

ZD Ye 葉仲德
 CK Wong 黃仲佳
 P Li 李 蓬
 Y Xie 謝 雍

The role of SARS-CoV protein, ORF-6, in the induction of host cell death

Key Messages

1. Overexpression of ORF-6 induced apoptosis.
2. Caspase-3 inhibitor and JNK inhibitor blocked ORF-6 induced apoptosis.
3. The protein level of ER chaperon protein, GRP94, was up-regulated when ORF-6 was overexpressed.
4. ORF-6 induced apoptosis via caspase-3-mediated, ER stress and JNK dependent pathways.

Introduction

In 2003, Hong Kong experienced an outbreak of severe acute respiratory syndrome (SARS) and incurred huge economic and social losses. Immunohistochemistry and *in situ* hybridisation of organs from deceased SARS patients revealed that the virus was not only in the lungs and intestines, but also in the liver, distal convoluted renal tubules, sweat glands, parathyroid glands, pituitary gland, pancreas, adrenal glands, and the cerebrum. The aetiological agent for this disease was a novel member of the coronavirus (SARS-CoV) family, with limited sequence homology to other coronaviruses.¹⁻⁸ Coronaviruses are enveloped, plus-stranded RNA viruses that lead to respiratory diseases akin to those caused by avian coronavirus and infectious bronchitis virus. The SARS-CoV encodes 23 putative proteins including four typical structural proteins: the spike, nucleocapsid, membrane, and envelope proteins.^{4,7} These four proteins contribute to the host's immune response, as has been observed with many other transmissible viruses, such as gastroenteritis coronavirus, bronchitis virus, porcine respiratory coronavirus, and mouse hepatitis virus. In addition to the structural proteins, replicase 1a ORF, replicase 1b ORF and eight novel ORFs have been identified.

Eight SARS-CoV-encoded proteins have been shown to induce apoptosis.⁹ These suggest that apoptosis may play an important role in helping with virus dissemination *in vivo*, minimising the inflammatory reaction and evasion of the host's defence mechanisms. Studies of its pathology have revealed diffuse alveolar damage as the most notable feature in persons who died of SARS. In SARS patients, apoptosis occurs in the alveolar epithelial cells. Four SARS-CoV accessory proteins have been shown to induce apoptosis, ORF-3a (also known as U274, SARS X1, or ORF-3), ORF-3b (also known as ORF-4), ORF-7a (also known as U122, SARS X4, or ORF-8) and ORF-8a.¹⁰⁻¹² We report the characterisation of another SARS-CoV group-specific gene product encoded by ORF-6 (also known as X3, ORF-7), which contains 63 amino acids (equivalent to nucleotides 27,074 - 27,265 in Tor2 genome sequence).⁴ This small protein has no significant sequence homology to other proteins, and has been identified as an ER/Golgi membrane localised protein.¹³

We report that (1) there was overexpression of ORF-6 induced apoptosis; (2) caspase-3 was activated in the presence of ORF-6; (3) apoptosis induced by ORF-6 and ORF-7a was blocked by caspase-3 inhibitor, z-DEVD, and the JNK (c-Jun N-terminal kinase) inhibitor; and (4) ORF-6 and ORF-7a up-regulated levels of ER chaperone protein and 94kDa glucose-regulated protein (GRP94). All these features suggest that in addition to ORF-3a, ORF-3b and ORF-7a, ORF-6 is a new player involved in SARS-CoV-induced apoptosis and both ORF-6 and ORF-7a share similar pathways to induce apoptosis.

