Video Article

Applying an Inducible Expression System to Study Interference of Bacterial Virulence Factors with Intracellular Signaling

Christian Berens[1,2], Stephanie Bisle[3], Leonie Klingenbeck[3], Anja Lührmann[3]

[1]Department Biologie, Friedrich-Alexander-Universität

[2]Institut für Molekulare Pathogenese, Friedrich-Loeffler-Institut

[3]Mikrobiologisches Institut - Klinische Mikrobiologie, Immunologie und Hygiene, Universitätsklinikum Erlangen

Correspondence to: Anja Lührmann at anja.luehrmann@uk-erlangen.de

URL: http://www.jove.com/video/52903
DOI: doi:10.3791/52903

Keywords: Infection, Issue 100, Apoptosis, Bax, Caspase 3, *Coxiella burnetii*, Doxycycline, Effector protein, Inducible expression, stable cell line, Tet system, Type IV Secretion System

Date Published: 6/25/2015

Citation: Berens, C., Bisle, S., Klingenbeck, L., Lührmann, A. Applying an Inducible Expression System to Study Interference of Bacterial Virulence Factors with Intracellular Signaling. *J. Vis. Exp.* (100), e52903, doi:10.3791/52903 (2015).

Abstract

The technique presented here allows one to analyze at which step a target protein, or alternatively a small molecule, interacts with the components of a signaling pathway. The method is based, on the one hand, on the inducible expression of a specific protein to initiate a signaling event at a defined and predetermined step in the selected signaling cascade. Concomitant expression, on the other hand, of the gene of interest then allows the investigator to evaluate if the activity of the expressed target protein is located upstream or downstream of the initiated signaling event, depending on the readout of the signaling pathway that is obtained. Here, the apoptotic cascade was selected as a defined signaling pathway to demonstrate protocol functionality. Pathogenic bacteria, such as *Coxiella burnetii*, translocate effector proteins that interfere with host cell death induction in the host cell to ensure bacterial survival in the cell and to promote their dissemination in the organism. The *C. burnetii* effector protein CaeB effectively inhibits host cell death after induction of apoptosis with UV-light or with staurosporine. To narrow down at which step CaeB interferes with the propagation of the apoptotic signal, selected proteins with well-characterized pro-apoptotic activity were expressed transiently in a doxycycline-inducible manner. If CaeB acts upstream of these proteins, apoptosis will proceed unhindered. If CaeB acts downstream, cell death will be inhibited. The test proteins selected were Bax, which acts at the level of the mitochondria, and caspase 3, which is the major executioner protease. CaeB interferes with cell death induced by Bax expression, but not by caspase 3 expression. CaeB, thus, interacts with the apoptotic cascade between these two proteins.

Video Link

The video component of this article can be found at http://www.jove.com/video/52903/

Introduction

The virulence of many Gram-negative bacterial pathogens depends on specialized secretion systems to hijack eukaryotic host cells. Bacteria use these secretion systems to inject bacterial virulence proteins (effectors) into the host cell to modulate a variety of cellular and biochemical activities. The study of effector proteins has not only provided remarkable insight into fundamental aspects of host/pathogen interactions but also into the basic biology of eukaryotic cells[1]. Modulation of host cell apoptosis has been shown to be an important virulence mechanism for many intracellular pathogens, and a number of effector proteins modulating apoptosis have been identified[2-9]. However, their precise molecular mechanisms of activity remain elusive in many cases.

Apoptosis, a form of programmed cell death, plays an important role in immune responses to infection[10]. Two main pathways leading to apoptosis have been identified: targeting the mitochondria (intrinsic apoptosis) or direct transduction of the signal via cell death receptors at the plasma membrane (extrinsic apoptosis). The intrinsic or mitochondria-mediated cell death pathway is triggered by intracellular signals and involves the activation of Bax and Bak, two pro-apoptotic members of the Bcl-2 family. This family is composed of pro- and anti-apoptotic regulator proteins that control cell death[11-14]. Activation of apoptosis leads to oligomerization of Bax and Bak followed by subsequent permeabilization of the mitochondrial outer membrane, resulting in cytochrome C release into the cytoplasm. Cytochrome C release initiates activation of the effector caspases 3 and 7 through activation of caspase 9 in the apoptosome[15]. This leads to proteolysis of selected substrates that, among others, results in the exposure of phosphatidylserine on the cell surface[16] and frees a dedicated DNase that fragments chromatin[17,18].

In order to determine where within the apoptotic cascade an individual effector protein interferes, an inducible expression system was employed[19]. Regulatory systems for conditional expression of transgenes have been an invaluable tool in analyzing a protein's function within the cell or its importance for tissue, organ and organism development, as well as during initiation, progression and maintenance of disease[20-23]. Typically, inducible control systems, such as the Tet system[24] employed here, form an artificial transcription unit (see **Figure 1**). One component is an artificially engineered transcription factor called tTA (tetracycline-dependent transcription activator), formed by fusion of the bacterial transcription repressor TetR[25] to a mammalian protein domain that mediates transcriptional activation or silencing [24,26]. The second component

is a hybrid promoter, termed TRE (tetracycline-responsive element), consisting of a eukaryotic minimal promoter, containing at least a TATA-box and a transcription initiation site, joined to multiple repeats of the cognate DNA-binding site for TetR, *tetO*[24,25]. The third component is the natural ligand of TetR, tetracycline or one of its derivatives, such as anhydrotetracycline or doxycycline[25]. Upon ligand addition to the culture medium, TetR loses its affinity for *tetO* and dissociates from the TRE. As a result, transcription of the target gene is abolished. Transgene expression can, thus, be tightly controlled in a time- and dose-dependent manner in both cell culture and in animals[20,23,24]. With tTA, transgene expression occurs constitutively, except in the presence of a tetracycline. This can be a disadvantage in the study of cytotoxic or oncogenic proteins because tetracycline first has to be removed from the system, before transgene expression occurs and the target protein‘s effects on the cell can be monitored. This can be time-consuming and is not always complete, especially in transgenic animals[27]. To address this limitation, a TetR mutant with an inverse response to the presence of doxycycline was used to generate a new transcription factor, rtTA (reverse tTA)[28]. It only binds to the TRE and, concomitantly, activates transcription in the presence of doxycycline. Residual leakiness of the system, *i.e.*, transgene expression in the absence of TRE-bound transcription factor, originating either (i) from position effects at a genomic integration site, (ii) from the TRE itself[29], or (iii) from non-specific binding of tTA/rtTA[28], was addressed by introducing an additional transcriptional silencer, termed tTS (tetracycline-dependent transcriptional silencer)[30] to the system. It forms a dual regulator network together with rtTA (see **Figure 1**). In the absence of doxycycline, tTS binds to TRE and actively shuts down any remaining transcription. In the presence of doxycycline, tTS dissociates from TRE and rtTA binds simultaneously inducing expression of the target gene. This additional layer of stringency is often necessary to express highly active cytotoxic proteins[31-34].

