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RESEARCH PAPER

## The inhibition of the apoptosis pathway by the *Coxiella burnetii* effector protein CaeA requires the EK Repetition motif, but is independent of survivin

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### ABSTRACT

*Coxiella burnetii* is an obligate intracellular bacterium that causes Query (Q) fever, a zoonotic disease. It requires a functional type IV secretion system (T4SS) which translocate bacterial effector proteins into the host cell cytoplasm and thereby facilitates bacterial replication. To date, more than 130 effector proteins have been identified, but their functions remain largely unknown. Recently, we demonstrated that one of these proteins, CaeA (CBU1524) localized to the host cell nucleus and inhibited intrinsic apoptosis of HEK293 or CHO cells. In the present study we addressed the question whether CaeA also affects the extrinsic apoptosis pathway.

Ectopic expression of CaeA reduced extrinsic apoptosis and prevented the cleavage of the executioner caspase 7, but did not impair the activation of initiator caspase 9. CaeA expression resulted in an up-regulation of survivin (an inhibitor of activated caspases), which, however, was not causal for the anti-apoptotic effect of CaeA. Comparing the sequence of CaeA from 25 different *C. burnetii* isolates we identified an EK (glutamic acid/ lysine) repetition motif as a site of high genetic variability. The EK motif of CaeA was essential for the anti-apoptotic activity of CaeA. From these data, we conclude that the *C. burnetii* effector protein CaeA interferes with the intrinsic and extrinsic apoptosis pathway. The process requires the EK repetition motif of CaeA, but is independent of the upregulated expression of survivin.

### ARTICLE HISTORY

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apoptosis; bacterial pathogenesis; *Coxiella burnetii*; survivin; type IV secretion system

### Introduction

*C. burnetii* is a Gram-negative, obligate intracellular bacterium which is the causative agent of Q fever, a zoonotic disease with worldwide prevalence except for New Zealand.<sup>1</sup> Humans get infected by inhalation of infectious material transmitted from domestic livestock. Infection by a single bacterium can result in disease.<sup>2</sup> Most frequently, Q-fever remains asymptomatic or causes only a mild flu-like illness. However, the infection can also lead to interstitial pneumonia, hepatitis or severe chronic disease, which usually presents in form of an endocarditis.<sup>1</sup> Due to the increase in worldwide Q-fever cases over the last decade *C. burnetii* is considered as an emerging pathogen.<sup>3</sup>

Once *C. burnetii* has entered the human body by inhalation it is taken up by mononuclear phagocytes into a phagosome.<sup>4</sup> The *C. burnetii*-containing vacuole (CCV) matures to an acidic, phagolysosomal-like

parasitophorous vacuole that is permissive for bacterial replication.<sup>5–7</sup> *C. burnetii* requires a functional Dot/Icm type IV secretion system (T4SS) for the establishment of the replicative CCV.<sup>8–10</sup> The T4SS translocate bacterial virulence factors, termed effector proteins, into the host cell cytoplasm.<sup>11</sup> Importantly, it was shown that *C. burnetii* requires the T4SS to prevent host cell death.<sup>9</sup> To date, more than 130 putative *C. burnetii* effector proteins have been identified,<sup>12</sup> some of which have anti-apoptotic activity,<sup>13–15</sup> whereas the majority still awaits functional characterization.

Apoptosis, a form of programmed cell death, is part of the intrinsic immune defense.<sup>16</sup> It allows removal of damaged or infected cells in the absence of inflammation<sup>17</sup> and is a central mechanism of peripheral immune tolerance.<sup>18</sup> Two main pathways lead to apoptosis induction: The extrinsic and the intrinsic apoptosis pathway. The extrinsic pathway is triggered by ligand binding to

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death receptors and adaptor proteins, which activates caspases (cysteinyll aspartate proteases). The intrinsic pathway involves activation of Bax and Bak, which is regulated by the Bcl-2 protein family.<sup>19,20</sup> This protein family comprises both positive (BH3-only) and negative (Bcl-2-like) regulators of apoptosis. The ratio of positive and negative regulators expressed in a cell determines whether Bax and Bak are activated. Once activated, Bax and Bak oligomerize and permeabilize the mitochondrial membrane, resulting in the release of small molecules like cytochrome c and activation of caspase 9 through assembly of the apoptosome.<sup>21</sup> Activated caspase 9 leads to cleavage and, thus, activation of the key downstream executors of apoptosis: caspase 3 and caspase 7.<sup>22</sup> As proteolysis is irreversible, activation of caspases must be tightly regulated. One known mechanism deployed to protect the cell from death receptor and mitochondrial apoptosis is the inhibition of activated caspases by inhibitor of apoptosis proteins (IAPs). In humans the IAPs comprise 8 family members: NAIP, cIAP1, cIAP2, XIAP, survivin, Bruce, ML-IAP and ILP2.<sup>23</sup> While it was first believed that all of the IAPs can bind caspases, we now know that *in vivo* only XIAP functions as a physiological inhibitor of caspases.<sup>24,25</sup> The other IAPs require the interaction with cooperative partners to inhibit caspases and thereby apoptosis.<sup>24</sup>

Several pathogens have evolved mechanisms to modulate host cell apoptosis, which has emerged as a crucial determinant of virulence. Some bacteria actively induce host cell death to escape from an microbicidal, phagosomal environment or to overcome barriers.<sup>26,27</sup> In contrast, obligate intracellular pathogens frequently inhibit apoptosis (e. g. by use of T3SS or T4SS effector proteins) to prevent premature host cell death and to generate a habitat for replication.<sup>28,29</sup> *C. burnetii* is no exception as it utilizes its T4SS to inhibit host cell apoptosis.<sup>9</sup> So far, 3 anti-apoptotic effector proteins (AnkG, CaeA and CaeB) have been identified.<sup>14,15,30</sup> However, their precise molecular activity has still to be unraveled.

The *C. burnetii* effector protein CaeA localizes to the nucleus when produced in mammalian cells<sup>8</sup> and displays anti-apoptotic activity.<sup>14</sup> In this study we found that CaeA does not interfere with the activation of caspase 9 but prevents the cleavage of caspase 7, indicating that CaeA interferes with the apoptotic cascade between the initiator caspase 9 and the executioner caspase 7. Interestingly, CaeA expression resulted in an upregulation of survivin, an IAP known to inhibit activated caspases. However, siRNA experiments revealed that the anti-apoptotic activity of *C. burnetii* was not mechanistically linked to the increased level of survivin. By comparing the sequence of CaeA from 25 *C. burnetii* isolates we identified an EK repetition motif, consisting of

alternating glutamic acid and lysine residues, as a site of genetic variability. The EK motif of CaeA was required for anti-apoptotic activity, but not for the intracellular localization of CaeA.