Results and discussion

Overexpression of ORF-6 protein induces apoptosis

In the search for SARS proteins that could induce apoptosis, ORF-6 and ORF-7a were amplified by polymerase chain reaction, and restriction sites BamHI and XhoI were introduced at the 5' and 3' end, respectively. The fragments of ORF-6 and ORF-7a were sub-cloned into mammalian expression vector GFP-N1. GFP-ORF-6 was transfected into Vero E6 and COS-7 cells. We observed that ORF-6

Hong Kong Med J 2010;16(Suppl 4):S22-6

Department of Biology, Hong Kong University of Science and Technology
 ZD Ye, CK Wong, P Li, Y Xie
 Protein Science Laboratory of the Ministry of Education, Department of Biological Sciences and Biotechnology, Tsinghua University
 ZD Ye, P Li

RFCID project number: 01030832

Principal applicant and corresponding author:
 Dr Yong Xie
 Department of Biology, Hong Kong University of Science and Technology, Hong Kong SAR, China
 Tel: (852) 2358 7340
 Fax: (852) 2358 1559
 Email: boyxie@ust.hk

was able to induce apoptosis when overexpressed in Vero E6 and COS-7 cells (Fig 1). Approximately one third of the cells died when 2 μg of ORF-6 DNA was transfected into cells. The number of apoptotic cells increased when more ORF-6 DNA was transfected into the cells (Fig 1). The death rates were comparable to rates caused by the overexpression of Bax, a well-known pro-apoptotic member of the Bcl-family, and ORF-7a, a SARS protein that has been shown to induce apoptosis.¹¹ GFP-N1 served as the negative control, as the transfection of 5 μg of GFP-N1 DNA did not induce apoptosis. These results showed that the ORF-6 protein did induce apoptosis.

Overexpression of ORF-6-induced apoptosis is caspase-3 dependent

ORF-7a induces apoptosis via a caspase-3-dependent pathway.¹¹ To determine if cell death induced by ORF-6 is also caspase-3 dependent, a caspase-3-specific inhibitor, z-DEVD, was used to block caspase-3 activation in the cells. In the absence of z-DEVD, approximately 60% of the cells underwent apoptosis when ORF-6 and ORF-7a were transiently transfected into the Vero E6 cells. However, when ORF-6 or ORF-7a were overexpressed in z-DEVD pretreated cells, the percentage of apoptotic cells was significantly decreased to approximately 20% (Fig 2a). In