Using this tightly controlled dual-regulator system, the apoptotic cascade can be initiated at a defined step allowing analysis of whether the given effector protein can interfere with apoptosis induction. This method can not only be used to study the anti-apoptotic activity of bacterial effector proteins but also for the inducible expression of pro-apoptotic or toxic proteins, or for dissecting interference with other signaling pathways.

Protocol

1. Generation of Stable Cell Lines Expressing the Protein of Interest

1. Prepare media by adding heat-inactivated FCS and 1% Penicillin/Streptomycin to commercially available Dulbecco´s Modified Eagle Medium (DMEM) supplemented with GlutaMAX-I, Pyruvate, and 4.5 g/L D-Glucose.
2. Cultivate HEK293 cells in media at 37 °C in 5% CO_2. Sub-cultivate cells every third day. Remove the media and resuspend the cells in 15 ml fresh media. Pipette 1 ml into a new 75 cm^2 cell culture flask and add 14 ml media.
3. Analyze the cell number by using a hemocytometer.
4. Seed HEK293 cells in a 6-well plate at a density of 2 x 10^5 cells per well and incubate for 24 hr.
5. For transfection use the protocol provided by the manufacturer of the transfection reagent. Transfect cells with pEGFP-C2 or pEGFP-C2-CaeB using the transfection reagent. Prior to use, warm up the transfection reagent and the media required to RT. Use a 3:1 ratio of transfection reagent to DNA.
    1. In detail, pipette 1.5 µl of transfection reagent directly into 50 µl of serum-free DMEM medium. For complex formation, pipette 0.5 µg of DNA encoding GFP or GFP-CaeB to the transfection reagent-containing mixture and incubate for 15 min at RT.
    2. Transfect cells by adding the reaction mixture in a drop-wise manner and incubate at 37 °C in 5% CO_2 for 24 hr.
6. Add 1 mg/ml geneticin for selection of GFP-positive clones at 37 °C in 5% CO_2.
7. After 6 days, isolate single cells by flow cytometry sorting in a 96-well plate harboring medium and HEK293 supernatant in a 1:1 ratio. Generate HEK293 supernatant from cultured confluent HEK293 cells that were centrifuged for 15 min at 4,966 x g.
8. On the following day, replace culture medium with medium containing 1.5 mg/ml geneticin. Change media once a week. If the cells form a confluent monolayer, transfer them to a 24-well plate. Sub-cultivate cells twice a week in a ratio of 1:5 in media containing 1.5 mg/ml geneticin. Finally, analyze the percentage of GFP-positive cells by immunoblot analysis and flow cytometry.

2. Analysis of Stable Cell Lines by Immunoblot Analysis

1. Remove the supernatant and wash the adhered cells once with warm PBS. Resuspend the cells in 100 µl of 2x Laemmli sample buffer, heat for 5 min at 95 °C and store at -20 °C.
2. Load approximately 4 x 10^4 cells per sample on a 12% SDS-PAGE gel. Additionally, load 6 µl of a commercial protein ladder as a molecular weight marker. Run gels in a gel electrophoresis system under a constant voltage of 180 V for 45-60 min with 1x Laemmli running buffer.
3. Blot proteins onto PVDF membranes (pore size of 0.45 µm) at a constant voltage of 16 V for 60 min using a Trans-Blot SD Semi-Dry Transfer Cell.
4. Block membranes in 10 ml of 5% nonfat dry milk in PBS/0.05% Tween 20 (MPT) for 1 hr at RT.
5. Dilute 4 µl of anti-GFP antibody in 4 ml of MPT and incubate membranes O/N at 4 °C.
6. Perform three 10 min washing steps with 10 ml of PBS/0.05% Tween 20.
7. Incubate membranes with 0.8 µl of horseradish peroxidase-conjugated secondary antibodies in 4 ml of MPT.
8. After 1 hr incubation at RT, wash membranes three times with PBS/0.05% Tween 20 (as described in 2.6) and detect horseradish peroxidase activity with a commercial kit according to the manufacturer’s protocol. Apply standard equipment for film development to visualize protein bands.

3. Analyze the Cells Using a Flow Cytometer.

1. Resuspend cells in PBS. Use HEK293 cells as negative control to adjust forward scatter (FSC) and side scatter (SSC) so that the cells are on scale. Draw a gate on living cells by excluding cell doublets, aggregates and cell debris.
2. Adjust the photomultiplier tube (PMT) gain so that the unstained cells are on the far left of the histogram for the FL1 channel (488 nm argon laser).

3. To analyze the fluorescence intensity of HEK293-GFP and HEK293-GFP-CaeB cells open the histogram of the FL1 channel and acquire 10,000 events on the gated population.
4. Analyze the data using commercial FACS analysis software.

4. Transfection of the Stable Cell Line with the Inducible Expression Vector System

1. Seed HEK293 cells stably expressing GFP or GFP-CaeB in a 12-well plate at a density of 1 x 10^5 cells/well.
2. 19 hr post-seeding, co-transfect the cells with regulator plasmid and response plasmid either encoding Bax or activated caspase 3.
    1. Co-transfect the stable HEK293 cells with 100 ng of pWHE125-P regulator plasmid (**Figure 2**) and 100 ng of the response plasmids pWHE655-hBax or pWHE655-revCasp3 (**Figure 3**) and 0.4 µl of polyethylenimine.
    2. Prepare the DNA and polyethylenimine transfection reagent each in 75 µl of Opti-MEM medium and incubate for exactly 5 min at RT. For complex formation, incubate both mixtures together for 15 min at RT.
3. Add the polyethylenimine/DNA solution drop-wise to the cells and incubate at 37 °C in 5% CO_2.

5. Induction of Apoptosis

1. 5 hr post-transfection, induce expression of the pro-apoptotic proteins by addition of 1 µg/ml doxycycline to the culture medium. Incubate cells for 18 hr at 37 °C in 5% CO_2.

