Virology 527 (2019) 146–158

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

The feline calicivirus leader of the capsid protein causes survivin and XIAP downregulation and apoptosis

Oscar Salvador Barrera-Vázquez[a], Clotilde Cancio-Lonches[a], Olivia Hernández-González[a], Bibiana Chávez-Munguia[a], Nicolás Villegas-Sepúlveda[b], Ana Lorena Gutiérrez-Escolano[a,*]

[a] Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados del IPN, Av. IPN 2508. Col. San Pedro Zacatenco, México, D.F CP 07360, Mexico.
[b] Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del IPN, México City, Mexico

ARTICLE INFO

Keywords:
Calicivirus
Feline calicivirus
LC
Apoptosis
IAPs
Survivin
XIAP
Smac/DIABLO

ABSTRACT

Calicivirus infection causes intrinsic apoptosis, leading to viral propagation in the host. During murine norovirus infection, a reduction in the anti-apoptotic protein survivin has been documented. Here we report that in feline calicivirus infection, a downregulation of the anti-apoptotic proteins survivin and XIAP occur, which correlates with the translocation of the pro-apoptotic protein Smac/DIABLO from the mitochondria to the cytoplasm and the activation of caspase-3. Inhibition of survivin degradation by lactacystin treatment caused a delay in apoptosis progression, reducing virus release, without affecting virus production. However, the overexpression of survivin caused a negative effect in viral progeny production. Overexpression of the leader of the capsid protein (LC), but not of the protease-polymerase NS6/7, results in the downregulation of survivin and XIAP, caspase activation and mitochondrial damage. These results indicate that LC is responsible for the induction of apoptosis in transfected cells and most probably in FCV infection.

1. Introduction

The *Caliciviridae* are a family of non-enveloped positive-sense, single stranded RNA viruses, ubiquitous in the environment, that represent an important cause of disease in humans and in many other vertebrates. The *Caliciviridae* family contains five genera: *Norovirus, Sapovirus, Lagovirus, Vesivirus* and *Beco/Nebovirus*; and three proposed genera, Recovirus, Valovirus and Bavovirus (Farkas et al., 2008; L'Homme et al., 2009; Wolf et al., 2011). *Norovirus, Sapovirus,* and Recovirus cause gastroenteritis in humans (Smits et al., 2012), and can also infect a number of animals. Noroviruses in particular are considered the leading cause of outbreaks and sporadic cases of human viral gastroenteritis worldwide (Atmar, 2010; Lopman et al., 2016; Monroe, 2011). On the other hand, *Vesiviruses, Lagoviruses,* and *Beco/Neboviruses* primarily infect animals and cause a variety of diseases (Thiel and König, 1999).

During calicivirus replication, apoptosis is required for a successful infection, (Al-Molawi et al., 2003; Alonso et al., 1998; Alvarez-Sanchez et al., 2015; Bok et al., 2009; Natoni et al., 2006; Sosnovtsev et al., 2003), and even though it is well known that mitochondrial or intrinsic pathways are triggered, the molecular mechanisms involved in this process are still poorly understood. One of the stimuli that trigger the intrinsic apoptosis pathway is the translocation of the pro-apoptotic Bcl-2 family member Bax, into the mitochondria membrane, which results in the release of several mitochondrial proteins such as cytochrome C into the cytosol (Elmore, 2007). Cytochrome C binds to the protease-activating factor (Apaf) 1, to form the apoptosome, which recruits and activates the initiator caspase-9, and allows the activation of executor caspases such as caspase-3, − 6, and − 7, targeting a broad spectrum of cellular proteins and ultimately resulting in cell death (McIlwain et al., 2013). The direct inhibition of nascent active caspases is achieved by the protein inhibitors of apoptosis (IAPs); a group of suppressors of apoptosis, that confer protection from dead-inducing stimuli through its direct interaction with caspases (Salvesen and Duckett, 2002). Among them, the X-linked inhibitor of apoptosis protein (XIAP) is one of the most potent inhibitors of apoptosis (Deveraux et al., 1999; Huang et al., 2001). XIAP, cIAP1 and cIAP2, can directly inhibit certain caspases (Vaux and Silke, 2005), while survivin, the smallest IAP, interacts and stabilizes cofactor molecules such as XIAP to specifically inhibit caspase-9 activation; thus, survivin downregulation correlates with the intrinsic apoptosis (Chen et al., 2000; Pyrko et al., 2006).

The release of the cytochrome C to the cytosol is accompanied by the efflux of the pro-apoptotic molecule second mitochondrial activator

* Corresponding author.
E-mail address: alonso@cinvestav.mx (A.L. Gutiérrez-Escolano).

https://doi.org/10.1016/j.virol.2018.11.017
Received 27 July 2018; Received in revised form 27 November 2018; Accepted 27 November 2018
Available online 04 December 2018
0042-6822/

of caspases/direct IAP binding protein with low pI (Smac/DIABLO) in response to apoptotic stimuli. Mature Smac/DIABLO binds and neutralizes the caspase-inhibitory properties of XIAP and several other IAPs (Adrain et al., 2001). The ability of XIAP to repress active caspase-9 within the apoptosome complex is overcome by displacement of XIAP from caspase-9 by Smac/DIABLO.

Evidence of the translocation and activation of some of these molecules involved in the establishment of apoptosis such as translocation of Bax into the mitochondria, release of cytochrome C into the cytosol, and activation of caspase-9 and-3 have been documented to occur during FCV and MNV infection (Al-Molawi et al., 2003; Bok et al., 2009; Natoni et al., 2006; Sosnovtsev et al., 2003). In this regard, survivin, a member of the IAPs family, is significantly downregulated during MNV infection (Bok et al., 2009; Herod et al., 2014). Survivin downregulation occurs in an inverse relation with the genome replication and correlates with the activation of caspases; thus, suggesting that this event triggers the establishment of the intrinsic apoptosis in MNV (Bok et al., 2009). Moreover, the expression of the complete MNV ORF1 induces survivin downregulation and apoptosis in a virus-free cell model (Herod et al., 2014). On the other hand, during FCV infection, the expression of the leader of the capsid protein (LC), encoded in the subgenomic RNA, and expressed as a part of the major capsid protein LC-VP1 precursor by the NS6/7 proteinase (Sosnovtsev et al., 1998), causes a cytopathic effect that consists in rounding of CrFK cells and the activation of caspases (Abente et al., 2013). However, the interplay of apoptogenic and anti-apoptotic proteins and their role in viral replication is not yet fully understood.

In this study, we report that as observed during MNV, survivin is downregulated in FCV infection. Moreover, we also found that FCV causes the downregulation of XIAP and translocation of Smac/DIABLO into the cytosol of the infected cells, suggesting their contribution to the establishment of the intrinsic mitochondrial pathway during calicivirus infection. Since survivin has different roles during the cell cycle, its downregulation induced by FCV may have effects not only during apoptosis but also on viral replication. When cells were infected with FCV in the presence of the apoptosis inhibitor lactacystin, that prevents survivin degradation by the proteasome, a reduction of viral release was observed, in accordance to the role of apoptosis in virus spreading (Alvarez-Sanchez et al., 2015). However, overexpression of survivin reduced viral protein synthesis and virus production, suggesting that this molecule has the ability to regulate early events during FCV replication. Furthermore, to determine the viral molecule(s) responsible of survivin downregulation, both NS6/7 and LC proteins were transiently expressed in CrFK cells. Even though the expression of both proteins caused a CPE, cell rounding phenotype and survivin downregulation together with caspase activation and PARP processing were only observed in cells transfected with LC protein, indicating that this protein is required for apoptosis establishment.

2. Materials and methods

2.1. Materials and methods

2.1.1. Cells and virus infection

Crandell-Rees feline kidney (CrFK) cells obtained from the American Type Culture Collection (ATCC) (Rockville, MD) were grown as described previously (Hernandez et al., 2016). In this study, CrFK cells were infected with the FCV F9 strain. Virus titer were determined by plaque assay as reported (Escobar-Herrera et al., 2007).

2.2. Western blot analysis

Mock and FCV infected cells were washed with PBS, lysed in Laemmli sample buffer, and boiled for 10 min. The proteins were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 10% skimmed milk for 2 h and incubated overnight at 4 °C with the following antibodies: anti-survivin, anti-PARP, and anti-caspase-3 (Cell Signaling Technology); anti-XIAP, anti-nucleolin C23 (H-250), anti-Smac/DIABLO (Santa Cruz Biotechnology), and anti-caspase-9 (Santa Cruz Biotechnology), anti-actin (kindly donated by Dr. Manuel Hernández, Cinvestav, México), anti-FCV NS6/7 or NS3, and anti-MNV NS7 (kindly donated by Dr. Ian Goodfellow, University of Cambridge, UK), anti-Tim23 (Abcam) and anti HSC70 (Sigma), anti-pAm-Cyan (Anti-RCFP polyclonal Pan antibody, Clontech) anti-AnxA2 (Santa Cruz Biotechnology) and anti-DsRed2 (Santa Cruz Biotechnology). The blots were washed with 0.05% Tris-buffered saline (TBS)-Tween, incubated for 2 h with the appropriate secondary antibodies and developed using chemiluminescence (PIERCsE). Quantification of protein levels was achieved by measuring band intensities in the scanned images using ImageJ software (http:/rsb.info.nih.gov/ij) and expressed as arbitrary units.

2.3. Immunofluorescence assays

CrFK cells were grown overnight on glass cover slips and infected with FCV at an MOI of 5. At the indicated times the cells were treated with cytoskeleton buffer for 5 min and permeabilized in 4% formaldehyde-triton X100 solution for 5 min at room temperature (RT). The samples were washed 3 times with PBS for 5 min, blocked with 0.5% gelatin in PBS for 40 min at RT, washed 3 times with PBS for 5 min, and incubated with the corresponding primary antibody at 4 °C overnight. Samples were washed three times with cold phosphate buffer (PBS) for 5 min, and incubated with the appropriate secondary antibodies (Invitrogen) for 1 h at RT. The samples were washed 3 times with PBS, and incubated with 1 mg/ml of 4´6´-diamidino-2-phenylindole (DAPI) for 2 min. The samples were washed 6 times with PBS and 3 times with distilled water. The samples were treated with Vecta-Shield liquid mounting media (Vector Laboratories A.C.) and analyzed using a Zeiss LSM-700 confocal microscope. For mitochondria staining, cells were labeled in vivo by adding 50 nM Mito Tracker Deep Red into serum-free medium for 15 min at RT before fixing.

2.4. Preparation of mitochondrial fractions

Mock, FCV infected, and transfected CrFK cells with pAm-Cyan, Wt-LC-pAm-Cyan, and Mut-LC-pAm-Cyan were harvested at 3, 5, and 7 h. The cells were processed using a Mitochondria Isolation Kit for Cultured Cells (Thermo Scientific) according to the manufacturer's instructions.