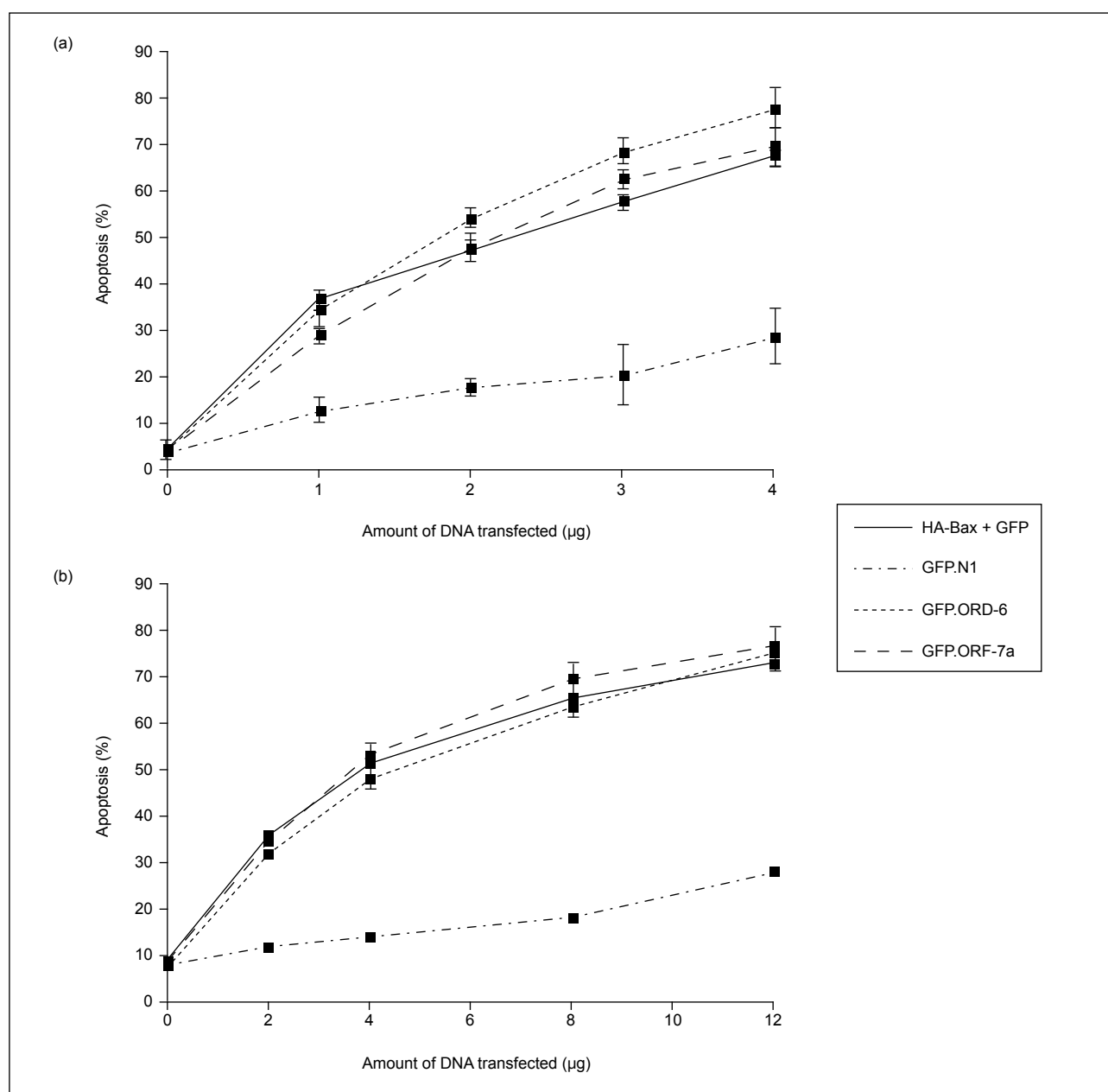


Fig 1. Overexpression of ORF-6-induced apoptosis in different cell lines

(a) COS-7 and (b) Vero E6 cells were transiently transfected with GFP and HA-Bax, GFP-N1, GFP-ORF-6 and GFP-ORF-7a for 24 hours. The nuclei of the cells were stained by Hoechst for 15 minutes. The number of healthy cells was counted under fluorescence microscopy with no DNA condensation and fragmentation. The percentage of apoptotic cells was calculated by the number of healthy cells over the total number of transfected cells. Experiments were repeated three times. Standard deviations are shown

parallel, caspase-3 activities were also monitored when the Vero E6 cells were transfected with ORF-6. The activity of caspase-3 can be detected by the amount of the 17kD active form of caspase-3 in cells by using caspase-3-specific antibodies. Both ORF-6 and ORF-7a induced caspase-3 activation, as the 17kD active form of caspase-3 was detected in both (Fig 2b). Cells without any treatment served as a negative control, and those treated with taurosporine served as a positive control. Similar results were obtained using 293T or COS-7 cells (data not shown). Our observation suggests that overexpression of ORF-6 induced apoptosis via a caspase-3-dependent pathway.

JNK inhibitor blocks ORF-6 and ORF-7a-induced apoptosis

One of the mechanisms for SARS protein-induced cell death is via the JNK pathway; JNK is phosphorylated in SARS-CoV-infected Vero E6 cells, and the JNK inhibitor

(SP600125) can block SARS-CoV-infected Vero E6 cell-induced apoptosis. We investigated whether the JNK inhibitor could block ORF-6- and ORF-7a-induced apoptosis in Vero E6 cells. Interestingly, apoptosis induced by ORF-6 and ORF-7a was blocked by the JNK inhibitor. The blocking efficiency was similar to that of z-DEVD, as only approximately 10% of cells underwent apoptosis (Fig 2c). The JNK inhibitor was also able to block ORF-6 and ORF-7a in 293T and COS-7 cells (data not shown). These results suggest that the JNK inhibitor was able to block overexpression of ORF-6- or ORF-7a-induced apoptosis.