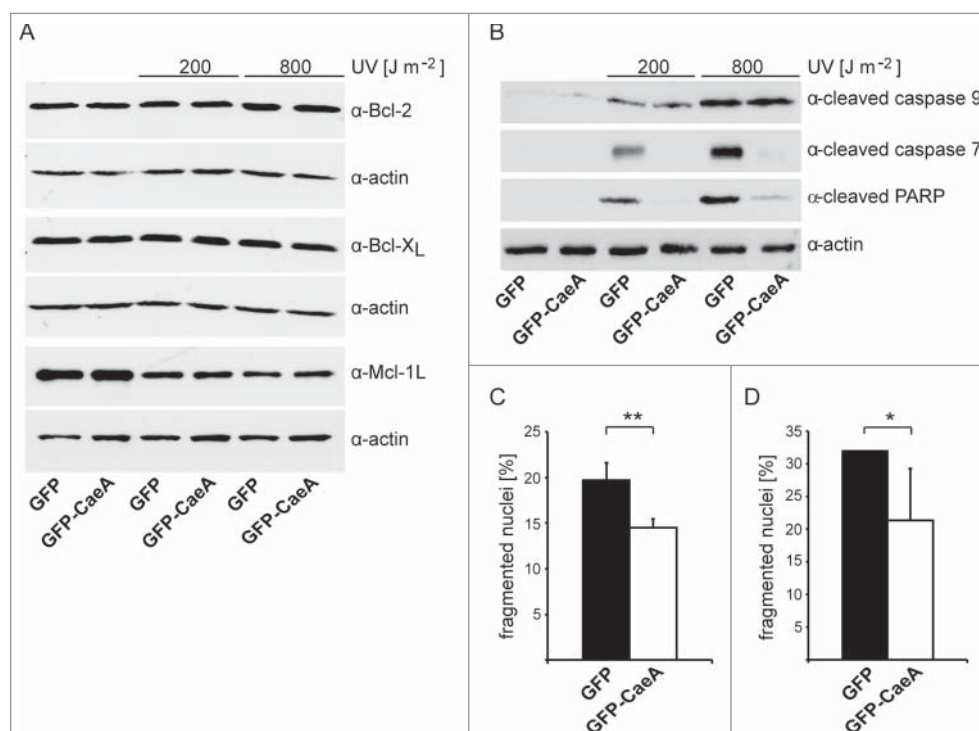
## Results

### *CaeA expression prevents apoptosis at the executioner caspase level*

An increase in the anti-apoptotic Bcl-2-like steady-state protein level results in cell survival. Therefore, we analyzed whether the expression of CaeA (derived from the *C. burnetii* Nine Mile [NM] II strain) results in an altered protein level of anti-apoptotic Bcl-2-family members. Thus, HEK293 cells stably expressing GFP or GFP-CaeA were treated with different UV-light intensities. UV-light induces DNA damage and subsequently intrinsic apoptosis.<sup>31</sup> Immunoblot analysis revealed a similar relative abundance of the anti-apoptotic Bcl-2-like proteins Bcl-2 and Bcl-x<sub>L</sub> under all conditions tested (Fig. 1A). In contrast, we detected decreased Mcl-1 protein levels after UV-light treatment, which, however was also true for both GFP-CaeA expressing and GFP control cells. Next, we analyzed whether CaeA inhibits activation of initiator and executioner caspases after UV-light treatment. Apoptosis-induction was measured by assaying the presence of cleaved poly ADP-ribose polymerase (PARP). Proteolytic cleavage of nuclear PARP inactivates DNA repair activity and is a marker for the terminal stages of apoptosis.<sup>32</sup> As shown in Figure 1B, GFP-expressing cells treated with UV-light displayed caspase 9 and caspase 7 cleavage as well as PARP cleavage. In contrast, cells expressing GFP-CaeA were refractory to UV-induced proteolytic cleavage of caspase 7 and PARP, but not to proteolytic cleavage of caspase 9. This result suggests that CaeA interferes with the apoptotic cascade downstream of caspase 9 activation and upstream of caspase 7 activation.

### *CaeA also inhibits extrinsic apoptosis*

As our data suggested that CaeA inhibits apoptosis at the executioner caspase level, we assumed that CaeA not only inhibits intrinsic apoptosis but also extrinsic apoptosis. Fas-ligand and tumor necrosis factor (TNF) in combination with cycloheximide (CHX) are known inducers of the extrinsic apoptosis pathway.<sup>33</sup> Thus, we induced extrinsic apoptosis with TNF and CHX in HeLa cells ectopically producing GFP or GFP-CaeA and measured nuclear fragmentation visualized by DAPI staining. This assay was used as it is a fast and robust assay. Importantly, we used the TUNEL assay to validate our nuclear fragmentation assay. As shown



**Figure 1.** CaeA prevents activation of executioner caspase 7. HEK293 cells stably expressing GFP or GFP-CaeA were exposed to UV-light ( $200 \text{ J/m}^2$  or  $800 \text{ J/m}^2$ ) and incubated for 6 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and probed with antibodies against (a) Bcl-2, Bcl- $\text{x}_\text{L}$ , Mcl-1 and actin as loading control or (b) cleaved caspase 9, cleaved caspase 7, cleaved PARP and actin as loading control. The result of one representative experiment out of 3 independent experiments with similar results is shown. (c) HeLa cells were transiently transfected with plasmids encoding GFP as control or GFP-CaeA followed by treatment with TNF ( $20 \text{ ng/ml}$ ) and cycloheximide ( $6 \mu\text{g/ml}$ ) for 5 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . The cells were fixed, permeabilized and the nuclei were stained with DAPI. The nuclear morphology of GFP-expressing cells was scored. Data represent average values  $\pm$  SD of 100 nuclei counted per sample from GFP-expressing cells from 3 independent experiments. (d) HeLa-Fas cells were transiently transfected with plasmids encoding GFP as control or GFP-CaeA followed by treatment with  $0.125 \mu\text{g/ml}$  anti-Fas IgG for 5 h. The cells were fixed, permeabilized and the nuclei were stained with DAPI. The nuclear morphology of GFP-expressing cells was scored. Data represent average values  $\pm$  SD of 100 nuclei counted per sample from GFP-expressing cells from 3 independent experiments.

in Figure 1C the expression of GFP-CaeA protected the cells significantly from TNF plus CHX-induced apoptosis compared to cells expressing GFP alone. To determine whether CaeA expression also inhibits Fas-ligand induced apoptosis, HeLa cells stably expressing the Fas receptor (HeLa-Fas) and transiently expressing GFP or GFP-CaeA were treated with Fas-ligand. As expected, CaeA expression significantly inhibited Fas-ligand-induced extrinsic apoptosis visualized by DAPI staining (Fig. 1D). Importantly, expression of GFP or GFP-CaeA without cell death induction did not influence viability of HeLa and HeLa-Fas cells (data not shown). Together these data indicate that CaeA protects cells from both intrinsic and extrinsic apoptosis.