2.5. Lactacystin treatment

CrFK cells were treated with 25 μM Lactacystin (Santa Cruz Biotechnology), in MEM medium-fetal bovine serum free for 1 h before infection; then, lactacystin was removed and after 1 h of viral adsorption, lactacystin was added and infection was allowed for the indicated times. The viability of lactacystin treated cells was assessed with the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTT) (Promega).

2.6. Plasmids construction

DNA fragment corresponding to the complete feline survivin sequence was amplified from CrFK cells cDNA, by using PFU DNA polymerase (Thermo Fisher Scientific) and a pair of primers: FW5´-AGTC AGAATTCATGGGCGCTTCGTCGTTG-3´, and RV5´- ACTCCGGATCCGC CTCCAGGGCCGCCAGCTGC-3´. The sequences of primers include *Eco*RI and *Bam*HI recognition sites. The amplicon was cloned into the cloning vector pJET (Thermo Fisher Scientific), and sub-cloned into the *Eco*RI and *Bam*HI enzyme sites of the pAm-Cyan (Clontech Laboratories, Inc) vector to generate the plasmid pAm-Cyan-survivin. For the expression of the FCV LC protein, a cDNA form nts 5314–5685 was amplified by PCR form a plasmid containing the complete FCV Urbana strain genome

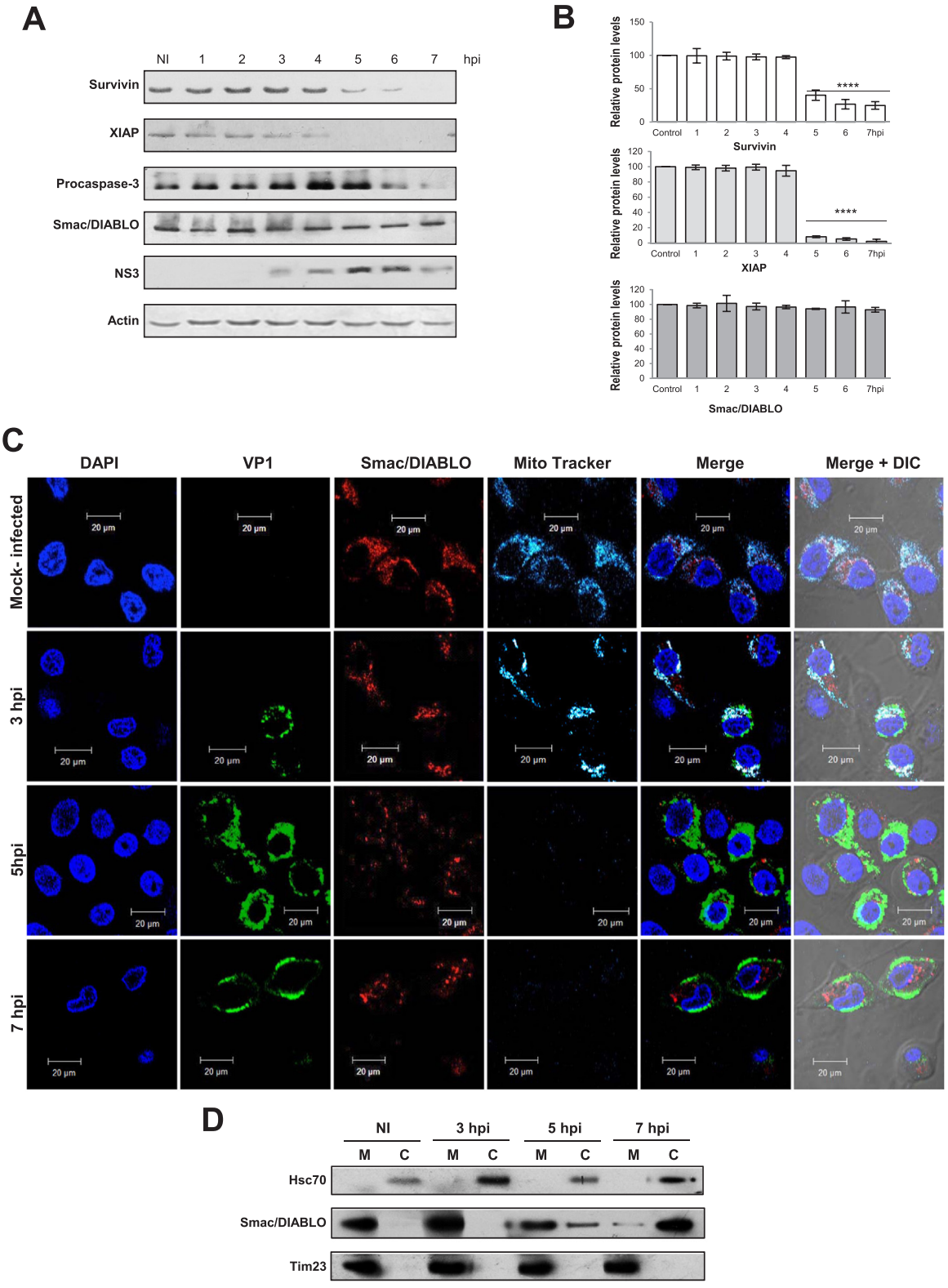

Fig. 1. Survivin, XIAP, and Smac/DIABLO changes during FCV infection. A) Total protein extracts from mock or FCV infected cells at an MOI of 5, were obtained at 1, 2, 3, 4, 5, 6, 7 hpi and subjected to SDS-PAGE. Protein expression was analyzed by western blotting using specific antibodies. NS3 indicates virus infection, procaspase-3 processing indicates caspase activation; actin was used as the loading control. B) Survivin, XIAP, and Smac/DIABLO band intensities of the scanned images were quantified using ImageJ software and expressed as arbitrary units. Standard deviations were obtained from duplicates of at least 3 independent assays. Values of P = < 0.05 (****), calculated using GraphPad Prism 7.00 are indicated. C) Mock-infected and FCV infected cells at an MOI of 5, for 3, 5, and 7 h were immunostained with an anti-Smac/DIABLO (red), and an anti-VP1 (green) antibodies, followed by Alexa Fluor 594 (red) and Alexa fluor 488 (green) staining respectively. Mitotracker and DAPI were used to stain nuclei (blue) and mitochondria (turquoise). The cells were analyzed using a Zeiss LSM 700 confocal microscope. The images depict single confocal slices taken from z-stacks. The data shown are representative of at least 3 independent experiments. D) Mitochondrial and cytoplasmic extracts obtained from mock-infected (control) and FCV infected cells at 3, 5, and 7 hpi were subjected to SDS-PAGE and Smac/DIABLO subcellular localization was analyzed by western blotting using specific antibodies. Hsc70 and Tim23 were used as cytoplasmic and mitochondrial fraction controls. Anti-mouse and anti-rabbit HRP secondary antibodies were used as indicated, and revealed by chemiluminescence.

(kindly donated by Dr. Kim Green) using PFU DNA polymerase (Thermo Fisher Scientific) and a pair of primers: FW5´-ACTGGCTCGA GATGTGCTCAACCTGCGC-3´ and RV5 ´- ACTGGCTCGAGATGTGCTCA ACCTGCGC-3´. The sequences of primers include *Xho*I and *Hin*dIII recognition sites. The amplicon was cloned into the cloning vector pJET (Thermo Fisher Scientific), and subcloned into the *Xho*I and *Hin*dIII restriction sites of the pAm-Cyan (Clontech Laboratories, Inc) vector to generate plasmid WT-LC-pAm-Cyan. Mut-LC-pAm-Cyan was generated by site direct mutagenesis of Wt-LC-pAm-Cyan using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies), and a pair of primers: FW5'-GATAATCCACTTATGTGCGCTTATCCTGAATTGCTCCC-3' and REV5'-GGGACGAATTCAGGATAAGCGCACATAAGTGGATTATC-3', following manufacturers instructions. The clones were screened by sequence analysis to verify that all plasmids contained the correct sequence.

2.7. Transient transfections

Subconfluent monolayers of CrFK cells were transfected in 6 well dishes with 3.5 μg of plasmids Cherry and Cherry-NS6/7 (kindly donated by Dr. Ian Goodfellow, University of Cambridge UK) pAm-Cyan, survivin-pAm-Cyan, Wt-LC-pAm-Cyanor Mut-LC-pAm-Cyan using Lipofectamine® 2000 Reagent (Thermo Fisher Scientific) according to manufacturer´s instructions during 24 or 48 h.

2.8. Transmission electron microscopy (TEM)

For gold immunolabeling experiments, CrFK cells at 24 h post-transfection with pAm-Cyan and Wt-5424, or at 5 h of infection with FCV at an MOI of 5, were washed three times with PBS. Cells were fixed with 4% PFA and 0.5% glutaraldehyde in PBS for 1 h at room temperature (RT) and dehydrated with increasing concentrations of ethanol. Samples were embedded in LR White resin (London Resin Co) and polymerized for 48 h under UV irradiation at 4 °C. Thin sections of 60 nm were obtained and mounted on nickel grids, and incubated overnight (ON) with the anti-CRFP polyclonal Pan antibody (Clontech) or anti-VP1 antibody (Santa Cruz Biotechnology) (1:5) and for 1 h with the anti-rabbit or anti-mouse IgGs antibodies conjugated to 20 nm gold particles (Ted Pella Inc., Redding, CA, USA; 1:50). Antibodies were diluted in PBS with 5% fetal bovine serum. After incubation with antibodies samples were contrasted with uranyl-acetate and lead citrate before being examined in a Joel JEM-1011 transmission electron microscope.

3. Results

3.1. The anti-apoptotic proteins survivin and XIAP are downregulated during FCV infection

The extensive CPE that occurs during calicivirus infection is in part, the result of the induction of the mitochondrial apoptosis pathway (Natoni et al., 2006; Roberts et al., 2003; Sosnovtsev et al., 2003; Willcocks et al., 2004). Even though the functional importance of this process during virus replication, it is not completely understood; evidence suggests that caliciviruses induce apoptosis to facilitate the dissemination of viral progeny in the host (Alonso et al., 1998; Alvarez-Sanchez et al., 2015; Bok et al., 2009). During infection, several cellular changes as the presence of pyknotic nuclei, DNA degradation, loss of mitochondrial membrane permeability, release of cytochrome C into the cytosol, and activation of caspases have been documented (Natoni et al., 2006; Roberts et al., 2003; Sosnovtsev et al., 2003); however, little is known about the regulation of pro- and anti-apoptotic proteins. During MNV infection, survivin, is downregulated at transcriptional and translational level (Bok et al., 2009), suggesting that activation of caspases occur as a consequence of the instability of survivin, a potent endogenous caspase inhibitor, thereby promoting the onset of apoptosis (Bok et al., 2009).