ORF-6 and ORF-7a induce ER stress

ORF-6 is localised in the ER/Golgi membrane, which is consistent with our immunostaining result for ORF-6 and ORF-7a in COS-7 cells (data not shown).¹³ Since both ORF-6 and ORF-7a are ER localised, we suspect that they induce apoptosis through the ER stress pathway.

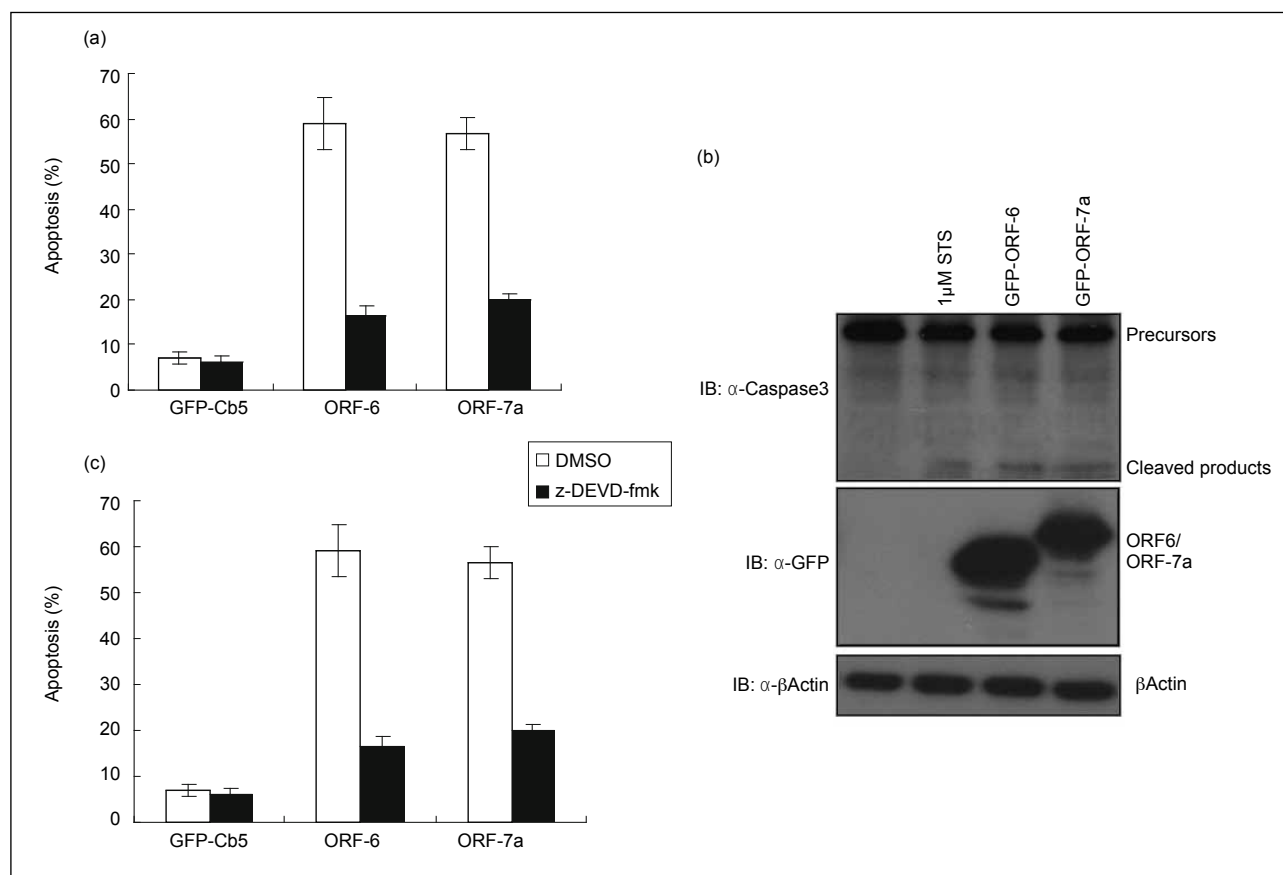


Fig 2. Overexpression of ORF-6-induced apoptosis is caspase-3 and JNK dependent

(a) Caspase-3 inhibitor (z-DEVD) blocks ORF-6-induced apoptosis. Vero E6 cells were incubated with either dimethyl sulfoxide or 50 μ M z-DEVD-fmk for 30 minutes before they were transiently transfected with 3 μ g of GFP-Cb5, GFP-ORF-6 and GFP-ORF-7a for 24 hours. The nuclei of the cells were stained by Hoechst for 15 minutes. The number of healthy cells was counted under fluorescence microscopy with no DNA condensation and fragmentation. The percentage of apoptotic cells was calculated by the number of healthy cells over the total number of transfected cells. Experiments were repeated three times and the standard deviations are shown. (b) Overexpression of ORF-6- and ORF-7a induces caspase-3 activation. Vero E6 cells were transiently transfected with GFP-ORF-6 and GFP-ORF-7a for 24 hours. Cell lysates were normalised to 2 μ g/ml by lysis buffer and subjected to Western Blot with α -GFP, α -caspase-3 and α -Actin. For positive controls, cells were treated with 1 μ M of staurosporine for 8 hours. Cells without any treatment were served as negative control. (c) JNK inhibitor blocked ORF-6-induced apoptosis. Vero E6 cells were incubated with either dimethyl sulfoxide or 40 μ M JNK inhibitor for 30 minutes before they were transiently transfected with 3 μ g of GFP-Cb5, GFP-ORF-6 and GFP-ORF-7a for 24 hours. Cell counts were done as mentioned in (b)