### **CaeA expression results in an up-regulation of survivin**

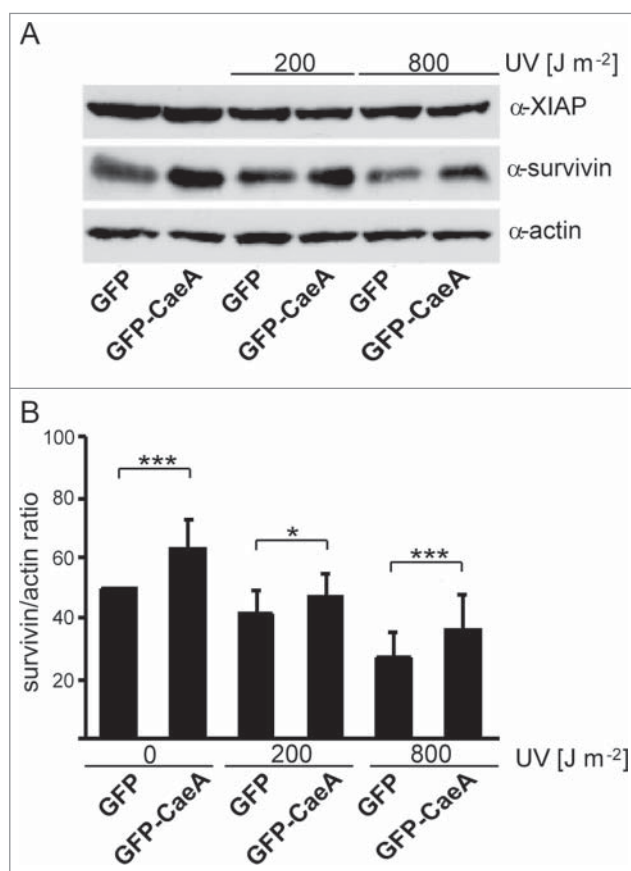
Apoptotic cell death can be prevented by inhibitor of apoptosis proteins (IAPs). Therefore, we assessed the steady state level of the IAPs XIAP and survivin. XIAP can bind

directly to caspase 3, 7 and 9, protecting them from proteolytic cleavage.<sup>34</sup> Survivin is the smallest member of the IAP family<sup>35</sup> and regulates several cellular processes, including apoptosis.<sup>36</sup> We also aimed to analyze the expression level of the other members of the IAP family. However, probably due to low expression level we were unable to detect these IAP at protein and mRNA level.

Thus, HEK293 cells stably expressing GFP or GFP-CaeA were treated with different UV-light intensities. Immunoblot analysis revealed an upregulation of survivin in GFP-CaeA expressing cells as compared to GFP expressing cells, while XIAP was not altered (Fig. 2A). This effect of CaeA was detected during steady-state conditions as well as after UV exposure of the cells (Fig. 2A and 2B).

### **CaeA-induced inhibition of apoptosis is not dependent on survivin up-regulation**

As CaeA expression caused inhibition of apoptosis and upregulation of survivin, we investigated whether the

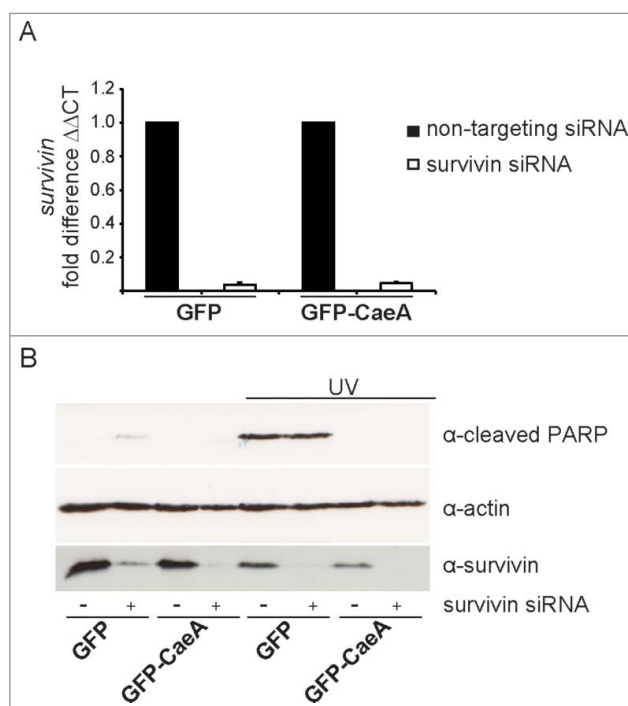


**Figure 2.** CaeA leads to survivin up-regulation. HEK293 cells stably expressing GFP or GFP-CaeA were exposed to UV-light (200 J/m<sup>2</sup> or 800 J/m<sup>2</sup>) and incubated for 6 h. (a) Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and probed with antibodies against survivin, XIAP and actin as loading control. The result of one representative experiment out of 3 independent experiments with similar results is shown. (b) Densitometric analysis of the survivin/actin ratio was performed using ImageJ. The data shown represent average values  $\pm$  SD from at least 3 independent experiments. \* $P < 0.05$  \*\*\* $P < 0.001$

increased protein expression of survivin accounts for the anti-apoptotic effect of CaeA. To this end, we transfected HEK293 cells stably expressing GFP or GFP-CaeA with survivin siRNA, which efficiently depleted the cells of *survivin* mRNA (Fig. 3A) and survivin protein (Fig. 3B). After exposure of the cells to UV light, CaeA was equally effective in protecting the cells from PARP cleavage and apoptosis in the presence and in the absence of survivin (Fig. 3B). From these data we conclude that inhibition of apoptosis by CaeA is not mediated by the upregulation of survivin.

### Sequence analysis of *C. burnetii* strains reveals a genetic variability of CaeA

CaeA has a molecular weight of 25.1 kDa and contains a predicted coiled-coil region that spans from aa 25 to 117



**Figure 3.** Survivin knock-down does not affect CaeA-induced apoptosis-inhibition. HEK293 cells stably expressing GFP or GFP-CaeA were transfected with 50nM non-targeting siRNA (–) or human survivin siRNA (+). (a) 48 hours post-transfection total isolated RNAs were reverse transcribed using SuperScript II reverse transcriptase according to the manufacturer's protocol and a qRT-PCR was performed with oligonucleotides specific for *survivin* and *hpbgd* as a house keeping gene. The  $\Delta\Delta C_t$  values were calculated for the fold difference of survivin siRNA-treated cells to non-targeting siRNA-treated cells using the  $2^{-\Delta\Delta C_t}$  method. (b) 48 hours post-transfection cells were treated with or without UV light (800 J/m<sup>2</sup>) and incubated for 6h at 37°C in 5% CO<sub>2</sub>. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and probed with antibodies against cleaved PARP, survivin and actin as loading control. The result of one representative experiment out of 3 independent experiments with similar results is shown.

and 2 nuclear localization signals (NLS).<sup>8,14</sup> Comparison of genomes from different *C. burnetii* isolates pointed to a significant plasticity in the repertoire of T4SS effector proteins.<sup>8</sup> With respect to single effector proteins, differences in the DNA sequence might help to elucidate their function. Therefore, we analyzed the sequence of *caeA* from 25 different strains of *C. burnetii* (Table 1). As shown in Figure 4, the sequence analysis revealed a striking similarity of all strains except for a 5' gene locus starting from base pair 148 that contains GAA AAG/A repeats. This segment, which is part of the coiled-coil region, encodes alternating glutamic acid (E) and lysine (K) residues. It is present in all strains analyzed, but the number of EK repeats varied between 3 and 13. In addition, 2 single base deletions occurred. Twenty of the sequenced strains showed a single base deletion that