To determine if survivin downregulation is a common feature during calicivirus infection, we investigated if as in MNV, survivin levels were downregulated during FCV infection. Thus, CrFK cells were infected with FCV at MOI of 5, and survivin protein was determined in total cell extracts by western blotting using a specific anti-survivin antibody (Fig. 1). Similar amounts of survivin protein were observed in mock as well as in infected cells at early times post infection (Fig. 1A); however, a reduction in survivin protein was observed form 5 and up to 7 hpi in FCV infected cell extracts (Fig. 1A), indicating that as in MNV infection (Bok et al., 2009; Herod et al., 2014), survivin is also downregulated in FCV infection. The 59.7%, 73.2%, and 75.1% decrease of survivin levels at 5, 6, and 7 hpi, (Fig. 1B), were statistically significant. This reduction correlates with the presence of increasing amounts of the non-structural (NS) 3 protein and the activation of caspase-9 and -3, supporting that apoptosis activation occurs during FCV infection.

Given that survivin protein stabilizes the anti-apoptotic protein XIAP, its regulation was also analyzed during FCV infection. XIAP levels showed a post-infection time dependent reduction from 5 h (Fig. 1B). The 91.4%, 94.81%, and 97.87% reduction of XIAP levels at 5, 6, and 7 hpi respectively were statistically significant (Fig. 1B).

3.2. Smac/DIABLO is translocated from the mitochondria to the cytosol during FCV infection

Since it has been described that the mitochondrial apoptogenic protein Smac/DIABLO, an inhibitor of IAPs, is released to the cytoplasm during apoptosis, and binds to IAPs to regulate its function as caspase inhibitors, it was of our interest to determine if its expression or subcellular localization was modulated during FCV infection. To determine whether Smac/DIABLO expression was modified during infection, total extracts from CrFK cells infected hourly up to 7 h were obtained and analyzed by western blotting (Fig. 1B). No changes in the expression of Smac/DIABLO were observed during infection (Figs. 1A and 1B). Because Smac/Diablo is normally located in the mitochondrial membrane and is translocated into the cytoplasm during apoptosis, its subcellular localization was analyzed during infection by immunofluorescence and western blotting.

The subcellular localization of this protein was analyzed by immunofluorescence using confocal microscopy at 3, 5, and 7 hpi (Fig. 1C). In mock-infected as well as in infected CrFK cells for 3 h, Smac/DIABLO showed a subcellular localization pattern that matches with the mitochondrial marker Mito Tracker Deep Red (Fig. 1C), with colocalization rates of 0.648 and 0.7 respectively. However, in the infected cells at 5 and 7 h, this pattern is altered and the colocalization rate was reduced less than 0.1, as analyzed by Pearson coefficient in the Zeiss Zen 2010 software program, suggesting that this protein is translocated from the mitochondria to the cytoplasm in the infected cells. To further corroborate that Smac/DIABLO was present in the cytoplasm of the infected cells, cytosolic proteins were separated from membrane (mitochondria)-associated proteins and its presence in each fraction was analyzed by western blotting (Fig. 1D). At 3 hpi, the localization of Smac/DIABLO was still mitochondrial, as in the mock-infected cells (MI); however, its presence in the cytoplasmic fraction was clearly observed at 5 and 7 hpi. The detection of Hsc70 and Tim23 were used to confirm purity of cytosolic and mitochondria fractions respectively. These results confirm the observations obtained by confocal microscopy that indicate that Smac/DIABLO is translocated from the mitochondria to the cytoplasm during FCV infection.

3.3. Treatment with lactacystin prevents the downregulation of survivin and delays apoptosis during FCV infection

It is well known that in addition to its role in apoptosis (Nakao et al., 2006), survivin is also involved in many cellular processes such as cell cycle, angiogenesis (Conway et al., 2003; Fernández et al., 2014; Li

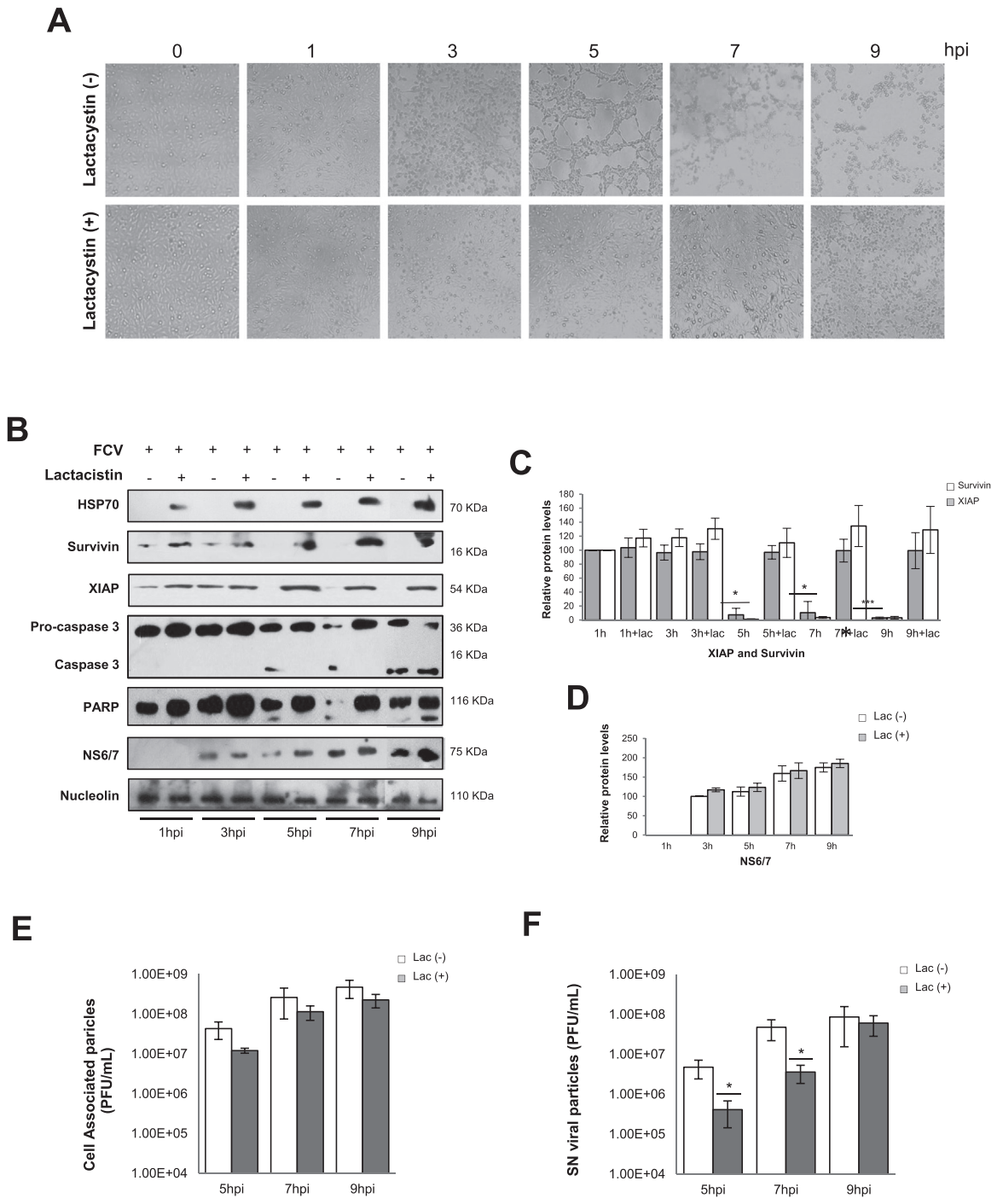

Fig. 2. Lactacystin treatment delays Survivin downregulation and affects FCV release from CrFK cells. A) Infected cells with FCV at an MOI of 5, were treated and untreated with the proteasome inhibitor lactacystin and the CPE was evaluated by light microscopy. B) Total protein extracts from mock-infected or infected cells were obtained at 1, 3, 5, 7, and 9 h, and subjected to SDS-PAGE. Survivin and XIAP expression was analyzed by western blotting using specific antibodies. NS6/7 indicates virus infection; procaspase-3 and PARP processing indicate caspase-3 activation; HSP70 was used as a proteasomal inhibition marker; and nucleolin was used as the loading control. C) and D) Band intensities of the scanned images were quantified using ImageJ software and expressed as arbitrary units. Standard deviations were obtained from duplicates of at least 3 independent assays. E) Viral particles from cell-associated and F) supernatants fractions were obtained at 5, 7, and 9 hpi and assessed by plaque assay. Standard deviations were obtained from duplicates of at least 3 independent assays. Values of P = < 0.05 (*), calculated using GraphPad Prism 7.00 software are indicated.

et al., 2017, 2016; Sanhueza et al., 2015), cell proliferation and migration (Hehlgans et al., 2013; Kawasaki et al., 2001; Koike et al., 2011; Li et al., 2015; McKenzie et al., 2010; Shiraki et al., 2000). Thus, its downregulation induced by FCV may have effects not only in apoptosis involved in virus release (Tayyari et al., 2011), but also on viral replication. To determine if survivin is involved in the regulation of FCV replication, CrFK cells treated with the proteasome inhibitor lactacystin, that prevents degradation of different members of the IAP family including survivin (Dohi et al., 2004; Yang et al., 2000; Zhao et al., 2000), were infected, and its effect in virus replication was analyzed by light microscopy and western blot (Fig. 2).

CrFK cells were treated or not with 25 μM lactacystin for 1 h and

infected with FCV at an MOI of 5, for 1, 3, 5, 7, and 9 h in the presence of lactacystin. A delay in the CPE was observed in the cells treated with lactacystin (Fig. 2A, lower panel) in comparison to untreated cells, where the progression of infection occurred as previously described (Fig. 2A, upper panel). To determine the expression of survivin and XIAP in the above conditions, cell extracts were prepared and the protein levels were analyzed by western blotting using specific antibodies. As expected, treatment with lactacystin prevented the down-regulation of both survivin and XIAP from 5 and up to 7 hpi (Fig. 2B). Moreover, procaspase-3 and PARP protein processing were also inhibited, indicating that in these conditions, apoptosis is delayed. At 9 hpi a statistically significant reduction of survivin and XIAP protein levels and procaspase-3 and PARP processing was observed in the absence or presence of lactacystin, demonstrating apoptosis progression in both conditions (Figs. 2B and 2C). Even though lactacystin treatment caused a delay in apoptosis up to 7 hpi, the viral protein levels (NS6/7) were similar to the ones observed in the untreated infected cells at all the times post infection tested (Figs. 2B and 2D), suggesting that viral translation was not affected by the treatment.