GRP94 is an ER-resident molecular chaperone protein, for which the expression level is increased by ER-stress.¹⁴ We compared the protein level of GRP94 in order to check whether ER stress occurred in cells transfected with ORF-6 and ORF-7a. In such cells, the GRP94 protein level increased compared to cells without transfection (Fig 3a). However, the protein level was lower than in cells treated with 1 μ M Thapsigargin, a chemical specific for induction of ER stress. In parallel, we conducted dose-dependent experiments by transfecting different amounts of ORF-6 and ORF-7a into cells. Consistently, the endogenous GRP94 protein level increased when the amount of ORF-6 or ORF-7 increased (Figs 3b and 3c). Our observation suggests that overexpression of ORF-6 and ORF-7a could induce apoptosis via the ER-stress pathway.

SARS-CoV encodes 23 putative proteins and in this context eight novel ORFs have been identified. Five of these (ORF-3a, ORF-3b, ORF-6, ORF-7a and ORF-8a) induce apoptosis when they are overexpressed in cells.^{10-12,15,16} However, the responsible signalling pathway remains

elusive. It has been shown that in SARS-CoV-infected Vero E6 cells, JNK is phosphorylated and that apoptosis was inhibited by both JNK and PI3K inhibitors.¹⁷ We found that the JNK inhibitor inhibits both ORF-6- and ORF-7a-induced apoptosis. We therefore determined that ORF-6 and ORF-7a in SARS-CoV may be responsible for inducing phosphorylation of JNK, which in turn leads to apoptosis.

Acknowledgments

The study was supported by the Research Fund for the Control of Infectious Diseases, Food and Health Bureau, Hong Kong SAR Government (#01030832). We thank Dr Zhenguo Wu from Hong Kong University of Science and Technology for GFP-ORF6 and GFP-ORF7a constructs.