**Table 1.** *Coxiella burnetii* strains used in this study.

Cluster	Isolate	Host	Material	Clinic	Origin	Year
I	F-3	human	mitral valve	endocarditis	Lyon, France	1978
I	F-4	human	blood, mitral valve	endocarditis	Marseille, France	1978
I	Namibia	goat	—	—	Windhoek, Namibia	1991
I	Z 3574-1/92	sheep	milk	—	Berlin, Germany	1992
II	19/34	goat	fetus, afterbirth	—	Bissingen/Teck, Germany	2010
II	23/2	sheep	fetus	—	Jork, Germany	2011
II	30/14	sheep	afterbirth	—	Jork, Germany	2011
II	W-3	fallow deer	afterbirth	abort	Gäufelden, Germany	1997
II	W-4	sheep	afterbirth	abort	Aidlingen, Germany	1996/97
II	Z 104/94	sheep	afterbirth	—	Dillenburg, Germany	1994
II	Z 346/99	sheep	afterbirth	abort	Sarstedt, Germany	1999
II	Z 3464/92	goat	afterbirth	abort	Pohlheim, Germany	1992
II	Z 3468-5/92	sheep	placenta	—	Niedersachsen, Germany	1992
II	Z 3478/92	sheep	organs lamb	abort	Pohlheim, Germany	1992
II	Z 4485/93	sheep	afterbirth	abort	Dillenburg, Germany	1993
III	NM II	tick	—	—	Montana, USA	1938
IV	Z 66/96	goat	organs lamb	abort	Driedorf, Germany	1996
V	Z 232-3/02	cattle	placenta	fertility disorder in livestock	Bobingen, Germany	2002
V	Z 502/99	cattle	placenta	—	—	1999
V	Z 3568/92	cattle	organs calf	abort	Niedersachsen, Germany	1992
VI	71/3	goat	afterbirth	—	Altkirchen, Germany	2010
VI	98/2	cattle	organ material	—	Altenberga, Germany	2010
VI	Z 488/94	cattle	amniotic liquor, afterbirth	abort	Lauterbach, Germany	1994
VI	Z 3351/92	cattle	placenta	abort	Hannover, Germany	1992
VII	Z 3567/92	cattle	afterbirth, maw	abort	Niedersachsen, Germany	1992

provoked a frame shift and the introduction of a premature ochre stop codon at base pairs 61–63. An additional second single base deletion, downstream of this stop codon, was present in 8 of these isolates. Consequently, *caeA* represents a presumptive pseudogene in 20 of the 25 isolates analyzed in this study. Based on these *in silico* analyses only the *C. burnetii* strain NM I, which contains 6 EK repeats, and 4 further isolates, which all contain 3 EK repeats, are predicted to express a functional CaeA molecule. These five isolates originated from different regions and host species. While strain F-3 was obtained in France from a patient with endocarditis and therefore appears to be associated with chronic Q-fever, strain F-4 was isolated in France from an acute Q-fever patient<sup>37</sup> and the NM strain originated from a tick in Montana, USA.<sup>1</sup> The strain Namibia was obtained from an infected goat in Namibia and the strain Z3574-1/92 was derived from milk of an infected ewe in Germany; in both cases there was no information available on the clinical status of the host animals.

Taken together, the *caeA* gene of *C. burnetii* shows a considerable genetic polymorphism in a region which encodes an EK-repetition motif.

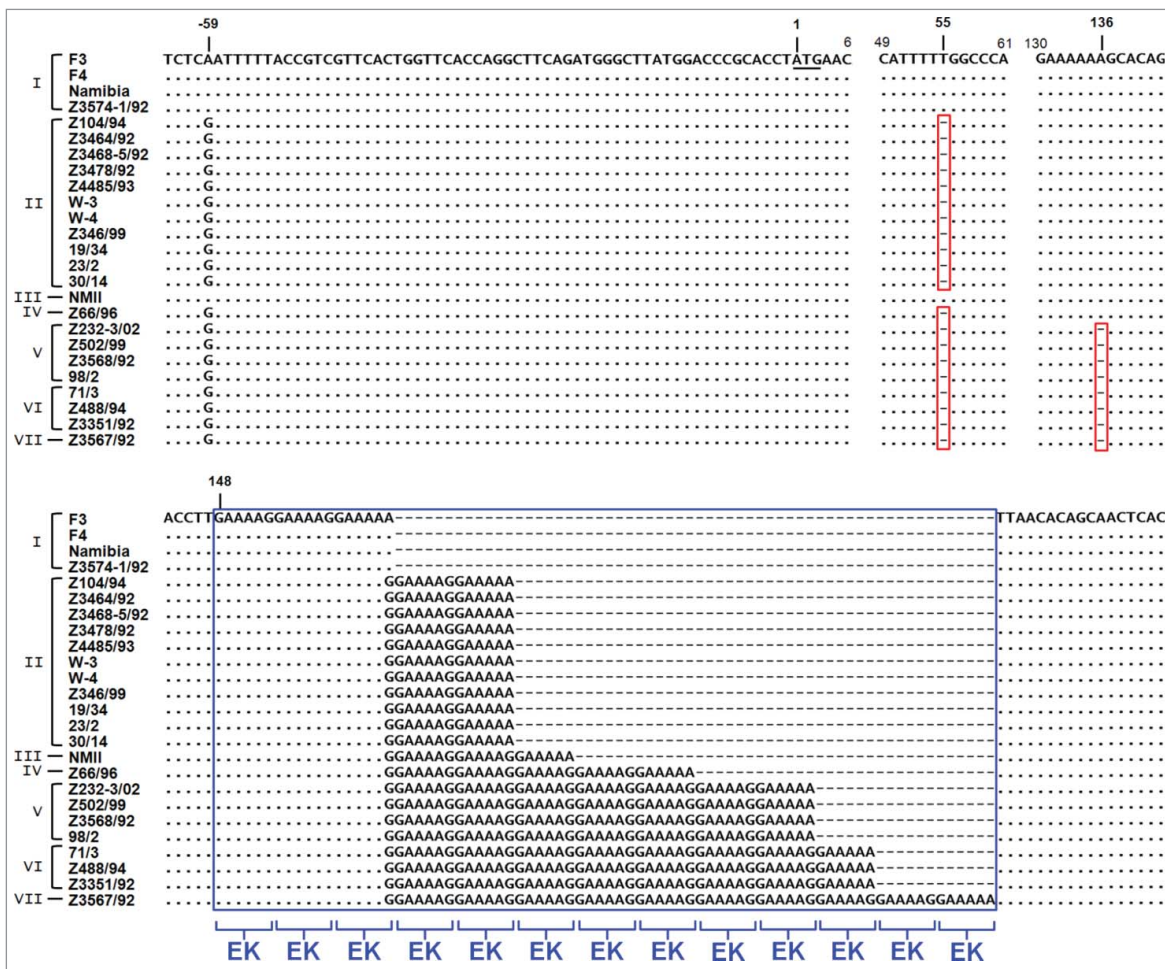
### The EK repetition motif is required for the anti-apoptotic activity of CaeA

To assess the functional role of the EK motif, a HEK293 cell line that stably expressed GFP-CaeA lacking the EK repeat (GFP-CaeAΔEK) was established. This cell line

was analyzed by flow cytometry together with the already established HEK293 cells stably expressing GFP or GFP-CaeA. As shown in Figure 5A, around 90% of the cells in all 3 stable cell lines expressed GFP. Next, the anti-apoptotic activity of CaeA lacking the EK repeats was tested. While HEK293-GFP cells showed caspase 7 and PARP cleavage after UV-light exposure, HEK293-GFP-CaeA cells were protected from caspase 7 and PARP cleavage (Fig. 5B and 5C), confirming the results in Figure 1B. In contrast, HEK293-GFP-CaeAΔEK showed increased levels of caspase 7 and PARP cleavage after UV-light treatment, suggesting that CaeA lacking the EK repeats was unable to protect cells from apoptosis. Importantly, ectopically expressed GFP-CaeAΔEK still exhibited a proper intracellular localization under steady-state conditions and after apoptosis induction. As depicted in Figure 6, both GFP-CaeA and GFP-CaeAΔEK showed nuclear speckle-like localization with and without apoptosis-induction. Thus, the EK repetition motif enables CaeA to interfere with the apoptosis cascade, but is not required for the intracellular localization of CaeA.