3.4. Survivin prevents apoptosis and affects FCV release of from CrFK cells

Due to the absence of apoptosis up to 7 h of infection in conditions were survivin is not degraded as a consequence of the treatment with lactacystin, we wanted to determine if survivin degradation might have an effect in viral production or viral release. Thus, we performed a plaque assay of the viral particles produced in cell associated and supernatant fractions at 5, 7, and 9 hpi from cells untreated and treated with lactacystin (Figs. 2E and 2F). In the presence of lactacystin, no significant reduction of viral particles production in the cell-associated fraction was observed when comparing with the viral production from non-treated cells (Fig. 2E). However, the viral titer from supernatants was significantly reduced at 5, and 7 hpi, with an increase in the supernatants at 9 hpi (Fig. 2F). Again, synthesis of NS3 observed at the different times post infection tested, showed similar amounts in the presence or absence of lactacystin (data not shown). Taken together these results indicate that endogenous levels of survivin do not affect FCV replication, but do reduce virus release, in concordance to the intrinsic apoptosis requirement for virus spread into the host as previously demonstrated.

3.5. Overexpression of survivin has a negative effect in FCV production

Since proteosome inhibition conditions, where similar levels of survivin to the ones observed in the mock-infected untreated cells resulted in a deficient virus release, we wanted to determine if the overexpression of survivin could affect FCV replication. Thus, CrFK cells were transfected with either survivin-pAm-Cyan or pAm-Cyan vector for 48 h and infected with FCV, and CPE and virus yield in cell associated and supernatant fractions was determined by light microscopy and plaque assay respectively (Fig. 3). Overexpression of survivin caused a delay in the CPE caused by FCV infection (Fig. 3A, lower panel) in comparison to the cells transfected with the pAm-Cyan alone (Fig. 3A, upper panel), As observed with lactacystin treatment, the virus production in the supernatants from survivin-pAm-Cyan vector transfected cells, was reduced almost 1 log at 5, and 7 hpi respectively, in comparison to the virus yield obtained from cells transfected with the pAm-Cyan vector alone (Fig. 3C). However, a statistically significant reduction in cell-associated particles at 5 and 7 hpi from cells transfected with survivin-pAm-Cyan was also observed (Fig. 3B), suggesting that overexpression of survivin has a negative effect in viral production. All these results taken together indicate that the absence of survivin favors FCV replication.

3.6. Survivin overexpression causes a reduction in FCV viral proteins production

Since FCV production was reduced during the over-expression of survivin, we wanted to determine which steps during FCV replication might be affected; thus, the levels of non-structural proteins in this condition were analyzed by western blot (Figs. 3D and 3E). CrFK cells were transfected with either survivin-pAm-Cyan or pAm-Cyan vectors for 48 h and infected with FCV at an MOI of 5; total protein extracts were obtained at 5, 7, and 9 hpi and NS6/7 protein levels were analyzed by western blot. CrFK cells overexpressing surviving (Cyan-survivin) showed a reduction of the non-structural viral protein NS6/7 (97.23%, 90.65% and 55.55%) levels at all 5, 7, and 9 hpi in comparison to the levels observed in the pAm-Cyan transfected cells (Fig. 3E). At the same conditions, XIAP and endogenous survivin protein levels were not affected by the viral infection, in concordance with the absence of procaspase-3 activation and PARP processing (Fig. 3D). These results indicate that the reduction of virus yield in conditions where survivin is overexpressed could be a consequence of the reduction of non-structural proteins production.

3.7. NS6/7 expression is associated with a CPE of CrFK transfected cells but not with the induction of apoptosis

Downregulation of survivin occurs during both MNV (Bok et al., 2009), and FCV replication, as seen here. Moreover, expression of the ORF1 polyprotein alone was sufficient to induce apoptosis which was characterized by the activation of caspase-9 and downregulation of survivin (Herod et al., 2014). However, no molecule has been reported to be responsible of survivin degradation during FCV infection.

To determine the viral factor(s) involved in apoptosis and survivin and XIAP downregulation during FCV infection, we analyzed two viral proteins: the protease-polymerase NS6/7, since different viral proteases are associated with apoptosis (Barco et al., 2000; Chau et al., 2007; Li et al., 2002; Lin et al., 2014; Shafee and AbuBakar, 2003; Zaragoza et al., 2006), and the LC protein that is associated with CPE in FCV infection (Abente et al., 2013).

Transfection of NS6/7-Cherry caused a disruption of the monolayer confluence at 48 hpt, in comparison with the cells transfected with the mCherry alone, where the cell monolayer remained unaltered, as observed by epifluorescence microscopy (Fig. 4A). However, no cell rounding was observed. Moreover, cell extracts from transfected cells were obtained and the integrity of several proteins associated with apoptosis induction was analyzed (Fig. 4B). No changes in survivin, XIAP, caspase-3 and PARP levels were detected in non infected as well as in m-Cherry and NS6/7-mCherry transfected cell extracts (Fig. 4B), while a reduction in survivin and XIAP levels as well as caspase-3 and PARP processing were clearly observed in the extracts form infected cells (Fig. 4B). These results indicate that NS6/7 expression is not associated with apoptotic triggering in transfected cells.

3.8. The LC protein expression is associated with XIAP and survivin downregulation in CrFK transfected cells

To analyze if LC protein from FCV is associated with the down-regulation of survivin and XIAP, CrFK cells were transfected with the Wt-LC-pAm-Cyan and the CPE (CPE) was evaluated by epifluorescence microscopy. As showed in Fig. 5A, expression of LC caused a characteristic CPE, which appeared as cell rounding, disruption of the monolayer and detachment of cells from the surface (Fig. 5A, middle panel), as previously described (Abente et al., 2013), and in comparison with the monolayer of cells transfected with pAm-Cyan alone, that remained unchanged (Fig. 5A, upper panel). Moreover, the expression of a mutant LC (Mut-LC-pAM-Cyan) that contain an alanine substitution at position C40 (C40A), that was previously reported to result on failure of cell rounding phenotype as well as a dramatic reduction of cytopathic

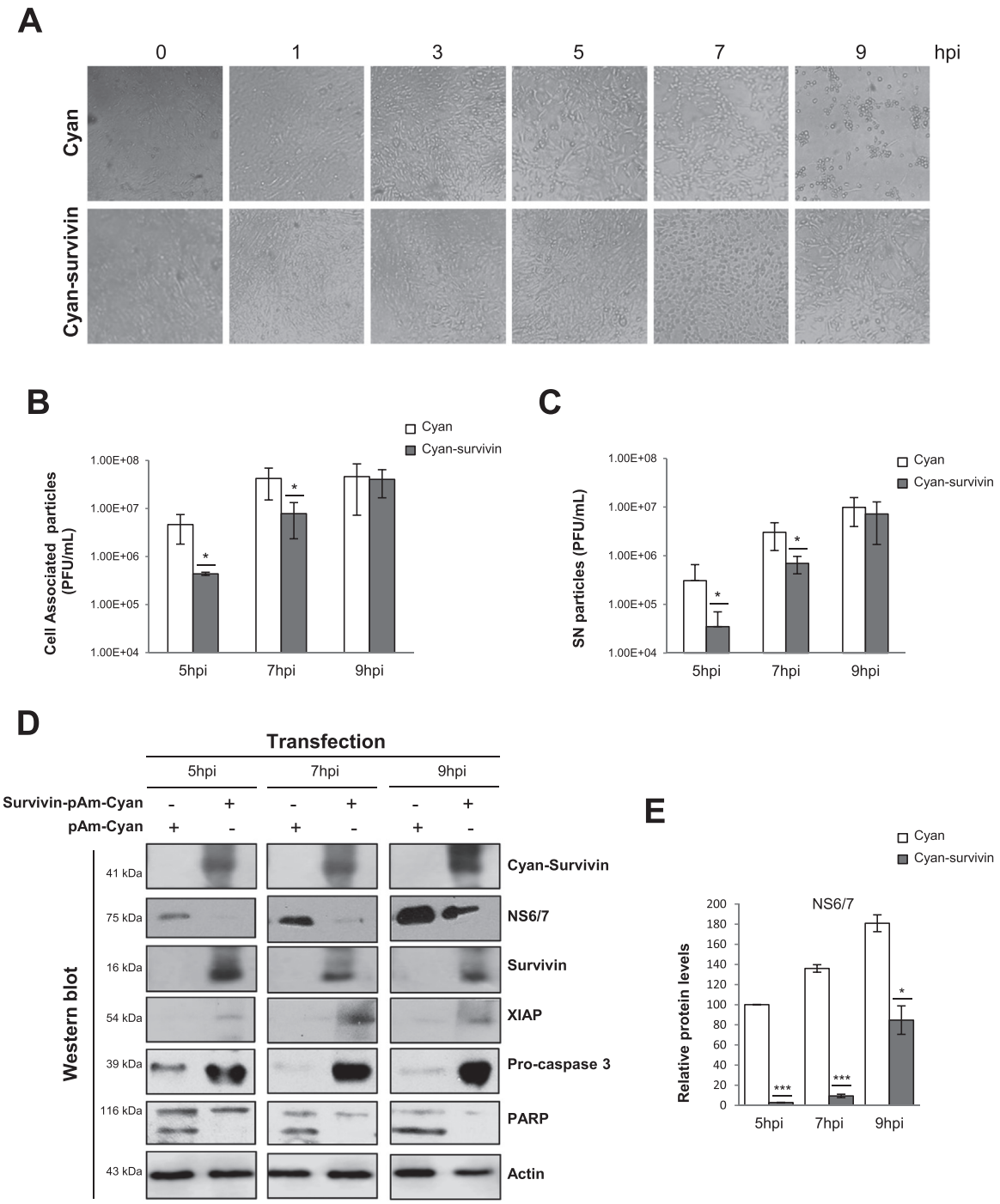

Fig. 3. Over-expression of survivin has a negative effect on FCV replication. A) CrFK cells were transfected with plasmids pAm-Cyan and survivin-pAm-Cyan and infected with FCV at an MOI of 5; the CPE was evaluated at 0, 1, 3, 5, 7, and 9 h by light microscopy. B) Viral particles from cell-associated and C) supernatants fractions were obtained at 5, 7, and 9 hpi and assessed by plaque assay. Standard deviations were obtained from duplicates of at least 3 independent assays. D) Total extracts from CrFK cells transfected for 48 h with plasmids pAm-Cyan and survivin-pAm-Cyan and infected with FCV at an MOI of 5 were subjected to SDS-PAGE. Cyan-survivin and NS6/7 expression was analyzed by western blotting using specific antibodies. Survivin and XIAP expression, and procaspase-3 and PARP processing indicates apoptosis. Actin was used as the loading control. E) NS6/7 band intensities of the scanned images were quantified using ImageJ software and expressed as arbitrary units. Standard deviations were obtained from duplicates of at least 3 independent assays. Values of P = < 0.05 (*), P = < 0.0001 (***), calculated using GraphPad Prism 7.00 are indicated.

virus recovery (Abente et al., 2013), did not caused a disruption of the monolayer, or detachment of cells from the surface; however, partial cell rounding cell phenotype was observed (Fig. 5A, lower panel). In addition, cell extracts from transfected cells were obtained and the integrity of survivin and XIAP proteins was analyzed (Fig. 5B). A strong reduction of survivin and XIAP levels were detected in Wt-LC-pAm-Cyan transfected cells as well as in infected cell extracts (Fig. 5C), in correlation with caspase-3 and PARP processing while no changes in survivin, XIAP, caspase-3 and PARP levels were detected in non infected as well as in pAm-Cyan and Mut-LC-pAM-Cyan transfected cell extracts (Fig. 5B). These results taking together indicate that LC expression alone triggers apoptosis in cells, with the concomitant changes such as the downregulation of survivin and XIAP, and the activation of caspase-3.