References

1. Drosten C, Gunther S, Preiser W, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003;348:1967-76.
2. Ksiazek TG, Erdman D, Goldsmith CS, et al. A novel coronavirus

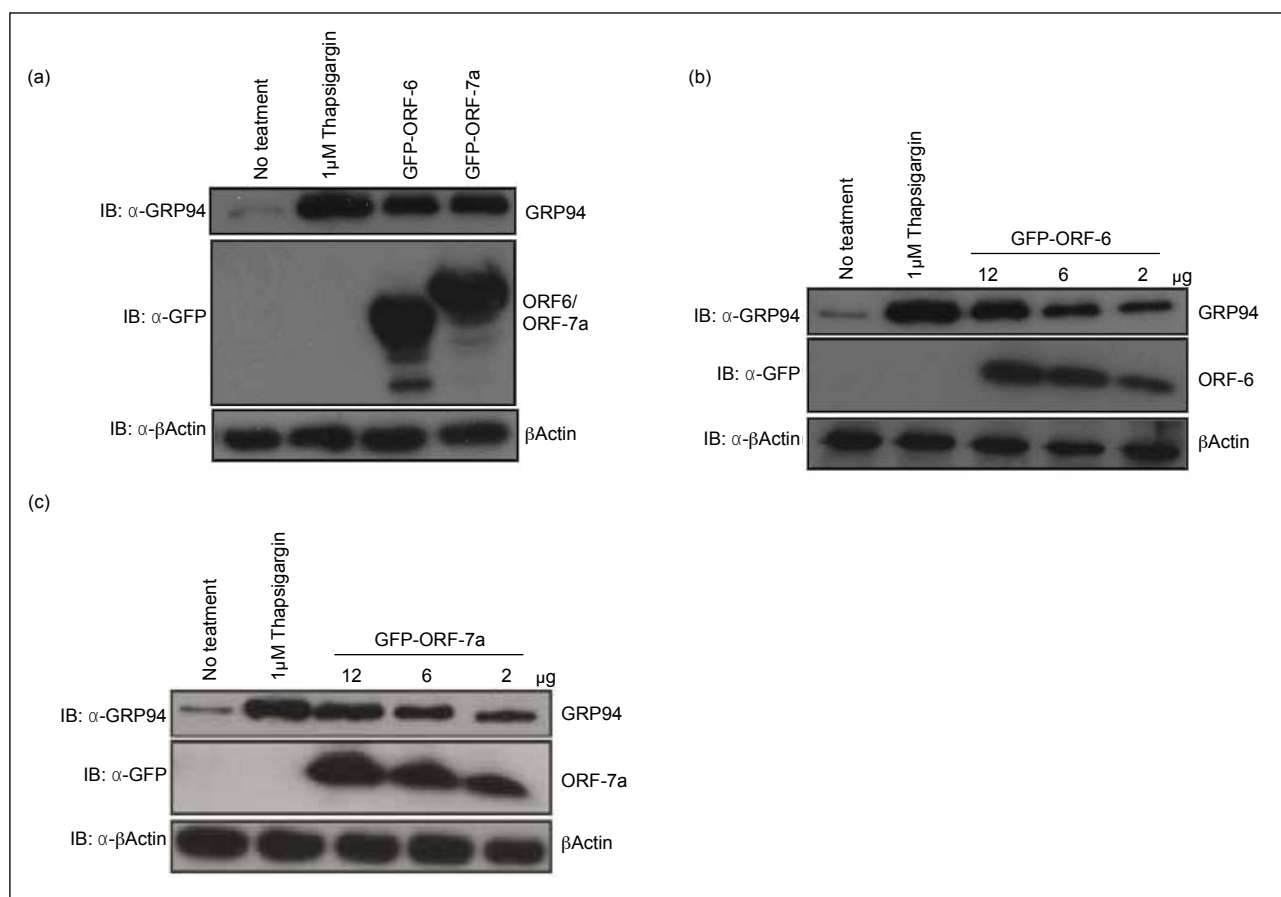


Fig 3. ORF-6 and ORF-7a induces ER stress

(a) Overexpression of ORF-6 and ORF-7a increases endogenous GRP94 protein level. Vero E6 cells were transiently transfected with 6 μ g of GFP-ORF-6 and GFP-ORF-7a for 24 hours. Cell lysates were normalised to 1 μ g/ μ l by lysis buffer and subjected to Western Blot with a-GFP, a-GRP94 and a-Actin. For positive controls, cells were treated with 1 μ M of Thapsigargin for 24 hours. Cells without treatment served as negative controls. (b) and (c) ORF-6 and ORF-7a increase endogenous GRP94 protein level in dose-dependent manner. Vero E6 cells were transiently transfected with 2 μ g, 6 μ g and 12 μ g of GFP-ORF-6 or GFP-ORF-7a for 24 hours. Cell lysates were normalised to 1 μ g/ μ l by lysis buffer and subjected to Western Blot with α -GFP, α -GRP94 and α -Actin. For positive controls, cells were treated with 1 μ M of Thapsigargin for 24 hours. Cells without treatment served as negative controls

- associated with severe acute respiratory syndrome. *N Engl J Med* 2003;348:1953-66.
3. Lee N, Hui D, Wu A, et al. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 2003;348:1986-94.
 4. Marra MA, Jones SJ, Astell CR, et al. The Genome sequence of the SARS-associated coronavirus. *Science* 2003;300:1399-404.
 5. Peiris JS, Chu CM, Cheng VC, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* 2003;361:1767-72.
 6. Poutanen SM, Low DE, Henry B, et al. Identification of severe acute respiratory syndrome in Canada. *N Engl J Med* 2003;348:1995-2005.
 7. Rota PA, Oberste MS, Monroe SS, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 2003;300:1394-9.