### CaeA-induced apoptosis inhibition varies with the number of EK repeats

Sequence analyses of 25 different isolates of *C. burnetii* showed that CaeA contains between 3 and 13 EK repeats. As stated above, only CaeA molecules containing either 3 or 6 EK repeats are expressed as functional effector proteins. To determine how many

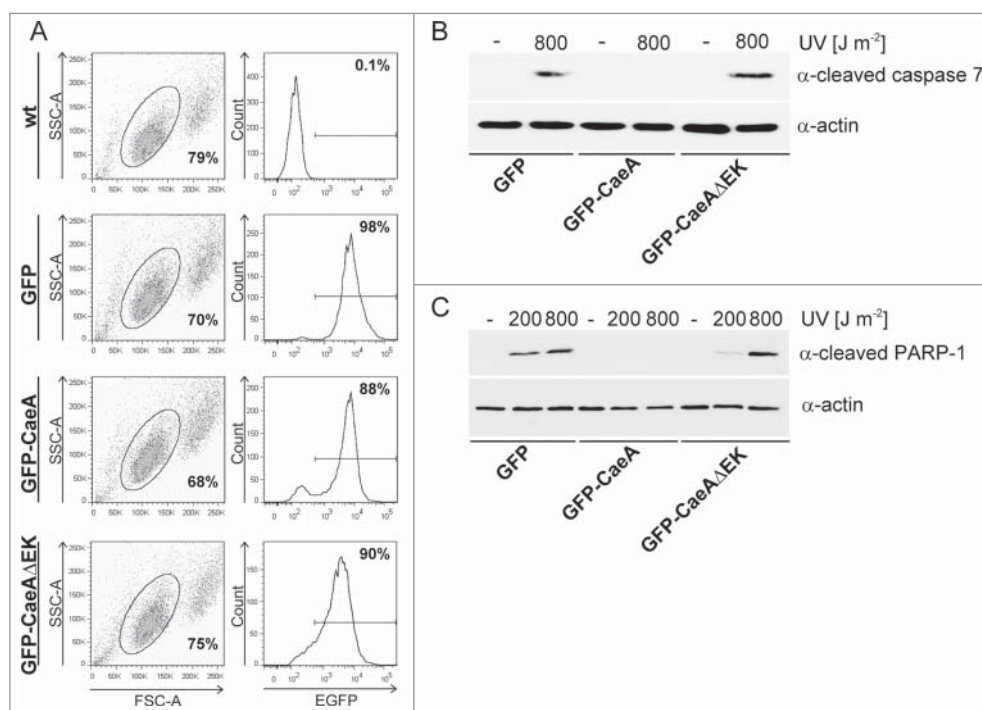


**Figure 4.** Genomic plasticity in the *caeA* gene. Genomic DNA of 25 *C. burnetii* isolates was prepared, the gene *caeA* and flanking regions was amplified and sequenced, and a multiple sequence alignment was performed. The illustrated alignment includes 63 bp upstream of the start codon (underlined) and the first 182–240 bp of the *caeA* open reading frame. Nucleotides matching in all isolates are marked by a dot and single base deletions are indicated by a dash in a red box. Non-displayed regions (positions 7–48 and 62–129) are identical in all isolates. Within the sequence of *caeA* each isolate contains a GAA AAG/A (EK) motif with a different number (3 to 13) of repetitions (blue box). Strains were grouped into 7 clusters (I to VII) according to their EK-repetition motif profile. The first base deletion (nt~55) results in a frame shift and premature stop codon 'TAA' at bp 61 to 63, the second base deletion (nt~136) similarly provokes a 'TAA' stop codon immediately downstream of the GAA AAG/A motif.

repetitions of the EK motif in CaeA are minimally required for efficient inhibition of apoptosis, we tested CHO cells ectopically expressing CaeA $\Delta$ EK with 2 (CaeA $\Delta$ EK+2EK) or 4 EK (CaeA $\Delta$ EK+4EK) repeats. CHO cells expressing wild-type NM II-derived CaeA were significantly protected from apoptosis, whereas cells expressing CaeA $\Delta$ EK were not (Fig. 7), confirming the results obtained in HEK293 cells (Fig. 5B and 5C). Importantly, expression of CaeA $\Delta$ EK+2EK also inhibited apoptosis, but significantly less than CaeA, while expression of CaeA $\Delta$ EK+4EK protected the cells as efficiently as wild-type NM II CaeA, which contains 6 EK repeats. From these findings we conclude that at least 4 EK repetitions are required for full anti-apoptotic activity of CaeA.

## Discussion

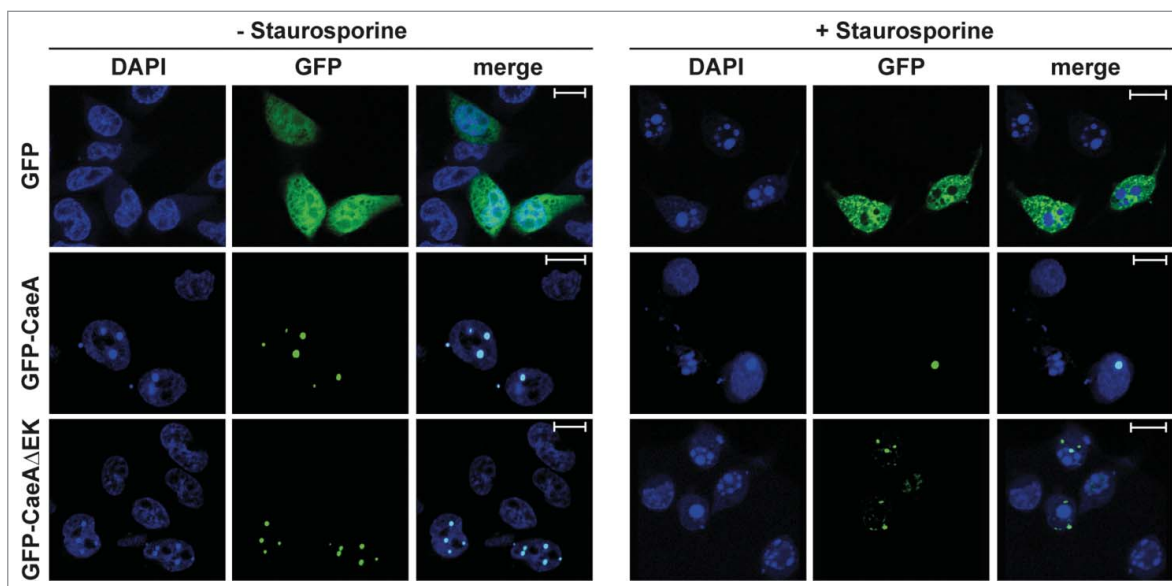
Apoptosis of infected host cells has been identified as an important element of the innate immune response contributes to the clearance of microorganisms cleared from the body. Especially obligate intracellular pathogens have evolved mechanisms to impede the process of host cell apoptosis, but the underlying molecular mechanisms are still incompletely understood. This is also the case for *C. burnetii*, for which inhibition of host cell apoptosis was first described in 2007.<sup>38,39</sup> The anti-apoptotic function of the *C. burnetii* effector proteins AnkG, CaeA and CaeB<sup>14,15</sup> suggests that proteins transported by the T4SS are important regulators of host cell survival.