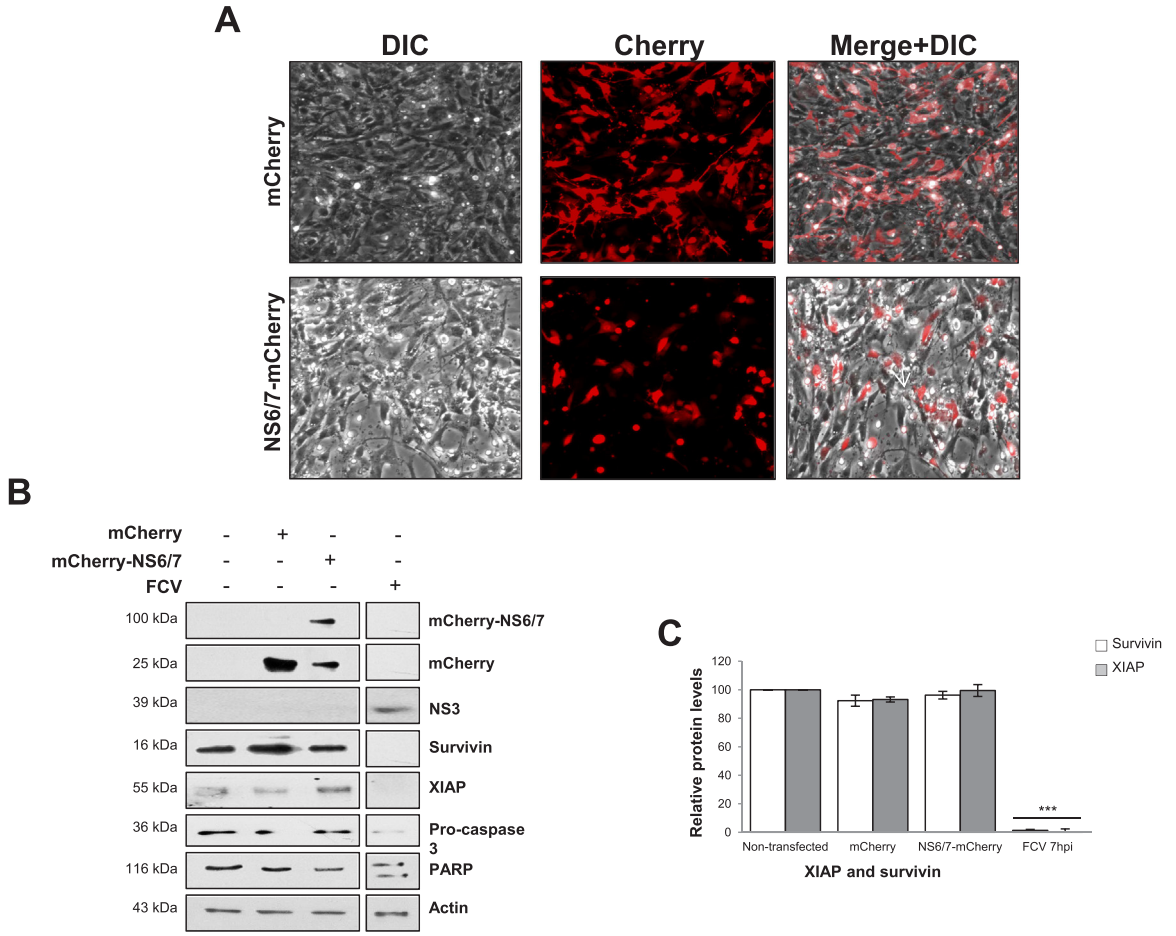

Fig. 4. NS6/7 causes a CPE but does not induce changes in survivin and XIAP protein levels. A) CrFK cells were transfected with mCherry and NS6/7-mCherry plasmids and the CPE was evaluated at 48 hpt by light microscopy. B) Total protein extracts from mock-infected, FCV infected cells at an MOI of 5 for 5 h, and transfected cells with mCherry and NS6/7-mCherry for 48 h were subjected to SDS-PAGE. Protein expression was analyzed by western blotting using specific antibodies. Survivin and XIAP proteins integrity was detected using specific antibodies. Procaspase-3 and PARP processing indicates caspase activation. NS3 indicates virus infection. mCherry and mCherry NS6/7 were detected with the anti-Cherry antibody Actin was used as the loading control. C) Survivin and XIAP band intensities of the scanned images were quantified using ImageJ software and expressed as arbitrary units. Standard deviations were obtained from duplicates of at least 3 independent assays. Values of P = < 0.05 (***), calculated using GraphPad Prism 7.00 are indicated.

3.9. Transfection of LC protein caused mitochondria damage

Since downregulation of survivin and XIAP are associated with the efflux of Smac/DIABLO from the mitochondria after a caspase-catalyzed event (Adrain et al., 2001), we wanted to determine if LC expression was associated with changes in mitochondria integrity. CrFK cells were transfected with Wt-LC-pAm-Cyan and pam-Cyan, and the integrity of mitochondria was evaluated by Immunoelectron microscopy to detect direct association of LC with the mitochondria in WT-LC-pAm-Cyan transfected cells and to study the mitochondria ultrastructure in mock and infected cells as well as in pAm-Cyan and WT-LC-pAm-Cyan transfected cells (Fig. 6). LC protein label was observed close to the mitochondria, and extensively in the mitochondria membrane as well as inside of mitochondria (Fig. 6B). Damage to the mitochondrial morphology, and a reduction of the electron density of the matrix and cristae folds was seen in LC transfected as well as in infected cells (Figs. 6B and 6D), in comparison with the mock-infected and the pAm-Cyan transfected cells, were mitochondria integrity was clearly observed (Figs. 6A and 6C); moreover, the presence of LC in the mitochondrial fraction was also corroborated by western blotting (Fig. 6E).

Besides the mitochondrial damage, LC transient overexpression also induced down regulation of survivin and XIAP, activation of procaspase-3 and PARP processing, as observed during infection. Thus, to determine if LC was responsible of Smac/DIABLO translocation from the mitochondria to the cytoplasm as in the viral infection, CrFK cells were transfected for 48 h with pAm-Cyan, Wt-LC-pAm-Cyan and the Mut-LC-pAm-Cyan, and cytoplasmic and mitochondrial fractions were obtained to analyze the presence of this pro-apoptotic protein by western blot. Smac/Diablo was observed in the mitochondrial fractions from the mock infected as well as the pAm-Cyan and Mut-LC-pAm-Cyan transfected cells, in comparison to the infected cells were Smac/DIABLO was detected in the cytoplasmic fraction (Fig. 6E). Moreover, in the Wt-LC-pAm-Cyan transfected cells, Smac/DIABLO was observed in both the mitochondria and the cytoplasmic fractions, indicating that LC expression triggers translocation of Smac/Diablo from the mitochondria to the cytosol as occurs during viral infection. These results correlate with the presence of WT-LC in both the mitochondrial and cytoplasmic fractions, suggesting that LC is associated to the mitochondria fraction. All these results taken together demonstrate that FCV LC protein associates with the mitochondria membranes and is responsible for the induction of the mitochondria membrane damage and the translocation of Smac/DIABLO to the cytosol as during infection.