6. Analysis of Host Cell Apoptosis by Immunoblot Analysis

1. Inspect cells prior to harvesting under the light microscope to check for cell death induction. Apoptotic cells are detectible by the presence of apoptotic bodies surrounding the dying cell.
2. Remove the supernatant and wash the adhered cells once with warm PBS. Resuspend the cells in 100 µl of 2x Laemmli sample buffer, heat for 5 min at 95 °C and store at -20 °C.
3. Load approximately 4 x 10^4 cells per sample on a 12% SDS-PAGE gel. Additionally, load 6 µl of a commercial protein ladder as a molecular weight marker. Run gels in a gel electrophoresis system under a constant voltage of 180 V for 45-60 min with 1x Laemmli running buffer.
4. Blot proteins onto PVDF membranes (pore size of 0.45 µm) at a constant voltage of 16 V for 60 min using a Trans-Blot SD Semi-Dry Transfer Cell.
5. Block membranes in 10 ml of 5% nonfat dry milk in PBS/0.05% Tween 20 (MPT) for 1 hr at RT.
6. Dilute 4 µl of anti-cleaved poly ADP-ribose polymerase (PARP) antibody in 4 ml of MPT and incubate O/N at 4 °C.
7. Perform three 10 min washing steps with 10 ml of PBS/0.05% Tween 20.
8. Incubate membranes with 0.8 µl horseradish peroxidase-conjugated secondary antibodies diluted in 4 ml MPT.
9. After 1 hr incubation at RT, wash membranes three times with PBS/0.05% Tween 20 (as described in 6.7) and detect horseradish peroxidase activity with a commercial kit according to manufacturer's protocol. Apply standard equipment for film development to visualize protein bands.
10. Remove bound antibodies by 30 min incubation at RT with 7 ml Western Blot Stripping Buffer.
11. After three washes with PBS/0.05% Tween 20 (as described in 6.7), probe the membranes with 4 µl of anti-actin antibody in 4 ml of MPT O/N at 4 °C.
12. After three washing steps with MPT (as described in 6.7), incubate the membranes with 0.8 µl of horseradish peroxidase-conjugated secondary antibodies diluted in 4 ml of MPT.
13. After 1 hr incubation at RT, wash membranes three times with PBS/0.05% Tween 20 (as described in 6.7 and detect horseradish peroxidase activity with a commercial kit according to the manufacturer's protocol. Apply standard equipment for film development to visualize protein bands.
    Note: All incubation steps were performed on a plate shaker for the indicated time and temperature.

Representative Results

First, HEK293 cell lines stably expressing the protein of interest (CaeB) as a GFP-fusion protein were established. As a control, HEK293 cell lines stably expressing GFP were also generated. Expression of GFP and GFP-CaeB was verified by immunoblot analysis. The representative immunoblot (**Figure 4A**) demonstrates stable and clearly detectable expression of GFP and GFP-CaeB. However, this assay cannot determine whether all cells express GFP or GFP-CaeB. Therefore, the stably transfected HEK293 cell lines were also analyzed by flow cytometry. As shown in **Figure 4B**, over 90% of the cells in both cell lines expressed GFP. Thus, these stable cell lines can be used to further analyze the function of CaeB. In the next step, the anti-apoptotic activity of CaeB was assayed by immunoblot analysis using an antibody against cleaved PARP. Proteolytic cleavage of nuclear PARP inactivates DNA repair activity and is a typical marker of the terminal stages of apoptosis. Cleavage of PARP is not detected in HEK293-GFP or HEK293-GFP-CaeB cells, demonstrating that neither the expression of GFP, nor of GFP-CaeB is toxic to the cells. However, when the cells were treated with staurosporine, a potent inducer of intrinsic apoptosis, cleavage of PARP was detected (**Figure 5**). Importantly, HEK293-GFP-CaeB cells exhibit reduced cleavage of PARP, indicating the anti-apoptotic activity of the *Coxiella burnetii* type IV secretion system effector protein CaeB[7].

To analyze at which step CaeB interferes with the apoptotic pathway, the Tet-On system was employed. In detail, HEK293-GFP or HEK293-GFP-CaeB cells were transfected with a regulator plasmid constitutively expressing a doxycycline-controlled dual repressor/activator setup (**Figure 2**) and a pTRE response plasmid encoding either full length human Bax or the activated form of human caspase 3, termed revCasp 3 (**Figure 3**). This system is tightly regulated, as demonstrated by the lack of PARP cleavage under non-inducing conditions (**Figure 6**). Addition of doxycycline to the transfected cells resulted in the expression of Bax or revCasp 3. If CaeB interferes with the apoptotic pathway downstream of Bax, then the apoptotic signal generated by expression and subsequent activation of Bax should be blocked by CaeB expression. Indeed, HEK293 cells stably expressing GFP-CaeB profoundly inhibit apoptosis induction by doxycycline-controlled Bax expression, while HEK293-GFP

cells displayed obvious PARP cleavage. This result indicates that CaeB blocks apoptosis induction downstream of Bax activation. Next, it was analyzed whether CaeB can interfere with caspase 3 activation — a late event in the apoptotic pathway. HEK293-GFP cells, as well as HEK293-GFP-CaeB cells, exhibit PARP cleavage (**Figure 6**), indicating that once the effector caspase 3 is activated, CaeB cannot prevent apoptosis from occurring. Taken together, these data demonstrate that CaeB acts between Bax and caspase 3 activation.

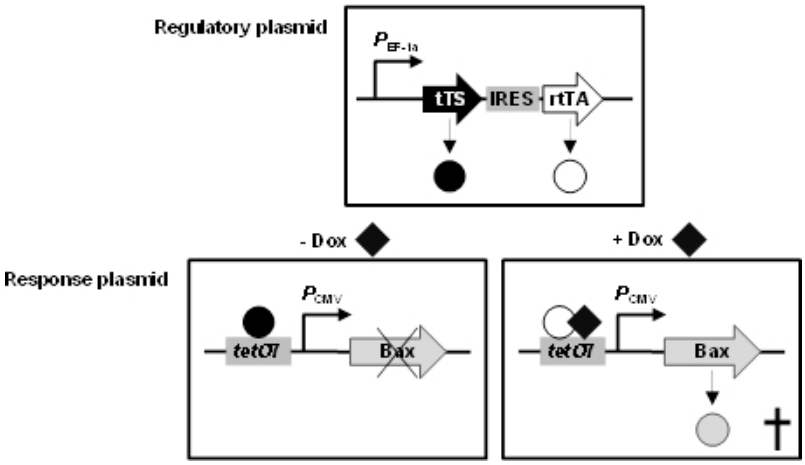

Figure 1. Schematic overview of the tetracycline regulatory system. The Tet-On system is composed of two different plasmids, the regulatory and response plasmids. The regulatory plasmid encodes the Tet-responsive transsilencer (tTS; filled circle) and the Tet-responsive reverse transactivator (rtTA, open circle). Both proteins are constitutively expressed under the control of the strong, constitutive elongation factor-1 alpha promoter (P_{EF-1a}). TTS and rtTA are chimeric transcription factors consisting of a eukaryotic transcriptional regulatory domain (silencer or activator, respectively) and a bacterial tetracycline repressor (TetR). TetR mediates DNA binding to seven *tet* operator (*tetO*) sequences on the response plasmid. *TetO* is located upstream of the inducible minimal cytomegalovirus promoter (P_{CMV}), together forming the Tet-responsive element (TRE). Under non-inducing conditions, tTS is bound to TRE preventing expression. After addition of doxycycline (Dox; filled diamond), rtTA interacts with Dox leading to conformational changes and activation. Activated rtTA replaces tTS on the response plasmid, allowing transcription of the respective target genes Bax and caspase 3, thus leading to apoptosis induction and cell death. Please click here to view a larger version of this figure.