**Figure 5.** The EK repetition motif is required for apoptosis inhibition. (a) HEK293 cells and HEK293 cells stably expressing GFP, GFP-CaeA and GFP-CaeA $\Delta$ EK were analyzed by flow cytometry. One representative experiment out of at least 3 independent experiments with similar results is depicted. (b and c) HEK293 cells stably expressing GFP, GFP-CaeA or GFP-CaeA $\Delta$ EK were exposed to the UV-light intensities indicated and incubated for 6 h at 37°C in 5% CO<sub>2</sub>. Proteins were separated by SDS-PAGE, transferred to a PVDF membrane and probed with antibodies against (b) cleaved caspase 7, cleaved PARP (c) and actin as loading control. The result of one representative experiment out of at least 3 independent experiments with similar results is shown.

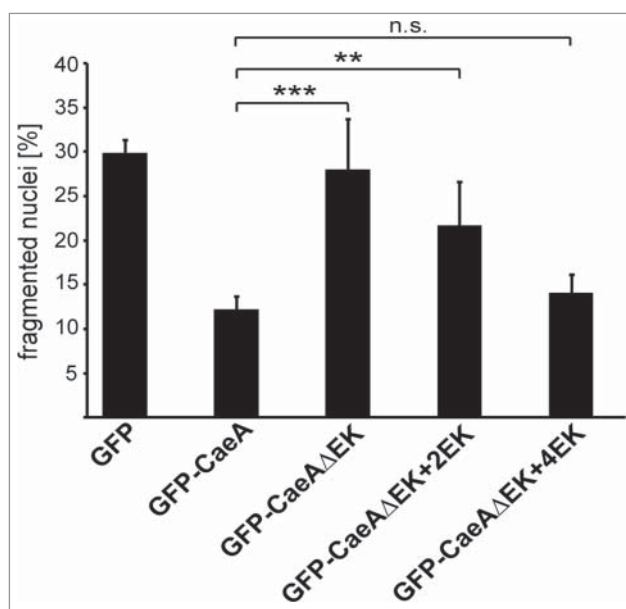
Due to a re-annotation of *C. burnetii* Nine Mile phase I (RSA493) CaeA and CaeB are now annotated as pseudogenes. CaeA (former CBU1524) is now part of CBU1523 and CaeB (former CBU1532) is now part of

CBU1531. However, RT-PCR analysis confirmed transcription of CaeA and CaeB and the presence of a functional T4SS translocation signal at its C-terminus.<sup>8</sup> Whether CaeA is only a fragment of CBU1523 or



**Figure 6.** Deletion of the EK repetition motif does not alter intracellular localization of CaeA. Representative immunofluorescence micrographs show CHO-FcR cells transiently transfected with GFP, GFP-CaeA and GFP-CaeA $\Delta$ EK. The cells were treated with or without 1  $\mu$ M staurosporine for 6 h at 37°C in 5% CO<sub>2</sub> followed by fixation, permeabilization and staining of the nuclei with DAPI (blue).





**Figure 7.** Four EK repeats are sufficient for inhibition of apoptosis. CHO-FcR cells transiently transfected with GFP, GFP-CaeA, GFP-CaeA $\Delta$ EK, GFP-CaeA $\Delta$ EK+2EK and GFP-CaeA $\Delta$ EK+4EK were treated with 1  $\mu$ M staurosporine for 6 h at 37°C in 5% CO<sub>2</sub>. The cells were fixed, permeabilized and the nuclei were stained with DAPI. The nuclear morphology of GFP-expressing cells was scored. Data represent average values  $\pm$  SD of 100 nuclei counted per sample from GFP-expressing cells from 5 independent experiments. \*\*P < 0.01, \*\*\*P < 0.001.

whether CBU1523 and CaeA (CBU1524) are 2 independent proteins is unknown and has not been experimentally tested. However, we hypothesize that CaeA is not a fragment of CBU1523 but rather a discrete protein, as the CBU1524 gene contains a potential Shine Dalgarno sequence upstream of the start codon, and an 114bp intergenic stretch, which separates the stop codon of CBU1523 and the start codon of CBU1524. This intergenic stretch is enough sequence space to contain transcriptional and translational start sites for CBU1524.

In this study, we investigated the determinants of the anti-apoptotic function of the *C. burnetii* effector protein CaeA. CaeA was originally described as an inhibitor of the intrinsic apoptosis pathway when endogenously expressed in HEK293 or CHO cells.<sup>14</sup> Our present results suggest that CaeA also protects from the induction of extrinsically triggered apoptosis (Fig. 1C and D). This indicates that CaeA might act at the intersection of both apoptosis pathways. In agreement with this assumption, cleavage of the effector caspase 7 was completely inhibited in presence of CaeA whereas cleavage of the upstream initiator caspase 9 was not (Fig. 1B). IAPs are known to inhibit activated caspases and to interfere at this late step of the apoptotic pathway.<sup>24</sup> Some pathogens exploit the anti-apoptotic function of IAPs in order to

manipulate the host apoptotic pathway and to promote host cell survival. Importantly, CaeA expression resulted in up-regulation of the steady-state protein level of the IAP survivin (Fig. 2). Several intracellular pathogens have been described to elicit an upregulation of *survivin* which subsequently improved host cell survival.<sup>40-42</sup> For instance, the intracellular protozoan *Cryptosporidium parvum* caused an increased expression of cIAP1, cIAP2, XIAP as well as survivin in HCT-8 epithelial colon cells, and survivin was shown to be important for apoptosis inhibition by the pathogen.<sup>41</sup> These observations contrast with our findings. While CaeA expression led to higher levels of survivin protein in the host cell, CaeA-mediated inhibition of apoptosis was independent of survivin (Fig. 3).