4. Discussion

Some members of the *Caliciviridae* family such as the rabbit

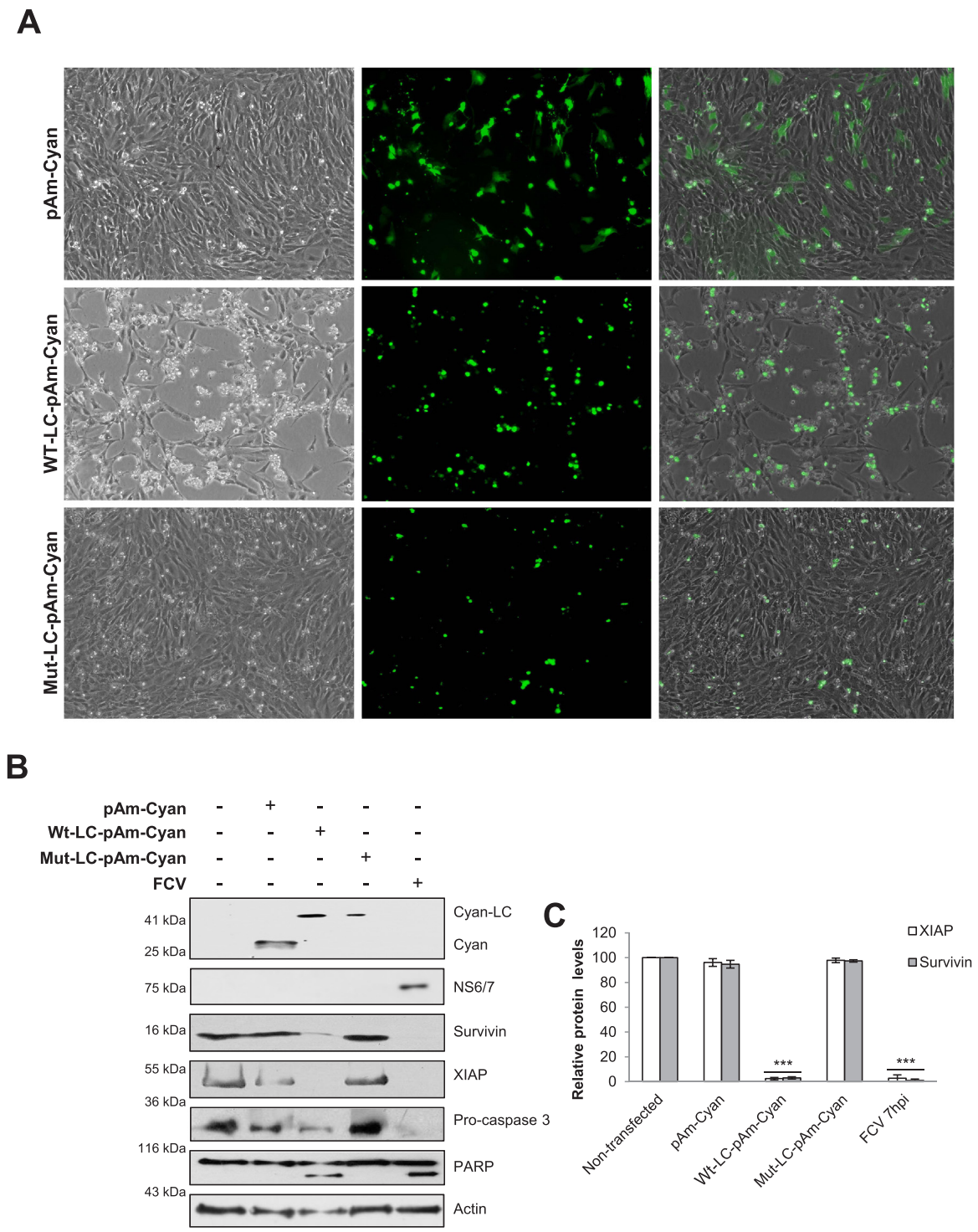

Fig. 5. LC down-regulates survivin and XIAP expression. A) CrFK cells were transfected with plasmids pAm-Cyan and Wt-LC-pAm-Cyan, and Mut-LC-pAm-Cyan and the CPE was evaluated at 48 hpt by light microscopy. B) Total protein extracts from mock-infected, FCV infected cells at an MOI of 5 for 5 h, and transfected cells with pAm-Cyan, Wt-LC-pAm-Cyan, and Mut-and LC-pAm-Cyan for 48 h were subjected to SDS-PAGE. Protein expression was analyzed by western blotting using specific antibodies. Survivin and XIAP proteins integrity was detected using specific antibodies. Procaspase-3 and PARP processing indicates caspase activation. NS6/7 indicates virus infection. Actin was used as the loading control. Cyan and Cyan–LC were detected with the anti-Cyan antibody. C) Survivin and XIAP band intensities of the scanned images were quantified using ImageJ software and expressed as arbitrary units. Standard deviations were obtained from duplicates of at least 3 independent assays. Values of P = < 0.05 (***), calculated using GraphPad Prism 7.00 are indicated.

hemorrhagic disease virus, (RHDV), FCV, and MNV have been important models for the study of the molecular mechanisms of calicivirus replication. Replication of these viruses result in an extensive CPE followed by cell death reminiscent of apoptosis (Bok et al., 2009; Jung et al., 2000; Natoni et al., 2006; Niedzwiedzka-Rystwej and Deptula, 2012; Roberts et al., 2003; Sosnovtsev et al., 2003). Even though the functional role of apoptosis in calicivirus replication is not completely understood, its role to facilitate viral progeny spread in the host has been documented (Alvarez-Sanchez et al., 2015; Olsen et al., 1996; Rodríguez-Grille et al., 2014).

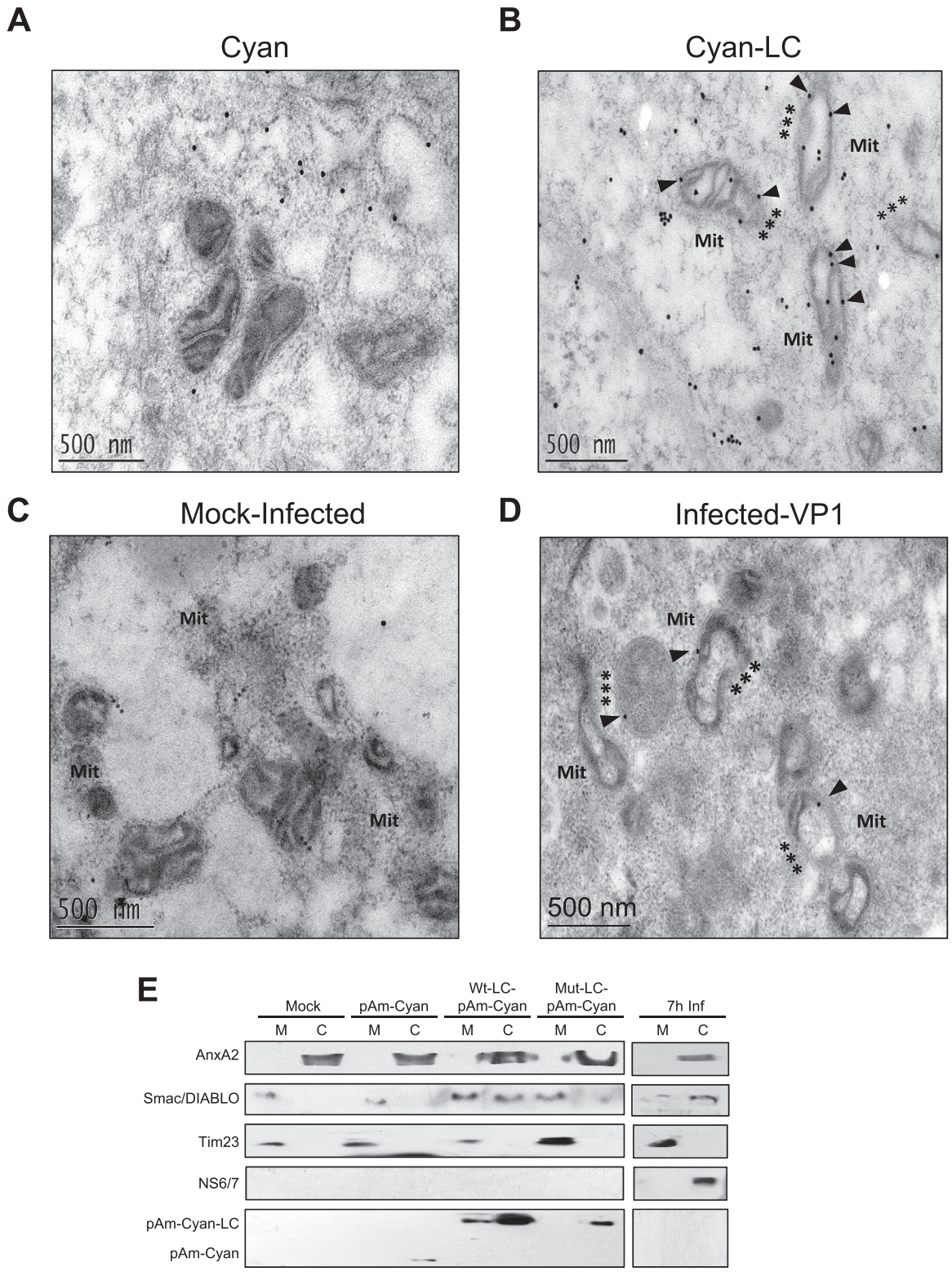

Fig. 6. FCV LC protein interacts with the mitochondria membrane and induces mitochondria membrane damage. Transmission electron microscopy images of the mitochondria integrity from pAm-Cyan (A) and LC-pAm-Cyan (B) transfected cells, mock (C) and FCV infected (D) cells. pAm-Cyan and LC-pAM-Cyan transfected cells show immunogold-labeled Cyan antibody not-associated (A) and associated inside the mitochondria and into the mitochondria membrane (arrowhead) (B). Immunogold-labeled VP1 protein antibodies were used to indicate FCV infection (D). Alterations to the mitochondrial morphology were observed as disintegration of the membrane (marked with ***). E) Mitochondrial and cytoplasmic extracts obtained from pAm-Cyan, Wt-LC-pAm-Cyan and Mut-LC-pAm-Cyan transfected cells and from Mock infected and infected cells for 7 h (controls) were subjected to SDS-PAGE and Smac/DIABLO, subcellular localization was analyzed by western blotting using specific antibodies. AnxA2 and Tim23 were used as cytoplasmic and mitochondrial fraction controls. Cyan antibody was used for detection of Cyan, and Cyan-LC. Anti-mouse and anti-rabbit HRP secondary antibodies were used as indicated, and revealed by chemiluminescence.

Induction of apoptotic morphological and biochemical changes of the mitochondrial pathway occur during MNV and FCV infection in cell culture, such as chromatin condensation, DNA fragmentation, release of cytochrome c into the cytosol, and caspase activation (Bok et al., 2009; Natoni et al., 2006). However, information about the role of pro- and anti-apoptogenic proteins during the progression of apoptosis is very limited. In this regard, Bok et al. (2009) reported that survivin, an inhibitory apoptosis protein is negatively regulated during MNV infection. The authors show that the decrease in survivin RNA and protein is detected from 12 hpi and continues up to 24 hpi. However, it is unknown if downregulation of survivin and other anti-apoptotic proteins is a common mechanism during calicivirus infection. We have found that during FCV, survivin is also downregulated from 5 hpi, suggesting that this downregulation may occur in all members of this family of viruses.

Survivin is a structurally unique IAP protein that has been implicated in protection from apoptosis and regulation of mitosis (Altieri, 2003). As survivin, XIAP is also a critical regulator of cell survival in tumors and in response of cell death stimulation. XIAP protein, is a potent inhibitor of apoptosis and its overexpression induced by different conditions such as serum deprivation and exposure to etoposide, suppresses apoptosis (Gill et al., 2009; Wei et al., 2008). In response to cell death stimulation, survivin physically associates with XIAP, and this complex promotes enhanced XIAP stability and synergistic inhibition of caspase-9 processing/activation, alone or in the context of apoptosome, and block apoptosis in vivo (Dohi et al., 2004). Here we found that survivin downregulation correlates with XIAP downregulation, in concordance with the fact, that in the absence of survivin, XIAP degradation occurs rapidly (Dohi et al., 2004), a basic consequence during apoptosis establishment.

One of the major antagonists of survivin and XIAP proteins is the pro-apoptotic molecule Smac/DIABLO, which as a dimer, sterically and/or competitively occludes caspases-3, − 7 and − 9 binding sites of XIAP (Flanagan et al., 2010), releasing them for driving activation and cell death (Arnt et al., 2002; Gao et al., 2007). Even though Smac/DIABLO is not able to degrade XIAP, its presence in the cytoplasm can induce survivin degradation and XIAP inhibition (McNeish et al., 2005; Yang and Du, 2004). In accordance with this, during FCV infection, we found that degradation of XIAP and survivin correlates with the translocation of Smac/DIABLO from the mitochondrial membrane to the cytosol, with the following activation of caspases activity that leads to apoptosis and viral spread through the host, thereby contributing to a successful infection.

