1 $\mu$g ml$^{-1}$) for 8 h before caspase 3 activation assays were performed. In the mock-infected cells, all caspase 3-positive cells were counted. However, in the experimental samples, only infected/transduced cells (which were positive for RV antigen or AcGFP) were counted in order to quantify caspase 3 activation levels.

All *P*-values were calculated using paired Student's *t*-tests. Asterisks indicate *P*-values of < 0.05 compared to the wild-type capsid, except where otherwise noted.

### Capsid and Bax co-immunoprecipitation

A549 cells were seeded at $1 \times 10^6$ cells in 100 mm dishes and transfected 1 day later with pCMV5-Capsid, pCMV5-S46A or pCMV5-S46D using Lipofectamine 2000 (Invitrogen). Two days post transfection, cells were lysed in 350 $\mu$l of lysis buffer [1% Igepal, 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1× Halt™ Phosphatase Inhibitor (Thermo Scientific) and 1× Complete™ Protease Inhibitor Cocktail (Roche)]. Insoluble debris was removed by centrifugation at 10 000 *g* for 10 min. Lysates were pre-cleared by incubating with 33% protein A sepharose slurry (15 $\mu$l) for an hour at 4°C after which 4 $\mu$g of antibody Rabbit anti-Bax (Abcam) pre-bound to 15 $\mu$l of protein A sepharose slurry in 1% casein was added for 4 h at 4°C. Immune-complexes on the beads were washed three times with lysis buffer before elution by boiling in gel loading buffer. Immunoprecipitates together with lysate (9%) control were separated on 12% acrylamide gels before immunoblotting with a mouse monoclonal antibody (Wolinsky *et al.*, 1991) to capsid (C-1) from Dr Jerry Wolinsky (University of Texas, Houston) and rabbit anti-Bax.

### Indirect immunofluorescence

A549 cells were seeded on coverslips and then transfected the next day with Transit-LT1 (Mirius). Two days after transfection, cells were fixed and indirect immunofluorescence was conducted as previously described (Ilkow *et al.*, 2011). Capsid proteins were detected using a mouse monoclonal (C-1). Rabbit antibodies to DCP1 (Abcam, Toronto, ON) and calnexin (Dr David Williams, University of Toronto) were used to detect P-bodies and endoplasmic reticulum respectively. To label mitochondria, cells were incubated with Mitotracker Red CMXRos (Molecular Probes®, Eugene, OR) 30 min before fixation. Images were acquired using a Quorum WaveFX-X1 Spinning Disc Confocal System with Volocity acquisition software.

## Acknowledgements

## References

Anderson, P., and Kedersha, N. (2009) RNA granules: post-transcriptional and epigenetic modulators of gene expression. *Nat Rev Mol Cell Biol* **10:** 430–436.

Beatch, M.D., and Hobman, T.C. (2000) Rubella virus capsid associates with host cell protein p32 and localizes to mitochondria. *J Virol* **74:** 5569–5576.

Beatch, M.D., Everitt, J.C., Law, L.J., and Hobman, T.C. (2005) Interactions between rubella virus capsid and host protein p32 are important for virus replication. *J Virol* **79:** 10807–10820.

Bhuvanakantham, R., Cheong, Y.K., and Ng, M.L. (2010) West Nile virus capsid protein interaction with importin and HDM2 protein is regulated by protein kinase C-mediated phosphorylation. *Microbes Infect* **12:** 615–625.

Cam, M., Handke, W., Picard-Maureau, M., and Brune, W. (2010) Cytomegaloviruses inhibit Bak- and Bax-mediated apoptosis with two separate viral proteins. *Cell Death Differ* **17:** 655–665.

Chantler, J.K., Ford, D.K., and Tingle, A.J. (1982) Persistent rubella infection and rubella-associated arthritis. *Lancet* **1:** 1323–1325.

Chantler, J.K., Tingle, A.J., and Petty, R.E. (1985) Persistent rubella virus infection associated with chronic arthritis in children. *N Engl J Med* **313:** 1117–1123.