Sequence analysis of *caeA* from 25 different *C. burnetii* isolates revealed a noticeable difference in a region consisting of a variable number of GAA AAG/A (EK) codons (Fig. 4). The EK repetition motif of CaeA represents a short tandem repeat (STR) with the 6 bases 'GAA AAG/A'. In general, STRs or 'microsatellites' are widely distributed in the genomes of eukaryotes and prokaryotes and are characterized by a sequentially repeated unit of one to 6 bp reaching up to 100 nt. Tandem repeats are hot spots for mutational events contributing to gene diversity and the evolutionary process. The high mutation rate and variability of tandem repeats is mainly explained by slipped-strand mispairing and a malfunctioning repair system during replication.<sup>43</sup> Consistently, the presence of diverse numbers of EK repeats among the analyzed strains is most likely due to events of slipped-strand mispairing during the replication of the hexanucleotide GAA AAG/A. Besides the variability in the number of GAA AAG/A repetitions, 2 single base deletions were found to contribute to the sequence polymorphism of *caeA* in the different strains. In isolates encoding 5, 8, 10, 11 or 13 GAA AAG/A repetitions a single base deletion occurred leading to a frameshift and premature stop. In these strains *caeA* presumably represents a pseudogene. Interestingly, in addition to this first single base deletion, only in isolates encoding a higher number of repetitions, i.e. 10 to 13 GAA AAG/A units, a second single base deletion was found. In the absence of the first single base deletion the second base deletion would result in a frameshift altering the EK motif to an irregular succession of lysine and arginine residues and provoking a premature stop codon directly downstream. Thus, the second base deletion would similarly ensure the expression of a truncated protein. However, every isolate harboring the second base deletion, already showed the first base deletion at position  $\sim$ 55 which *per se* is sufficient to cause the truncation of CaeA. Remarkably,

in addition to NM only 4 of all 25 analyzed isolates are also capable to express a non-truncated protein. All these isolates of *C. burnetii* contain 3 EK repeat units and include 2 human, one goat and one sheep isolate. Thus, one might speculate the presence of a low number of EK repeats is a prerequisite for the expression of a non-truncated CaeA.

In prokaryotic genomes STRs exhibit a high mutation rate of up to  $10^{-1}$ <sup>44</sup> which contributes to phenotypic variability and fast adaptation to environmental or host conditions as has been shown especially for pathogenic bacteria. Indeed, human pathogens such as *Neisseria gonorrhoeae* or *Haemophilus influenzae* have been shown to undergo phase variation and to regulate protein expression similar to a switch on and off mechanism by alteration of the number of 5 and 4 base pair repetition units, respectively.<sup>45</sup> Thus, the expression of *H. influenzae licA*, a gene that participates in the synthesis of lipoteichoic acid (LTA), undergoes a phase variation that is mediated by the varying number of a 4 base pair repetition unit.<sup>46</sup> In contrast, variations of the number of tri- or hexanucleotides (such as the CaeA EK repetition motif) do not influence the frame of a sequence and alter gene expression *per se*. Tri- or hexanucleotide tandem repeats seem to rather influence the function of a protein as variations of the repeat number may alter structural characteristics of a protein.<sup>47</sup> For instance, the activity of the *Salmonella enterica* serovar Typhimurium and *Escherichia coli* DNA mismatch repair protein MutL is lost by alteration of the number of the 3 hexanucleotide repeat units encoded in the ATP binding pocket of MutL.<sup>48,49</sup> As shown by Shaver *et al.* the deletion as well as the addition of one unit of the hexanucleotide repeat presumably interferes with ATP binding resulting in the inhibition of the ATP-dependent MutL function and an increased mutation rate.<sup>49</sup>

Regarding CaeA, the EK repetition lies within a predicted coiled-coil region, a domain that is organized in  $\alpha$ -helical bundles facilitating protein-protein interactions. The negatively and positively charged amino acids of the EK motif account for a charged region with hydrophilic character and presumably lead to the exposition of the domain to the protein surface. Thus, the EK motif might provide a protein interaction site within the coiled-coil region which accounts for the interaction with specific protein partners. Similar to the aforementioned MutL mutation, variation of the EK motif might influence the interaction and the function of the protein. In accordance with this hypothesis the EK repetition motif was shown to be essential for CaeA-mediated inhibition of apoptosis (Fig. 5B and 5C).

Taken together, we have demonstrated that the effector protein CaeA of *C. burnetii* is an efficient inhibitor of the extrinsic pathway of apoptosis. Furthermore, we defined the EK repetition motif of CaeA as a structural component that is crucial for the anti-apoptotic function of CaeA. Further studies will aim to identify the exact interaction partners of CaeA in the host cell.

## Materials and methods

### Reagents, cell lines and bacterial strains

Unless otherwise noted, chemicals were purchased from Sigma Aldrich. Complete Protease inhibitor cocktail mixture and X-tremeGENE 9 Transfection Reagent were from Roche. Staurosporine was from Cell Signaling. Cell lines were cultured at 37°C in 5% CO<sub>2</sub> in media containing 5% heat-inactivated fetal bovine serum (Biochrom). Chinese hamster ovary (CHO) fibroblasts were grown in minimal essential medium  $\alpha$  medium (Invitrogen) and stable human embryonic kidney (HEK293) cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) supplemented with 1.5 mg/ml G418 (Roth). To construct HEK293 cells stably expressing GFP-CaeA $\Delta$ EK the corresponding plasmid was transfected into HEK293 cells using X-tremeGENE 9 and selected by culturing in media supplemented with 1.5 mg/ml G418 (Roth) for 7 days. Single GFP-positive cells were sorted and grown for additional 10 to 14 days. *C. burnetii* strains used in this study are listed in Table 1. To generate plasmids encoding CaeA fusion proteins, genes were amplified by PCR, using *C. burnetii* Nine Mile phase II clone 4 as template and primers designed according to an earlier annotation of RSA493 (*C. burnetii* Nine Mile phase I).

### siRNA knock-down

The protocol used for siRNA transfection was adapted from Dharmacon's HeLa cell transfection protocol. One volume of siRNA buffer containing 50nM of non-targeting or survivin siRNA (Dharmacon) was incubated with one volume of serum-free DMEM high glucose containing 5  $\mu$ l/ml DharmaFECT-1 transfection reagent for 20 min at room temperature. Two volumes of DMEM high glucose supplemented with 20 % FBS containing  $5 \times 10^4$  HeLa cells were added. The cells were seeded in a 12-well plates and incubated at 37°C with 5% CO<sub>2</sub>. The transfection mix was replaced with 500  $\mu$ l of fresh media (DMEM high glucose supplemented with 10% FBS) 24 h post transfection. 3 days post transfection, the cells were washed with PBS and exposed to UV light (800 J/m<sup>2</sup>) in a transilluminator box (Stratagene). After

**Table 2.** Plasmids used in this study.

no	Plasmid	Reference
1	pCMV-HA-CaeA	Klingenbeck <i>et al.</i>
2	pCMV-HA-CaeAΔEK	this study
3	pEGFP-CaeAΔEK	this study
4	pCMV-HA-CaeAΔEK+2EK	this study
5	pCMV-HA-CaeAΔEK+4EK	this study
6	pEGFP-CaeAΔEK +2EK	this study
7	pEGFP-CaeAΔEK +4EK	this study

adding fresh media, the cells were incubated for 6 h at 37°C in 5% CO<sub>2</sub>. The samples were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). The membranes were probed with antibodies against survivin (2808, Cell Signaling), cleaved PARP (611038, BD Biosciences) and actin (A2066, Sigma-Aldrich). The proteins were visualized by using horseradish peroxidase-conjugated secondary antibodies (Dianova) and a chemiluminescence detection system (Thermo Scientific or Millipore).

### Plasmids and primers

Plasmid and primers used in this study are listed in Tables 2–4

### Plasmid construction

pEGFP-CaeAΔEK was generated by mutagenic PCR with primers 1 and 2 (Table 3) amplified from pCMV-HA-CaeA. Ligation of PCR product resulted in pCMV-HA-CaeAΔEK. This vector was restricted with BglII and KpnI, followed by ligation with like-wise restricted pEGFP-C2.

pEGFP-CaeAΔEK+2EK and pEGFP-CaeAΔEK+4EK were generated by mutagenic PCR with primers 1 and 3 or 1 and 4 (Table 3) amplified from pCMV-HA-CaeAΔEK. Ligation of the PCR product resulted in pEGFP-CaeAΔEK+2EK and pEGFP-CaeAΔEK+4EK. The vectors were restricted with BglII and KpnI, followed by ligation with like-wise restricted pEGFP-C2.