It has been described that following Smac/DIABLO release from the mitochondria, it is rapidly degraded by the proteasome by the action of XIAP, that functions as an ubiquitin-protein ligase in the ubiquitination of Smac/DIABLO (MacFarlane et al., 2002). Thus, it is possible that degradation of XIAP during FCV infection may be another factor that contributes to maintain Smac/DIABLO active to promote the degradation of other IAPs and to allow apoptosis progression. To this regard, the disruption of other anti-apoptotic molecules such as cIAP2 and cIAP1 during FCV apoptosis induction is currently under investigation.

Survivin stabilization using lactacystin, an anti-apoptotic compound that inhibits the proteasome, an thus the degradation of several members of the IAP family (Yang et al., 2000; Zhao et al., 2000), did not affected significantly FCV particles production; however, virus release from infected cells was reduced up to 7 hpi, corroborating previous reports from different research groups that indicate that apoptosis is involved in virus spread (Olsen et al., 1996; Rodríguez-Grille et al., 2014). Moreover, overexpression of survivin in CrFK cells, affected not only virus release, but also viral production; the particular decrease of viral proteins in this particular context, suggest that the overexpression of survivin may have a negative role in early stages of the infection. Weather survivin overexpression affects viral translation, or virus binding or entry is currently under investigation.

Once we corroborated that survivin and XIAP are downregulated during FCV infection, it was of our interest to determine which viral molecule was responsible of this downregulation. Two of the most interesting molecules to study were the 1) the protease-polymerase NS6/7, since viral proteases are commonly responsible for apoptosis regulation (Barco et al., 2000; Zaragoza et al., 2006); and 2) the LC protein, since it is essential for the production of viruses with characteristic CPE, which causes cell rounding in CrFK cells and the generation of rapidly spreading virus, and causes activation of caspases (Abente et al., 2013).

Transfection of NS6/7 active protein resulted in a disruption of the CrFK cell monolayer; however, it did not cause the typical rounding of CrFK cells showed in FCV infection (Abente et al., 2013). Moreover, NS6/7 transfection did not caused a reduction in the amount of survivin and XAIP; neither caspase activation and PARP degradation, indicating that NS6/7 does not cause apoptosis as a single viral protein, in contrast with other proteases (Barco et al., 2000; Chau et al., 2007; Li et al., 2002; Lin et al., 2014; Shafee and AbuBakar, 2003; Zaragoza et al., 2006). On the other hand, the expression of LC protein caused the disruption of the monolayer and the typical rounding of CrFK cells observed during FCV infection, as previously described (Abente et al., 2013); however, the mut-C40A-LC, did not cause the monolayer disruption although a partial cell rounding was observed. WT-LC but not mut-C40A-LC expression caused the change of Smac/DIABLO subcellular localization from the mitochondria to the cytoplasm and the downregulation of survivin and XIAP in concordance with caspase-3 and PARP processing. These results together with the presence of the LC in the mitochondria membrane and inside of the mitochondria, as well as the mitochondrial damage observed by EM strongly indicate that as a single protein, LC is responsible of apoptosis induction. Moreover, the CPE-inducing activity of LC and its apoptosis-inducing ability are dependent from each other.

The induction of intrinsic apoptosis is a common mechanism during calicivirus infection. Even though several parameters of apoptosis have been documented to occur during infection with members of different genera in the *Caliciviridae* family (Alonso et al., 1998; Bok et al., 2009; Jung et al., 2000; Natoni et al., 2006; Roberts et al., 2003; Sosnovtsev et al., 2003), the downregulation of the anti-apoptotic protein survivin had only been documented in MNV infection (Bok et al., 2009; Herod et al., 2014). Here, we show that both survivin and XIAP are downregulated during FCV infection, and during LC expression; thus, it is very likely that the downregulation of anti-apoptogenic proteins, and particularly survivin, that contribute to apoptosis induction, may be a common feature for all the members of the *Caliciviridae* family. Regarding the molecules involved in apoptosis establishment, LC protein is produced in the FCV infected cells as the consequence of the processing of the precursor capsid protein VP1, encoded from ORF2, a genetic feature unique to members of the genus; thus indicating that the viral proteins/molecules responsible for apoptosis induction in the distinct members of the *Caliciviridae* family must be different. In this regard, survivin downregulation and apoptosis induction in MNV infection is caused by the expression of ORF1 polyprotein (Herod et al., 2014), while the human norovirus GII-NTPase posses a pro-apoptotic activity, that can be enhanced by co-expression with Nterm or p22 protein (Yen et al., 2018).

These results taken together demonstrate that LC is responsible of apoptosis induction in transfected cells. Knowledge of specific viral proteins responsible for apoptosis induction that involve downregulation of molecules such as survivin and XIAP, is important, not only for understanding virus biology, but also to understand the importance of targeting multiple IAPs, such as survivin and XIAP, to enhance sensitivity of a variety of cancer types to apoptosis.

Acknowledgements

We thank Patricia Talamás, Rosa del Angel and Juan Ludert for helpful suggestions and Raul Bonilla for technical support. This

research was supported by Consejo Nacional de Ciencia y Tecnología. Country: México (CONACYT): Project number CB02500696.

References

Abente, E.J., Sosnovtsev, S.V., Sandoval-Jaime, C., Parra, G.I., Bok, K., Green, K.Y., 2013. The feline calicivirus leader of the capsid protein is associated with cytopathic effect. J. Virol. 87, 3003–3017.

Adrain, C., Creagh, E.M., Martin, S.J., 2001. Apoptosis-associated release of Smac/DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J. 20, 6627–6636.

Al-Molawi, N., Beardmore, V.A., Carter, M.J., Kass, G.E.N., Roberts, L.O., 2003. Caspase-mediated cleavage of the feline calicivirus capsid protein. J. General. Virol. 84, 1237–1244.

Alonso, C., Oviedo, J.M., Martín-Alonso, J.M., Díaz, E., Boga, J.A., Parra, F., 1998. Programmed cell death in the pathogenesis of rabbit hemorrhagic disease. Arch. Virol. 143, 321–332.

Altieri, D.C., 2003. Validating survivin as a cancer therapeutic target. Nat. Rev. Cancer 3, 46–54.

Alvarez-Sanchez, C., Cancio-Lonches, C., Mora-Heredia, J.E., Santos-Valencia, J.C., Barrera-Vazquez, O.S., Yocupicio-Monroy, M., Gutierrez-Escolano, A.L., 2015. Negative effect of heat shock on feline calicivirus release from infected cells is associated with the control of apoptosis. Virus Res. 198, 44–52.

Arnt, C.R., Chiorean, M.V., Heldebrant, M.P., Gores, G.J., Kaufmann, S.H., 2002. Synthetic Smac/DIABLO peptides enhance the effects of chemotherapeutic agents by binding XIAP and cIAP1 in situ. J. Biol. Chem. 277, 44236–44243.

Atmar, R.L., 2010. Noroviruses - State of the Art. Food Environ. Virol. 2, 117–126.

Barco, A., Feduchi, E., Carrasco, L., 2000. Poliovirus protease 3C(pro) kills cells by apoptosis. Virology 266, 352–360.

Bok, K., Prikhodko, V.G., Green, K.Y., Sosnovtsev, S.V., 2009. Apoptosis in murine norovirus-infected RAW264.7 cells is associated with downregulation of survivin. J. Virol. 83, 3647–3656.

Chau, D.H., Yuan, J., Zhang, H., Cheung, P., Lim, T., Liu, Z., Sall, A., Yang, D., 2007. Coxsackievirus B3 proteases 2A and 3C induce apoptotic cell death through mitochondrial injury and cleavage of eIF4GI but not DAP5/p97/NAT1. Apoptosis 12, 513–524.

Chen, J., Wu, W., Tahir, S.K., Kroeger, P.E., Rosenberg, S.H., Cowsert, L.M., Bennett, F., Krajewski, S., Krajewska, M., Welsh, K., Reed, J.C., Ng, S.C., 2000. Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. Neoplasia 2, 235–241.

Conway, E.M., Zwerts, F., Van Eygen, V., DeVriese, A., Nagai, N., Luo, W., Collen, D., 2003. Survivin-dependent angiogenesis in ischemic brain: molecular mechanisms of hypoxia-induced up-regulation. Am. J. Pathol. 163, 935–946.

Deveraux, Q.L., Leo, E., Stennicke, H.R., Welsh, K., Salvesen, G.S., Reed, J.C., 1999. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. EMBO J. 18, 5242–5251.

Dohi, T., Okada, K., Xia, F., Wilford, C.E., Samuel, T., Welsh, K., Marusawa, H., Zou, H., Armstrong, R., Matsuzawa, S., Salvesen, G.S., Reed, J.C., Altieri, D.C., 2004. An IAP-IAP complex inhibits apoptosis. J. Biol. Chem. 279, 34087–34090.

Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495–516.

Escobar-Herrera, J., Medina-Ramirez, F.J., Gutierrez-Escolano, A.L., 2007. A carboxymethyl-cellulose plaque assay for feline calicivirus. J. Virol. Methods 146, 393–396.

Farkas, T., Sestak, K., Wei, C., Jiang, X., 2008. Characterization of a rhesus monkey calicivirus representing a new genus of Caliciviridae. J. Virol. 82, 5408–5416.

Fernández, J.G., Rodríguez, D.A., Valenzuela, M., Calderon, C., Urzúa, U., Munroe, D., Rosas, C., Lemus, D., Díaz, N., Wright, M.C., 2014. Survivin expression promotes VEGF-induced tumor angiogenesis via PI3K/Akt enhanced β-catenin/Tcf-Lef dependent transcription. Mol. Cancer 13, 209.

Flanagan, L., Sebastia, J., Tuffy, L.P., Spring, A., Lichawska, A., Devocelle, M., Prehn, J.H., Rehm, M., 2010. XIAP impairs Smac release from the mitochondria during apoptosis. Cell Death Dis. 1, e49.

Gao, Z., Tian, Y., Wang, J., Yin, Q., Wu, H., Li, Y.M., Jiang, X., 2007. A dimeric Smac/Diablo peptide directly relieves caspase-3 inhibition by XIAP - Dynamic and cooperative regulation of xiap by Smac/Diablo. J. Biol. Chem. 282, 30718–30727.