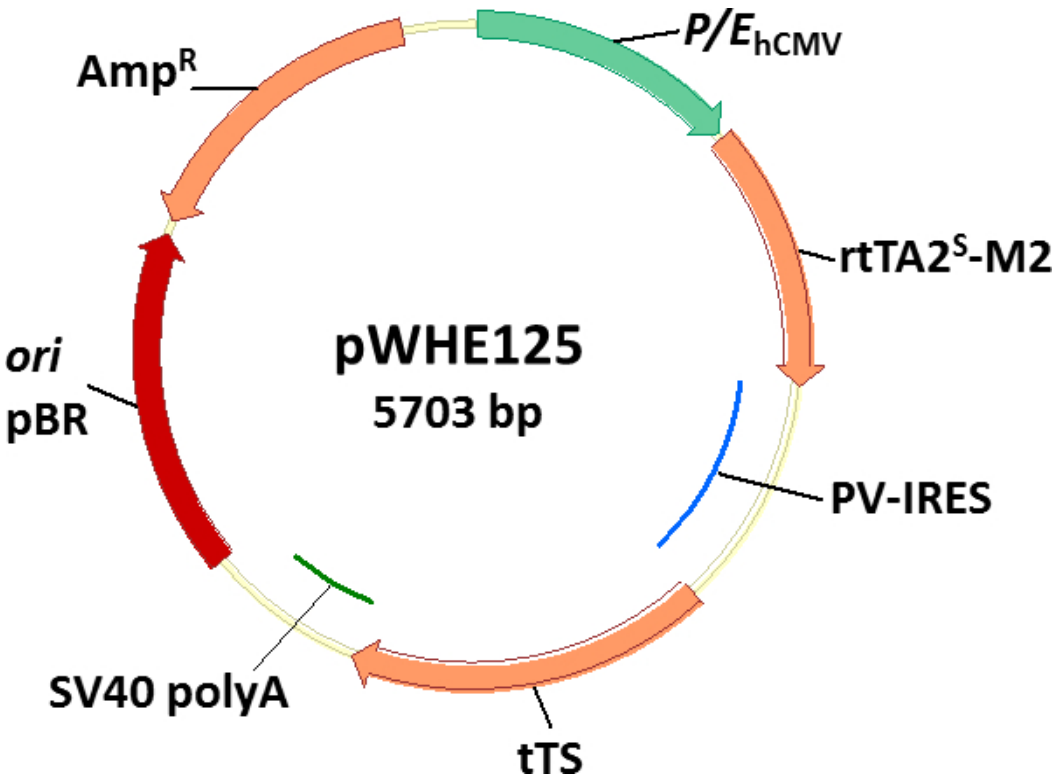

Figure 2. Schematic overview of the regulatory plasmid pWHE125. Elements essential for propagation in bacteria, including an origin of replication (*ori* pBR) and an antibiotic resistance cassette against ampicillin (Amp^R), and for expression of the Tet-transregulators, such as the strong, constitutive immediate early promoter/enhancer of the human Cytomegalovirus (P/E_{hCMV}), an internal ribosome binding site from poliovirus (PV-IRES) and a polyA-site from Simian virus 40 (SV40 polyA) are displayed. Please click here to view a larger version of this figure.

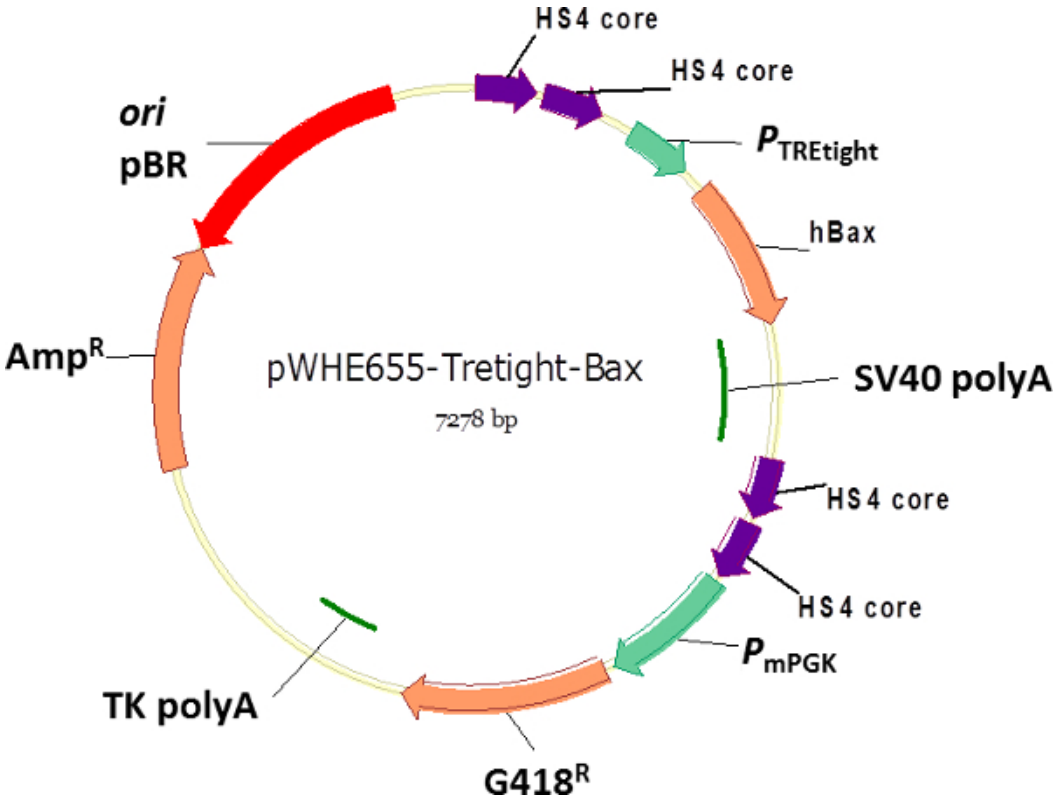

Figure 3. Schematic overview of the response plasmid. Elements essential for propagation in bacteria, including an origin of replication (*ori* pBR) and an antibiotic resistance cassette (Amp^R), and for inducible expression in eukaryotes, such as the transgene expression cassette with the Tet-dependent minimal promoter TREtight ($P_{TREtight}$), the gene of interest human Bax (*hBax*) and a polyA site from Simian virus 40 (SV40 polyA), are depicted. The transgene expression cassette is flanked by tandem direct repeats of two copies of the chicken HS4 insulator core sequence (HS4 core). Additionally, a G418 resistance cassette consisting of a murine phosphoglycerate kinase 1 promoter (P_{mPGK}), a neomycin resistance gene ($G418^R$) and a human thymidine kinase polyA site (TK polyA) is present. Please click here to view a larger version of this figure.