 8. Tsang KW, Ho PL, Ooi GC, et al. A cluster of cases of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 2003;348:1977-85.
 9. Tan YJ, Lim SG, Hong W. Regulation of cell death during infection by the severe acute respiratory syndrome coronavirus and other coronaviruses. *Cell Microbiol* 2007;9:2552-61.
 10. Fielding BC, Tan YJ, Shuo S, et al. Characterization of a unique group-specific protein (U122) of the severe acute respiratory syndrome coronavirus. *J Virol* 2004;78:7311-8.
 11. Tan YJ, Fielding BC, Goh PY, et al. Overexpression of 7a, a protein specifically encoded by the severe acute respiratory syndrome coronavirus, induces apoptosis via a caspase-dependent pathway. *J Virol* 2004;78:14043-7.
 12. Chen CY, Ping YH, Lee HC, et al. Open reading frame 8a of the human severe acute respiratory syndrome coronavirus not only promotes viral replication but also induces apoptosis. *J Infect Dis* 2007;196:405-15.
 13. Frieman M, Yount B, Heise M, Kopecky-Bromberg SA, Palese P, Baric RS. Severe acute respiratory syndrome coronavirus ORF6 antagonizes STAT1 function by sequestering nuclear import factors on the rough endoplasmic reticulum/Golgi membrane. *J Virol* 2007;81:9812-24.
 14. Zhang K, Kaufman RJ. Signaling the unfolded protein response from the endoplasmic reticulum. *J Biol Chem* 2004;279:25935-8.
 15. Law PT, Wong CH, Au TC, et al. The 3a protein of severe acute respiratory syndrome-associated coronavirus induces apoptosis in Vero E6 cells. *J Gen Virol* 2005;86:1921-30.
 16. Yuan X, Shan Y, Zhao Z, Chen J, Cong Y. G0/G1 arrest and apoptosis induced by SARS-CoV 3b protein in transfected cells. *Virol J* 2005;2:66.
 17. Mizutani T, Fukushi S, Murakami M, et al. Tyrosine dephosphorylation of STAT3 in SARS coronavirus-infected Vero E6 cells. *FEBS Lett* 2004;577:187-92.