Chattopadhyay, S., Marques, J.T., Yamashita, M., Peters, K.L., Smith, K., Desai, A., *et al.* (2010) Viral apoptosis is induced by IRF-3-mediated activation of Bax. *EMBO J* **29:** 1762–1773.

Chen, H., Gill, A., Dove, B.K., Emmett, S.R., Kemp, C.F., Ritchie, M.A., *et al.* (2005) Mass spectroscopic characterization of the coronavirus infectious bronchitis virus nucleoprotein and elucidation of the role of phosphorylation in RNA binding by using surface plasmon resonance. *J Virol* **79:** 1164–1179.

Chen, L., Zhang, S., Banerjee, A.K., and Chen, M. (2013) N-terminal phosphorylation of phosphoprotein of vesicular stomatitis virus is required for preventing nucleoprotein from binding to cellular RNAs and for functional template formation. *J Virol* **87:** 3177–3186.

Cheong, Y.K., and Ng, M.L. (2011) Dephosphorylation of West Nile virus capsid protein enhances the processes of nucleocapsid assembly. *Microbes Infect* **13:** 76–84.

Emara, M.M., and Brinton, M.A. (2007) Interaction of TIA-1/TIAR with West Nile and dengue virus products in infected cells interferes with stress granule formation and processing body assembly. *Proc Natl Acad Sci USA* **104:** 9041–9046.

Fros, J.J., Domeradzka, N.E., Baggen, J., Geertsema, C., Flipse, J., Vlak, J.M., and Pijlman, G.P. (2012) Chikungunya virus nsP3 blocks stress granule assembly by recruitment of G3BP into cytoplasmic foci. *J Virol* **86:** 10873–10879.

Goldmacher, V.S., Bartle, L.M., Skaletskaya, A., Dionne, C.A., Kedersha, N.L., Vater, C.A., *et al.* (1999) A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. *Proc Natl Acad Sci USA* **96:** 12536–12541.

Griff, I.C., Schekman, R., Rothman, J.E., and Kaiser, C.A. (1992) The yeast SEC17 gene product is functionally equivalent to mammalian alpha-SNAP protein. *J Biol Chem* **267:** 12106–12115.

Griffin, D.E., and Hardwick, J.M. (1997) Regulators of apoptosis on the road to persistent alphavirus infection. *Annu Rev Microbiol* **51:** 565–592.

Hobman, T.C. (2013) Rubella virus. In *Fields Virology*, 6th edn. Knipe, D.M., and Howley, P.M. (eds). Philadelphia, PA: Lippincott Williams & Wilkins, pp. 687–711.

Hofmann, J., Pletz, M.W., and Liebert, U.G. (1999) Rubella virus-induced cytopathic effect *in vitro* is caused by apoptosis. *J Gen Virol* **80** (Part 7): 1657–1664.

Ilkow, C.S., Mancinelli, V., Beatch, M.D., and Hobman, T.C. (2008) Rubella virus capsid protein interacts with poly(a)-binding protein and inhibits translation. *J Virol* **82:** 4284–4294.

Ilkow, C.S., Weckbecker, D., Cho, W.J., Meier, S., Beatch, M.D., Goping, I.S., *et al.* (2010) The rubella virus capsid protein inhibits mitochondrial import. *J Virol* **84:** 119–130.

Ilkow, C.S., Goping, I.S., and Hobman, T.C. (2011) The Rubella virus capsid is an anti-apoptotic protein that attenuates the pore-forming ability of Bax. *PLoS Pathog* **7:** e1001291.

Kubler, K., Gehrke, N., Riemann, S., Bohnert, V., Zillinger, T., Hartmann, E., *et al.* (2010) Targeted activation of RNA helicase retinoic acid-inducible gene-I induces pro-immunogenic apoptosis of human ovarian cancer cells. *Cancer Res* **70:** 5293–5304.

Law, L.J., Ilkow, C.S., Tzeng, W.P., Rawluk, M., Stuart, D.T., Frey, T.K., and Hobman, T.C. (2006) Analyses of phosphorylation events in the rubella virus capsid protein: role in early replication events. *J Virol* **80:** 6917–6925.