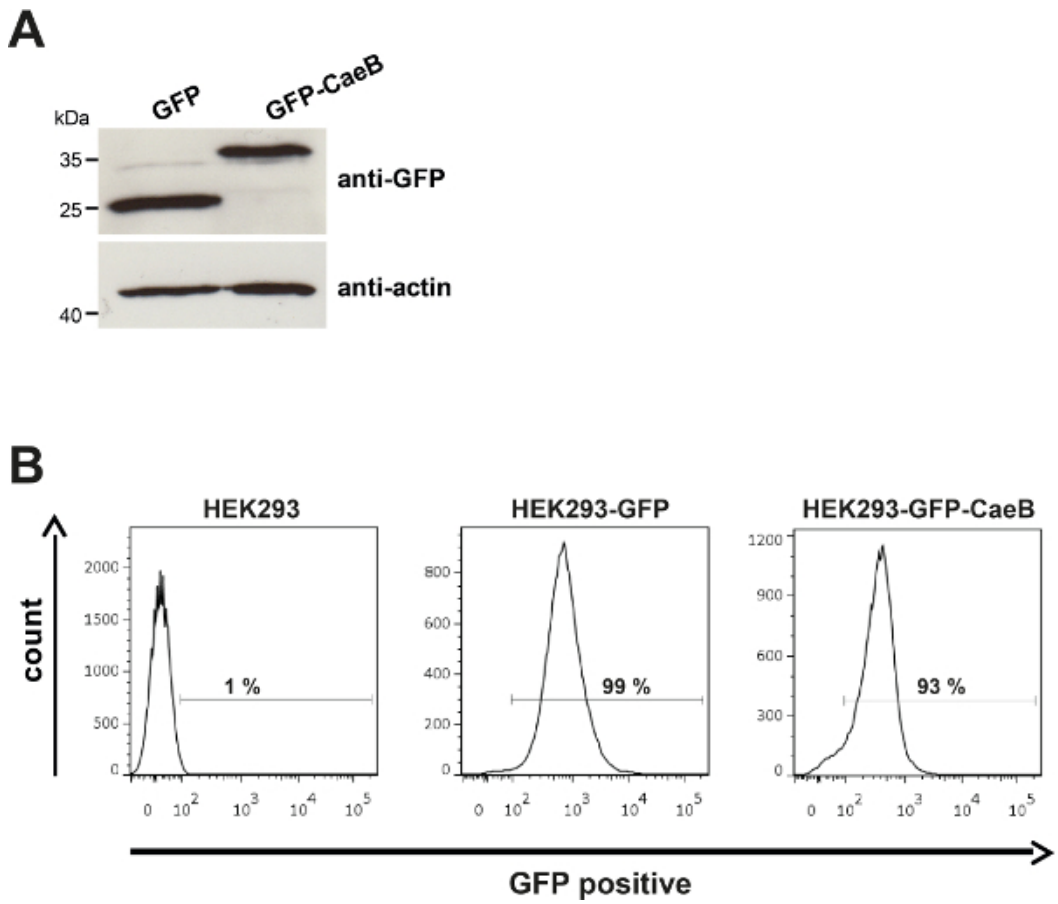

Figure 4. HEK293-GFP and HEK293-GFP-CaeB stably express green fluorescent proteins. (**A**) Whole-cell lysates of HEK293-GFP and HEK293-GFP-CaeB cells were immunoblotted for GFP to show stable expression. (**B**) Representative flow cytometry (FACS) analysis of HEK293-GFP and HEK293-GFP-CaeB cells is shown to demonstrate the intensity of the GFP expression. Please click here to view a larger version of this figure.

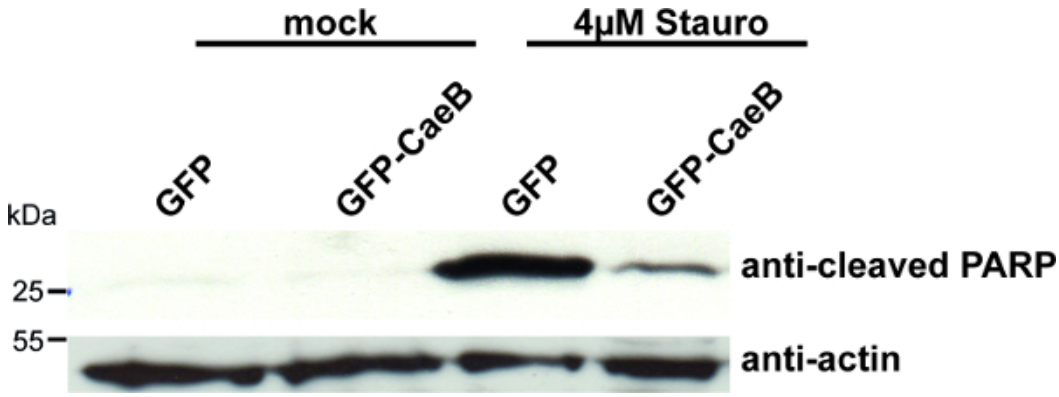

Figure 5. Effects of the expression of GFP-CaeB on staurosporine-induced apoptosis. HEK293-GFP and HEK293-GFP-CaeB cells were treated with 4 μM staurosporine for 6 hr to induce intrinsic apoptosis. Apoptosis was analyzed by cleaved PARP immunoblot analysis. PARP cleavage was reduced in HEK293-GFP-CaeB cells in comparison to HEK293-GFP cells. Please click here to view a larger version of this figure.