**Table 3.** PCR Primers used in this study.

no	Oligonucleotide	Sequence 5'-3'
1	caeAΔEK_fwd	TTAACACAGCAACTCACCGAAGAACAGGAGCGTTC
2	caeAΔEK_rev	AAGGTCTGTGCTTTTTCTTCGAGTCGCTGATAGTCATTG
3	caeAΔEK+2_rev	TTCTTTTCTTTTCAAGGTCTGTGCTTTTTCTTC
4	caeAΔEK+4_rev	TTCTTTTCTTTTCTTTTCTTTTCAAGGTCTGTGCTTTTTCTTC
5	caeA_3_fwd	AACTCGCAACCCATCCTCAAAAC
6	caeA_4_rev	GAAGAAAAAGCGCCATCCCTAAT

## Apoptosis assays

### Nuclear fragmentation assay

CHO cells were plated on coverslips in 24-well dishes at a density of  $2.5 \times 10^4$  cells/well. After an overnight incubation, cells were transfected with the plasmids indicated. 18 h post-transfection, the cells were incubated with staurosporine (1 μg/ml) for 6 h at 37°C in 5% CO<sub>2</sub>. The cells were fixed with 4 % paraformaldehyde (Alfa Aeser) in PBS (Biochrom) for 20min at room temperature, permeabilized with ice-cold methanol for 30sec and quenched with 50 mM NH<sub>4</sub>Cl (Roth) in PBS for 15min at room temperature. The cells were mounted using Pro-Long Gold with DAPI (Invitrogen) to visualize the nucleus.

### Immunoblotting

HEK293 cells stably expressing GFP, GFP-CaeA or GFP-CaeAΔEK were seeded in a 12-well plate at a density of  $3 \times 10^5$  cells/well. The cells were washed with PBS before exposure with the indicated UV-light intensities (Stratagene). After 6h incubation at 37°C in 5%CO<sub>2</sub> samples were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). The membranes were probed with antibodies directed against Bcl-2 (2870), Bcl-x<sub>L</sub> (2764), Mcl-1 (4572), cleaved caspase 7 (9491), cleaved caspase 9 (9501), XIAP (2045) and survivin (2808) all from Cell Signaling, cleaved PARP (611038, BD Biosciences) and actin (A2066, Sigma-Aldrich). The proteins were visualized by using horseradish peroxidase-conjugated secondary antibodies (Dianova) and a chemiluminescence detection system (Thermo Scientific or Millipore).

### TNF + CHX-induced extrinsic apoptosis

HeLa cells were plated on coverslips in 24-well dishes at a density of  $3.5 \times 10^4$  cells/well and were transfected with the plasmids indicated. 40 h post-transfection, the cells were incubated with 20 ng/ml TNF (Calbiochem) and 6 μg/ml cycloheximide (Sigma) for 5 h at 37°C in 5% CO<sub>2</sub>. The cells were fixed with 4 % paraformaldehyde (Alfa

**Table 4.** Sequencing Primers used in this study.

no	Oligonucleotide	Sequence 5'-3'
1	caeA_S1_rev	CTCCTGAAATTGCATCGCAA
2	caeA_S2_fwd	CCTTGAGCAAGTAATTAACC

Aeser) in PBS (Biochrom) for 20 min at room temperature, permeabilized with ice-cold methanol for 30 sec and quenched with 50 mM NH<sub>4</sub>Cl (Roth) in PBS for 15 min at room temperature. The cells were mounted using ProLong Gold with DAPI (Invitrogen) to visualize the nucleus.

### **Fas-ligand-induced extrinsic apoptosis**

HeLa-Fas cells<sup>50</sup> were plated on coverslips in 24-well dishes at a density of  $4 \times 10^4$  cells/well. After an overnight incubation, cells were transfected with the plasmids indicated. 18 h post-transfection, the cells were incubated with 0.125 μg/ml anti-Fas IgG (Millipore) for 5 h at 37°C in 5% CO<sub>2</sub>. The cells were fixed with 4% paraformaldehyde (Alfa Aeser) in PBS (Biochrom) for 20 min at room temperature, permeabilized with ice-cold methanol for 30 sec and quenched with 50 mM NH<sub>4</sub>Cl (Roth) in PBS for 15 min at room temperature. The cells were mounted using ProLong Gold with DAPI (Invitrogen) to visualize the nucleus.

### **Confocal microscopy**

$2 \times 10^4$  CHO cells were plated on coverslips and were transfected with the plasmids indicated. The cells were fixed with 4% paraformaldehyde (Alfa Aeser) in PBS (Biochrom), permeabilized with ice-cold methanol and quenched with 50mM NH<sub>4</sub>Cl (Roth) in PBS. The cells were mounted using ProLong Gold with DAPI (Invitrogen) to visualize the nucleus. Fluorescence was detected using a Carl Zeiss LSM 700 Laser Scan Confocal Microscope and a 64x objective. Images were analyzed using the ZEN2009 software (Jena, Germany).

### **FACS analysis**

Protein expression levels of HEK293 cells stably expressing GFP, GFP-CaeA and GFP-CaeAΔEK were analyzed by flow cytometry.

### **PCR, sequencing and sequence analysis**

PCR primers 5 and 6 (Table 3) for *caeA* were designed with Primer Select software implemented in Lasergene 9.0 (DNASTAR, Madison, WI). *caeA* was amplified

using 1x OptiBuffer (Bioline, London, United Kingdom), 1.4 mM MgCl<sub>2</sub>, 200 μM deoxynucleoside triphosphates (Bioline), 25pmol of each primer, 1.5 U Bio-X-Act Short DNA polymerase (Bioline), and 5 μl of DNA, in a final volume of 25 μl. The PCR was run with the following thermocycling profile: 5 min at 95°C; 1 min at 58°C; 35 cycles, each consisting of 1 min and 15 s at 72°C, 30 s at 95°C, and 30 s at 58°C; and a final elongation step of 10 min at 72°C. Cycle sequencing was performed using BigDye v1.1 chemistry according to the manufacturer's instructions (Applied Biosystems) (see Sequencing primers in Table 4), purified with DyeEx 96 plates (Qiagen) and electrophoresed on a 3130XL Genetic Analyzer (Applied Biosystems). Sequence analysis and polymorphism evaluation was performed using Lasergene 9.0 (DNASTAR, Madison, WI) and MEGA5 (Tamura *et al.*, 2011).

The sequences of *caeA* gene are available at GenBank under the accession numbers KT828680-KT828703.

### **Isolation of genomic *Coxiella burnetii* DNA**

Genomic DNAs of 25 paraformaldehyde-fixed or heat-killed *C. burnetii* isolates was prepared according to the manufacturer's protocol with the illustra bacteria genomic Prep Mini Spin Kit (GE Health Care), with 3×10 min incubation at 55°C in the beginning and a final elution with 30 μl H<sub>2</sub>O.

### **Statistical analysis**

An unpaired Student's t-test or Chi-square test was used for statistical analysis.

### **Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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