Law, L.M., Duncan, R., Esmaili, A., Nakhasi, H.L., and Hobman, T.C. (2001) Rubella virus E2 signal peptide is required for perinuclear localization of capsid protein and virus assembly. *J Virol* **75:** 1978–1983.

Law, L.M., Everitt, J.C., Beatch, M.D., Holmes, C.F., and Hobman, T.C. (2003) Phosphorylation of rubella virus capsid regulates its RNA binding activity and virus replication. *J Virol* **77:** 1764–1771.

Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94:** 491–501.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86:** 147–157.

McInerney, G.M., Kedersha, N.L., Kaufman, R.J., Anderson, P., and Liljestrom, P. (2005) Importance of eIF2alpha phosphorylation and stress granule assembly in alphavirus translation regulation. *Mol Biol Cell* **16:** 3753–3763.

Mangala Prasad, V., Willows, S.D., Fokine, A., Battisti, A.J., Sun, S., Plevka, P., *et al.* (2013) Rubella virus capsid protein structure and its role in virus assembly and infection. *Proc Natl Acad Sci USA* **110:** 20105–20110.

Mir, M.A., Duran, W.A., Hjelle, B.L., Ye, C., and Panganiban, A.T. (2008) Storage of cellular 5′ mRNA caps in P bodies for viral cap-snatching. *Proc Natl Acad Sci USA* **105:** 19294–19299.

Panas, M.D., Varjak, M., Lulla, A., Eng, K.E., Merits, A., Karlsson Hedestam, G.B., and McInerney, G.M. (2012) Sequestration of G3BP coupled with efficient translation inhibits stress granules in Semliki Forest virus infection. *Mol Biol Cell* **23:** 4701–4712.

Peng, S., Geng, J., Sun, R., Tian, Z., and Wei, H. (2009) Polyinosinic-polycytidylic acid liposome induces human hepatoma cells apoptosis which correlates to the up-regulation of RIG-I like receptors. *Cancer Sci* **100:** 529–536.

Phillips, C.A., Melnick, J.L., Yow, M.D., Bayatpour, M., and Burkhardt, M. (1965) Persistence of virus in infants with congenital Rubella and in normal infants with a history of maternal Rubella. *JAMA* **193:** 1027–1029.

Pugachev, K.V., and Frey, T.K. (1998) Rubella virus induces apoptosis in culture cells. *Virology* **250:** 359–370.

Suomalainen, M., Garoff, H., and Baron, M.D. (1990) The E2 signal sequence of rubella virus remains part of the capsid protein and confers membrane association *in vitro*. *J Virol* **64:** 5500–5509.

Surjit, M., Kumar, R., Mishra, R.N., Reddy, M.K., Chow, V.T., and Lal, S.K. (2005) The severe acute respiratory syndrome coronavirus nucleocapsid protein is phosphorylated and localizes in the cytoplasm by 14-3-3-mediated translocation. *J Virol* **79:** 11476–11486.

Urbanowski, M.D., and Hobman, T.C. (2013) The West Nile virus capsid protein blocks apoptosis through a phosphatidylinositol 3-kinase-dependent mechanism. *J Virol* **87:** 872–881.

Wolinsky, J.S., McCarthy, M., Allen-Cannady, D., Moore, W.T., Jin, R., Cao, S., *et al.* (1991) Monoclonal antibody-defined epitope map of expressed rubella virus protein domains. *J Virol* **65:** 3986–3994.

Yao, J., and Gillam, S. (1999) Mutational analysis, using a full-length rubella virus cDNA clone, of rubella virus E1 transmembrane and cytoplasmic domains required for virus release. *J Virol* **73:** 4622–4630.

You, J., Dove, B.K., Enjuanes, L., DeDiego, M.L., Alvarez, E., Howell, G., *et al.* (2005) Subcellular localization of the severe acute respiratory syndrome coronavirus nucleocapsid protein. *J Gen Virol* **86:** 3303–3310.