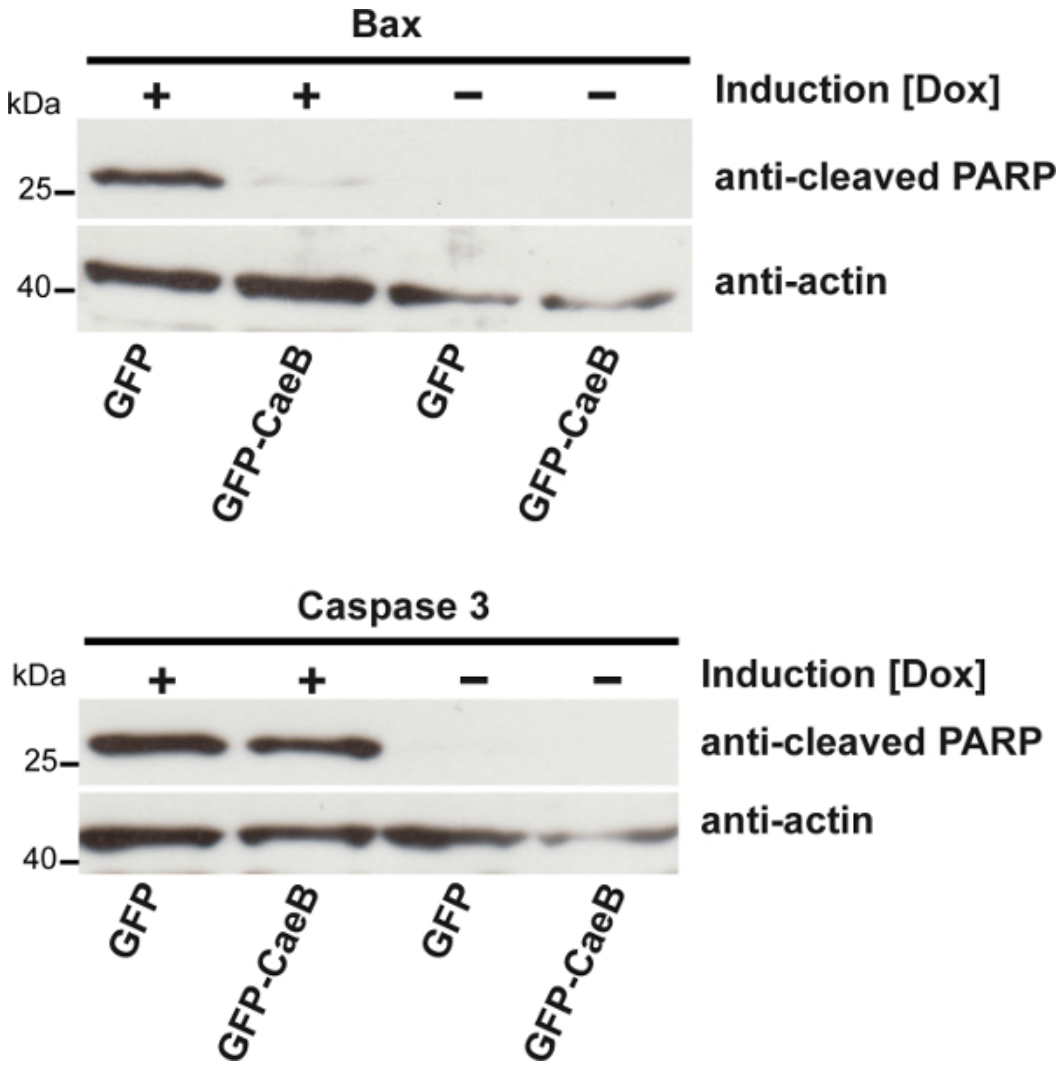

Figure 6. Usage of the TetOn system to narrow down the point of action of CaeB. (**A**) Apoptosis was induced by expression of Bax in HEK293-GFP and HEK293-GFP-CaeB cells. Apoptosis was analyzed by cleaved PARP immunoblot analysis. PARP cleavage was reduced in HEK293-GFP-CaeB cells in comparison to HEK293-GFP cells. (**B**) Apoptosis was induced by expression of revCasp 3 in HEK293-GFP and HEK293-GFP-CaeB cells. Apoptosis was analyzed by cleaved PARP immunoblot analysis. PARP cleavage was comparable in HEK293-GFP-CaeB and HEK293-GFP cells. Please click here to view a larger version of this figure.

Discussion

Many pathogenic bacteria harbor secretion systems to secrete or translocate bacterial effector proteins into the host cell. These effector proteins have the capacity to modulate processes and pathways in the host cell, allowing the bacteria to survive and replicate within their respective intracellular niche. Understanding the biochemical activities and the molecular mechanisms of the effector proteins will help towards a better understanding of pathogenicity and may help to develop new therapeutic tools to combat disease. Furthermore, as the effector proteins frequently exert their function by mimicking host cell activities, they can also be used to learn more about the biology of eukaryotic cells[1]. However, studying the biochemical activity of a single effector protein has proven to be complicated for several reasons: 1) functional redundancy among effector proteins, 2) the temporal regulation of engagement of the effector proteins, and 3) the effector protein working in context with another effector protein, interfering with its function. Therefore, the activity of an effector protein is commonly studied by overexpression studies. Unfortunately, the usage of transient overexpression of the effector protein also has several drawbacks. Thus, only single cell analysis assays can be employed, and the activity of the effector protein cannot be analyzed if the activity of the effector protein depends on the activity of another effector protein. To overcome at least the first drawback, stable cell lines can be used. In case the expression of the effector protein is toxic for the cell, this is not suitable. In this scenario, stable and inducible cell lines can be established using a similar system as we describe here for transient expression of apoptosis-inducing proteins (see below). However, our results (**Figure 5**) demonstrate that the expression of CaeB protects the cells from undergoing apoptosis and is therefore not toxic. There are a few options in dissecting at which step within the apoptotic cascade CaeB interferes with apoptosis induction. One possibility is to determine the host cell binding partner of CaeB. However, this is very challenging and the identification of the binding partner might not help to explain the point of action of CaeB within the apoptotic cascade. Another possibility is to use different apoptosis inducers, but CaeB inhibits apoptosis induction by two different apoptosis inducers, UV-light and staurosporine[7], indicating that this approach might also not lead to the identification of the point of action of CaeB. Another possibility in

narrowing down the point of action of CaeB, which was exploited here, is to use a system where the apoptosis signaling cascade can be induced at defined steps.

In case expression of the bacterial effector protein that was selected as protein of interest already interferes with cell physiology, its expression can also be placed under control of an inducible expression system, *e.g.*, such as the one used to express the target protein. Then, expression of both protein of interest and target protein will be concomitantly induced when the ligand is added to the cell culture allowing the investigator to monitor the cells for differential effects on cell physiology and signaling system output.

Clearly, expressing a protein of interest and trying to identify its point of action in a specific intracellular pathway by inducible expression of selected pathway proteins works best if the protein of interest interferes with the physiological activity of the selected pathway. In this case, readout is simply the lack of a signal normally associated with the pathway, such as cell death in the case of apoptotic signaling or activation of transcription in the case of Wnt signaling. In principle, proteins that activate a specific pathway can also be probed with this type of assay system. It only has to be set up differently, for example, using inducible expression of a si/sh/miRNA against a pathway protein and testing if the protein of interest can rescue pathway signaling. Alternatively, small molecule inhibitors of specific proteins can be used to interrupt signal transduction within a pathway and then to check for pathway rescue by inducible expression of a protein of interest. However, this type of approach will be experimentally more challenging than the protocol presented above.