Gill, C., Dowling, C., O'Neill, A.J., Watson, R.W.G., 2009. Effects of cIAP-1, cIAP-2 and XIAP triple knockdown on prostate cancer cell susceptibility to apoptosis, cell survival and proliferation. Mol. Cancer 8.

Hehlgans, S., Petraki, C., Reichert, S., Cordes, N., Rödel, C., Rödel, F., 2013. Double targeting of survivin and XIAP radiosensitizes 3D grown human colorectal tumor cells and decreases migration. Radiother. Oncol. 108, 32–39.

Hernandez, B.A., Sandoval-Jaime, C., Sosnovtsev, S.V., Green, K.Y., Gutierrez-Escolano, A.L., 2016. Nucleolin promotes in vitro translation of feline calicivirus genomic RNA. Virology 489, 51–62.

Herod, M.R., Salim, O., Skilton, R.J., Prince, C.A., Ward, V.K., Lambden, P.R., Clarke, I.N., 2014. Expression of the murine norovirus (MNV) ORF1 polyprotein is sufficient to induce apoptosis in a virus-free cell model. PLos One 9.

Huang, Y., Park, Y.C., Rich, R.L., Segal, D., Myszka, D.G., Wu, H., 2001. Structural basis of caspase inhibition by XIAP: differential roles of the linker versus the BIR domain. Cell 104, 781–790.

Jung, J.Y., Lee, B.J., Tai, J.H., Park, J.H., Lee, Y.S., 2000. Apoptosis in rabbit haemorrhagic disease. J. Comp. Pathol. 123, 135–140.

Kawasaki, H., Toyoda, M., Shinohara, H., Okuda, J., Watanabe, I., Yamamoto, T., Tanaka, K., Tenjo, T., Tanigawa, N., 2001. Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. Cancer 91, 2026–2032.

Koike, H., Morikawa, Y., Sekine, Y., Matsui, H., Shibata, Y., Suzuki, K., 2011. Survivin is associated with cell proliferation and has a role in 1a, 25-dihydroxyvitamin D3 induced cell growth inhibition in prostate cancer. J. Urol. 185, 1497–1503.

L'Homme, Y., Sansregret, R., Plante-Fortier, E., Lamontagne, A.M., Ouardani, M., Lacroix, G., Simard, C., 2009. Genomic characterization of swine caliciviruses representing a new genus of caliciviridae. Virus Genes 39, 66–75.

Li, M.-L., Hsu, T.-A., Chen, T.-C., Chang, S.-C., Lee, J.-C., Chen, C.-C., Stollar, V., Shih, S.-R., 2002. The 3C protease activity of enterovirus 71 induces human neural cell apoptosis. Virology 293, 386–395.

Li, S., Yang, Y., Ding, Y., Tang, X., Sun, Z., 2017. Impacts of survivin and caspase-3 on apoptosis and angiogenesis in oral cancer. Oncol. Lett. 14, 3774–3779.

Li, Y., Zhou, Y., Zheng, J., Niu, C., Liu, B., Wang, M., Fang, H., Hou, C., 2015. Downregulation of survivin inhibits proliferation and migration of human gastric carcinoma cells. Int. J. Clin. Exp. Pathol. 8, 1731.

Li, Z., Ren, W., Zeng, Q., Chen, S., Zhang, M., Zhao, Y., Cheng, J., Wang, X., 2016. Effects of survivin on angiogenesis in vivo and in vitro. Am. J. Transl. Res. 8, 270.

Lin, J.-C., Lin, S.-C., Chen, W.-Y., Yen, Y.-T., Lai, C.-W., Tao, M.-H., Lin, Y.-L., Miaw, S.-C., Wu-Hsieh, B.A., 2014. Dengue viral protease interaction with NF-κB inhibitor α/β results in endothelial cell apoptosis and hemorrhage development. J. Immunol. 193, 1258–1267.

Lopman, B.A., Steele, D., Kirkwood, C.D., Parashar, U.D., 2016. The vast and varied global burden of norovirus: prospects for prevention and control. PLoS Med. 13, e1001999.

MacFarlane, M., Merrison, W., Bratton, S.B., Cohen, G.M., 2002. Proteasome-mediated degradation of Smac during apoptosis: xiap promotes Smac ubiquitination in vitro. J. Biol. Chem. 277, 36611–36616.

McIlwain, D.R., Berger, T., Mak, T.W., 2013. Caspase functions in cell death and disease. Cold Spring Harb. Perspect. Biol. 5, a008656.

McKenzie, J.A., Liu, T., Goodson, A., Grossman, D., 2010. Survivin enhances motility of melanoma cells by supporting Akt activation and α5 integrin upregulation. Cancer Res. 0194, 2010.

McNeish, I.A., Lopes, R., Bell, S.J., McKay, T.R., Fernandez, M., Lockley, M., Wheatley, S.P., Lemoine, N.R., 2005. Survivin interacts with Smac/DIABLO in ovarian carcinoma cells but is redundant in Smac-mediated apoptosis. Exp. Cell Res. 302, 69–82.

Monroe, S.S., 2011. Control and prevention of viral gastroenteritis. Emerg. Infect. Dis. 17, 1347–1348.

Nakao, K., Hamasaki, K., Ichikawa, T., Arima, K., Eguchi, K., Ishii, N., 2006. Survivin downregulation by siRNA sensitizes human hepatoma cells to TRAIL-induced apoptosis. Oncol. Rep. 16, 389–392.

Natoni, A., Kass, G.E.N., Carter, M.J., Roberts, L.O., 2006. The mitochondrial pathway of apoptosis is triggered during feline calicivirus infection. J. General. Virol. 87, 357–361.

Niedzwiedzka-Rystwej, P., Deptula, W., 2012. Apoptosis of peripheral blood leukocytes from rabbits infected with non-haemagglutinating strains of rabbit haemorrhagic disease virus (RHDV). Vet. Immunol. Immunopathol. 149, 54–57.

Olsen, C.W., Kehren, J.C., Dybdahl-Sissoko, N.R., Hinshaw, V.S., 1996. bcl-2 alters influenza virus yield, spread, and hemagglutinin glycosylation. J. Virol. 70, 663–666.

Pyrko, P., Soriano, N., Kardosh, A., Liu, Y.T., Uddin, J., Petasis, N.A., Hofman, F.M., Chen, C.S., Chen, T.C., Schonthal, A.H., 2006. Downregulation of survivin expression and concomitant induction of apoptosis by celecoxib and its non-cyclooxygenase-2-inhibitory analog, dimethyl-celecoxib (DMC), in tumor cells in vitro and in vivo. Mol. Cancer 5.

Roberts, L.O., Al-Molawi, N., Carter, M.J., Kass, G.E.N., 2003. Apoptosis in cultured cells infected with feline calicivirus. Apoptosis: Signal. Pathw. Ther. Tools 1010, 587–590.

Rodríguez-Grille, J., Busch, L.K., Martínez-Costas, J., Benavente, J., 2014. Avian reovirus-triggered apoptosis enhances both virus spread and the processing of the viral nonstructural muNS protein. Virology 462, 49–59.

Salvesen, G.S., Duckett, C.S., 2002. IAP proteins: blocking the road to death's door. Nat. Rev. Mol. Cell Biol. 3, 401–410.

Sanhueza, C., Wehinger, S., Bennett, J.C., Valenzuela, M., Owen, G., Quest, A., 2015. The twisted survivin connection to angiogenesis. Mol. Cancer 14, 198.

Shafee, N., AbuBakar, S., 2003. Dengue virus type 2 NS3 protease and NS2B-NS3 protease precursor induce apoptosis. J. General. Virol. 84, 2191–2195.

Shiraki, K., Sugimoto, K., Fujikawa, K., Yamanaka, T., Takase, K., Nakano, T., 2000. Survivin promotes cell proliferation in human hepatocellular carcinoma. Gastroenterology 118, A906.

Smits, S.L., Rahman, M., Schapendonk, C.M.E., van Leeuwen, M., Faruque, A.S.G., Haagmans, B.L., Endtz, H.P., Osterhaus, A.D.M.E., 2012. Calicivirus from novel recovirus genogroup in human diarrhea, Bangladesh. Emerg. Infect. Dis. 18, 1192–1195.

Sosnovtsev, S.V., Prikhod'ko, E.A., Belliot, G., Cohen, J.I., Green, K.Y., 2003. Feline calicivirus replication induces apoptosis in cultured cells. Virus Res. 94, 1–10.

Sosnovtsev, S.V., Sosnovtseva, S.A., Green, K.Y., 1998. Cleavage of the feline calicivirus capsid precursor is mediated by a virus-encoded proteinase. J. Virol. 72, 3051–3059.

Tayyari, F., Marchant, D., Moraes, T.J., Duan, W., Mastrangelo, P., Hegele, R.G., 2011. **Identification of nucleolin as a cellular receptor for human** respiratory syncytial virus. Nat. Med. 17, 1132–1135.

Thiel, H.-J., König, M., 1999. Caliciviruses: an overview. Vet. Microbiol. 69, 55–62.

Vaux, D.L., Silke, J., 2005. IAPs, RINGs and ubiquitylation. Nat. Rev. Mol. Cell Biol. 6, 287.

Wei, Y.B., Fan, T.J., Yu, M.M., 2008. Inhibitor of apoptosis proteins and apoptosis. Acta Biochim. Biophys. Sin. 40, 278–288.

Willcocks, M.M., Carter, M.J., Roberts, L.O., 2004. Cleavage of eukaryotic initiation factor eIF4G and inhibition of host-cell protein synthesis during feline calicivirus infection.

J. Gen. Virol. 85, 1125–1130.
Wolf, S., Reetz, J., Otto, P., 2011. Genetic characterization of a novel calicivirus from a chicken. Arch. Virol. 156, 1143–1150.
Yang, Q.H., Du, C., 2004. Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells. J. Biol. Chem. 279, 16963–16970.
Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M., Ashwell, J.D., 2000. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 288, 874–877.
Yen, J.B., Wei, L.H., Chen, L.W., Chen, L.Y., Hung, C.H., Wang, S.S., Chang, P.J., 2018. Subcellular localization and functional characterization of GII.4 norovirus-encoded NTPase. J. Virol. 92.
Zaragoza, C., Saura, M., Padalko, E.Y., Lopez-Rivera, E., Lizarbe, T.R., Lamas, S., Lowenstein, C.J., 2006. Viral protease cleavage of inhibitor of kappaBalpha triggers host cell apoptosis. Proc. Natl. Acad. Sci. USA 103, 19051–19056.
Zhao, J., Tenev, T., Martins, L.M., Downward, J., Lemoine, N.R., 2000. The ubiquitin-proteasome pathway regulates survivin degradation in a cell cycle-dependent manner. J. Cell Sci. 113, 4363–4371.