The selection of the target gene to be probed in the interference assay is, in principle, straightforward. It is best to test as many of the proteins in the selected signaling pathway as possible to fine tune the identification of the protein of interest's site of interaction with the pathway. This should be done in an iterative fashion, because it is not always experimentally feasible or even possible to monitor all potential interaction partners in a single experiment. Therefore, it is best to first test several proteins spaced out evenly along the entire pathway and then to refine the experiment using the information gained in the first round. In signaling pathways, many proteins are modified post-translationally, either by a covalent modification, such as phosphorylation or sumoylation, or by a processing event, such as proteolytic cleavage. Consequently, the respective protein is either in a ground-state or in an activated form. If possible, it is best to test both forms of the protein for two reasons. One is that the activated form delivers a higher output signal[31], which presents a more stringent challenge for the protein of interest. In our proof-of-principle example, apoptotic signaling, the caspase 3 is activated by proteolytic cleavage and dimerization. This activation event can be simulated artificially by rearranging the unprocessed pro-caspase gene. The small subunit is artificially placed in front of the large subunit and both are joined by a linker. This is termed a "reverse caspase" and represents the activated form of the enzyme when expressed[35]. It was chosen due to its higher degree of activity. The second reason for probing both ground-state and activated forms of a pathway protein is because it allows one to distinguish if the protein of interest interferes with activation of the target protein, or if it acts downstream of the protein in the pathway. In the first case, cell death will be inhibited if the ground-state form is expressed, but not if the activated form is present. In the second case, neither protein will lead to apoptosis. Thus, testing both forms of a modified protein improves the resolution of the analytic system.

Cloning the target gene in the inducible expression vector requires several decisions. First, shall expression of the target gene occur only transiently or is it better if the vector is stably integrated into the genome of the host cell line? If only transient expression is required, a simple vector such as pUHC13-3[24], which contains a Tet-dependent promoter and a polyA site, or a bidirectional vector such as pBi-2 or pBi-3[36], which couples expression of the gene of interest to the expression of a reporter gene for easier monitoring of target gene expression, will be sufficient. If stable expression is preferred, a vector such as pWHE655[33] (**Figure 3**) should be used. It contains insulators that flank the transgene expression unit to prevent position effects at the genomic integration site from interfering with inducible transgene expression. It also contains a linked, but independently expressed, resistance cassette for G418 to facilitate the selection of vector integration events. The net result of these modifications is an increase in the number of clones with excellent regulatory properties[33,37,38]. cDNA sequences of target genes can be cloned into this vector by using the unique restriction sites for EcoRI and EcoRV[33]. Cloning of the vector expressing the reverse Caspase-3 cDNA was described in detail in reference 33. Cloning of the vector expressing human Bax was performed analogously. The human bax cDNA was amplified by PCR from the plasmid pVenus-Bax[39] using oligonucleotide primers that introduce restriction sites for EcoRI and EcoRV. After purification of the PCR fragment and restriction with the two enzymes, the fragment was ligated with likewise-restricted pWHE655, yielding pWHE655TREtight-Bax. Second, which minimal promoter is most appropriate for transgene expression? The original minimal promoter $P_{hCMV*-1}$[24] displays some cell-type specific leakiness[30], due in part to the presence of functional interferon-α inducible response elements[29]. This led to the development of second generation Tet-dependent promoters, termed ΔMtetO, TREtight and SG-TRE, with altered spacing and sequences between the *tetO* elements and with different minimal promoter sequences[33,40-42]. They display greatly reduced leakiness in the absence of doxycycline. Among these second generation promoters, ΔMtetO has the lowest transgene expression level in the presence of doxycycline[33]. TREtight mediates an intermediate and SG-TRE the highest level of transgene expression[33]. Further modifications have minimized background expression of the tetracycline responsive promoter even more[43,44]. Thus, the choice of a tetracycline-responsive promoter can be made according to the requirements or limitations of the respective transgene.

Independent of the type of experimental approach, tightness of expression of the target protein is important. For example, induction of apoptosis with activated forms of pro-apoptotic proteins leads to cell death, even when only minimal amounts of the respective target protein are expressed[31-33]. The required stringency is achieved by adding a tetracycline-dependent transsilencer to the conditional regulatory system[30]. It ensures that transcription from the inducible promoter is actively repressed, which is especially important when transient transfections are performed, since they are typically rather leaky in their transgene expression[30]. If signaling pathways that do not induce cell death are probed, the requirement for stringent regulation of target gene expression may be relaxed and inducible systems without a transsilencer could be used alternatively[43,44].

The inducible regulatory system used to probe the signaling pathway does not have to be the Tet system. Its major advantage is that it has been intensively used in mammalian cells for more than 20 years now and that it is the most highly developed and best characterized inducible control system. Nevertheless, there are at least 25 other conditional regulatory systems[45,46] that could be employed alternatively, or in addition to the Tet system. Two or three different inducible systems might be useful in probing convergent signaling pathways or for testing redundancy of either the target gene or the protein of interest.

The procedure presented here should work with transfection efficiencies that result in a robust and reliable output signal from the respective signaling pathway that is probed. Because the stable cell lines express the protein of interest in a homogenous manner (**Figure 4B**), all cells that

are transiently transfected with the inducible plasmid expressing the protein of interest are probed for interference of the protein of interest with pathway signaling.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) through the Collaborative Research Initiative 796 (SFB796) to A.L. and C.B., and through the ERA-NET PathoGenoMics 3rd call to A.L.

References

1. Galán, J. E. Common themes in the design and function of bacterial effectors. *Cell Host Microbe.* **5**, (6), 571-579 (2009).
2. Banga, S., *et al.* Legionella pneumophila inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family. *Proc Natl Acad Sci USA.* **104**, (12), 5121-5126 (2007).
3. Laguna, R. K., Creasey, E. A., Li, Z., Valtz, N., Isberg, R. R. A Legionella pneumophila-translocated substrate that is required for growth within macrophages and protection from host cell death. *Proc Natl Acad Sci USA.* **103**, (49), 18745-18750 (2006).
4. Schmid, M. C., *et al.* A translocated bacterial protein protects vascular endothelial cells from apoptosis. *PLoS Pathog.* **2**, (11), e115 (2006).
5. Nogueira, C. V., Carey, K. L., Roy, C. R. Inhibition of pathogen-induced apoptosis by a Coxiella burnetiitype IV effector protein. *Proc Natl Acad Sci USA.* **107**, (44), 18997-19001 (2010).
6. Rudel, T., Kepp, O., Kozjak-Pavlovic, V. Interactions between bacterial pathogens and mitochondrial cell death pathways. *Nat Rev Microbiol.* **8**, (10), 693-705 (2010).
7. Klingenbeck, L., Eckart, R. A., Berens, C., Lührmann, A. The Coxiella burnetii type IV secretion system substrate CaeB inhibits intrinsic apoptosis at the mitochondrial level. *Cell Microbiol.* **15**, (4), 675-687 (2012).
8. Raymond, B., *et al.* Subversion of trafficking, apoptosis, and innate immunity by type III secretion system effectors. *Trends Microbiol.* **21**, (8), 430-441 (2013).
9. Eckart, R. A., *et al.* Antiapoptotic activity of Coxiella burnetii effector protein AnkG is controlled by p32-dependent trafficking. *Infect Immun.* **82**, (7), 2763-2771 (2014).
10. Byrne, G. I., Ojcius, D. M. Chlamydia and apoptosis: life and death decisions of an intracellular pathogen. *Nat Rev Microbiol.* **2**, (10), 802-808 (2004).
11. Delft, M. F., Huang, D. C. S. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res.* **16**, (2), 203-213 (2006).
12. Willis, S. N., Adams, J. M. Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol.* **17**, (6), 617-625 (2005).
13. Shamas-Din, A., Kale, J., Leber, B., Andrews, D. W. Mechanisms of action of Bcl-2 family proteins. *Cold Spring Harb Perspect Biol.* **5**, (4), a008714 (2013).
14. Cory, S., Adams, J. M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer.* **2**, (9), 647-656 (2002).
15. Adams, J. M., Cory, S. Apoptosomes: engines for caspase activation. *Curr Opin Cell Biol.* **14**, (6), 715-720 (2002).
16. Suzuki, J., Denning, D. P., Imanishi, E., Horvitz, H. R., Nagata, S. Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. *Science.* **341**, (6144), 403-406 (2013).
17. Cory, S. Cell death throes. *Proc Natl Acad Sci USA.* **95**, (21), 12077-12079 (1998).
18. Enari, M., *et al.* A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature.* **391**, (6662), 43-50 (1998).
19. Fussenegger, M. The impact of mammalian gene regulation concepts on functional genomic research, metabolic engineering, and advanced gene therapies. *Biotechnol Prog.* **17**, (1), 1-51 (2001).
20. Felsher, D. W. Reversibility of oncogene-induced cancer. *Curr Opin Genet Dev.* **14**, (1), 37-42 (2004).
21. Maherali, N., *et al.* A high-efficiency system for the generation and study of human induced pluripotent stem cells. *Cell Stem Cell.* **3**, (3), 340-345 (2008).
22. Kozlova, E. N., Berens, C. Guiding differentiation of stem cells in vivo by tetracycline-controlled expression of key transcription factors. *Cell Transplant.* **21**, (12), 2537-2554 (2012).
23. Reijmers, L., Mayford, M. Genetic control of active neural circuits. *Front Mol Neurosci.* **2**, 27 (2009).
24. Gossen, M., Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA.* **89**, (12), 5547-5551 (1992).
25. Hillen, W., Berens, C. Mechanisms underlying expression of Tn10 .encoded tetracycline resistance. *Annu Rev Microbiol.* **48**, 345-369 (1994).
26. Deuschle, U., Meyer, W. K., Thiesen, H. J. Tetracycline-reversible silencing of eukaryotic promoters. *Mol Cell Biol.* **15**, (4), 1907-1914 (1995).
27. Robertson, A., Perea, J., Tolmachova, T., Thomas, P. K., Huxley, C. Effects of mouse strain, position of integration and tetracycline analogue on the tetracycline conditional system in transgenic mice. *Gene.* **282**, (1-2), 65-74 (2002).
28. Gossen, M., *et al.* Transcriptional activation by tetracyclines in mammalian cells. *Science.* **268**, (5218), 1766-1769 (1995).
29. Rang, A., Will, H. The tetracycline-responsive promoter contains functional interferon-inducible response elements. *Nucleic Acids Res.* **28**, (5), 1120-1125 (2000).
30. Freundlieb, S., Schirra-Müller, C., Bujard, H. A tetracycline controlled activation/repression system with increased potential for gene transfer into mammalian cells. *J Gene Med.* **1**, (1), 4-12 (1999).
31. Knott, A., *et al.* An optimized conditional suicide switch using doxycycline-dependent expression of human tBid. *Cancer Biol Ther.* **4**, (5), 532-536 (2005).
32. Danilchanka, O., *et al.* An outer membrane channel protein of Mycobacterium tuberculosis with exotoxin activity. *Proc Natl Acad Sci USA.* **111**, (18), 6750-6755 (2014).
33. Danke, C., *et al.* Adjusting transgene expression levels in lymphocytes with a set of inducible promoters. *J Gene Med.* **12**, (6), 501-515 (2010).

34. Maueröder, C., Chaurio, R. A., Platzer, S., Munoz, L. E., Berens, C. Model systems for rapid and slow induction of apoptosis obtained by inducible expression of pro-apoptotic proteins. *Autoimmunity.* **46**, (5), 329-335 (2013).
35. Srinivasula, S. M., *et al.* Generation of constitutively active recombinant caspases-3 and -6 by rearrangement of their subunits. *J Biol Chem.* **273**, (17), 10107-10111 (1998).
36. Baron, U., Freundlieb, S., Gossen, M., Bujard, H. Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res.* **23**, (17), 3605-3606 (1995).
37. Anastassiadis, K., *et al.* A predictable ligand regulated expression strategy for stably integrated transgenes in mammalian cells in culture. *Gene.* **298**, (2), 159-172 (2002).
38. Qu, Z., *et al.* Homogeneity and long-term stability of tetracycline-regulated gene expression with low basal activity by using the rtTA2S-M2 transactivator and insulator-flanked reporter vectors. *Gene.* **327**, (1), 61-73 (2004).
39. Spencer, S. L., Gaudet, S., Albeck, J. G., Burke, J. M., Sorger, P. K. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. *Nature.* **459**, (7245), 428-432 (2009).
40. Agha-Mohammadi, S., *et al.* Second-generation tetracycline-regulatable promoter: repositioned tet operator elements optimize transactivator synergy while shorter minimal promoter offers tight basal leakiness. *J Gene Med.* **6**, (7), 817-828 (2004).
41. Pluta, K., Luce, M. J., Bao, L., Agha-Mohammadi, S., Reiser, J. Tight control of transgene expression by lentivirus vectors containing second-generation tetracycline-responsive promoters. *J Gene Med.* **7**, (6), 803-817 (2005).
42. Hoffmann, A., Villalba, M., Journot, L., Spengler, D. A novel tetracycline-dependent expression vector with low basal expression and potent regulatory properties in various mammalian cell lines. *Nucleic Acids Res.* **25**, (5), 1078-1079 (1997).
43. Loew, R., Heinz, N., Hampf, M., Bujard, H., Gossen, M. Improved Tet-responsive promoters with minimized background expression. *BMC Biotechnol.* **10**, 81 (2010).
44. Heinz, N., *et al.* Retroviral and transposon-based tet-regulated all-in-one vectors with reduced background expression and improved dynamic range. *Hum Gene Ther.* **22**, (2), 166-176 (2011).
45. Toniatti, C., Bujard, H., Cortese, R., Ciliberto, G. Gene therapy progress and prospects: transcription regulatory systems. *Gene Ther.* **11**, (8), 649-657 (2004).
46. Ye, H., Fussenegger, M. Synthetic therapeutic gene circuits in mammalian cells. *FEBS Lett.* **588**, (15), 2537-2544 (2014).

