

RESEARCH ARTICLE

The Lumiptosome, an engineered luminescent form of the apoptosome can report cell death by using the same Apaf-1 dependent pathway

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ABSTRACT

Detection of the apoptosis signature becomes central in understanding cell death modes. We present here a whole-cell biosensor that detects Apaf-1 association and apoptosome formation using a split-luciferase complementary assay. Fusion of N-terminal (Nluc) and C-terminal (Cluc)-fragments of firefly luciferase to the N-terminus of human Apaf-1 was performed in HEK293 cells by using CRISPR-Cas9 technology. This resulted in a luminescent form of the apoptosome that we named 'Lumiptosome'. During Apaf-1 gene editing, a high number of knock-in events were observed without selection, suggesting that the Apaf-1 locus is important for the integration of exogenous transgenes. Since activation of caspase-9 is directly dependent on the apoptosome formation, measured reconstitution of luciferase activity should result from the cooperative association of Nluc-Apaf-1 and Cluc-Apaf-1. Time-response measurements also confirmed that formation of the apoptosome occurs prior to activation of caspase-3. Additionally, overexpression of the Bcl2 apoptosis regulator in transgenic and normal HEK293 cells confirmed that formation of the Lumiptosome depends on release of cytochrome *c*. Thus, HEK293 cells that stably express the Lumiptosome can be utilized to screen pro- and anti-apoptotic drugs, and to examine Apaf-1-dependent cellular pathways.

KEY WORDS: Apoptosis, Split luciferase, Apaf-1, Apoptosome, CRISPR-Cas9, Lumiptosome

INTRODUCTION

Programmed cell death, i.e. apoptosis, is a key regulatory event that is important for various cellular processes, including embryonal development, functioning of the immune system and cell turnover (Elmore, 2007; Wong, 2011). Defects in apoptosis can cause diseases, such as cell accumulation (cancer, restenosis) (Wong,

2011; Gerl and Vaux, 2005) or cell loss disorders (stroke, heart failure, neurodegeneration) (Kim and Kang, 2010; Ghavami et al., 2014). Thus, detection of apoptosis would help to evaluate therapy and screening of pro- and anti-apoptotic drugs. Cells undergoing intrinsic apoptosis processes show several biochemical changes, including translocation of phosphatidylserine (PS) from the cytoplasmic surface of the membrane to the cell surface (Zhang et al., 2018; Blankenberg et al., 1998; Johnstone et al., 2002). Apoptosis could be monitored through translocation of PS and binding of its negative charge to positive charge of annexin V. Unfortunately, exposure to PS also occurs during other biological processes, and numerous false-positive results have been reported (Demchenko, 2013; Ademchenko, 2012). DNA laddering as a feature of apoptosis is another way of apoptotic-cell assessment. But, according to previous reports, not only does this method include several stages but DNA cleavage cannot exclusively be used as an apoptosis marker. (Rahbar Saadat et al., 2015; Kraupp et al., 1995; Rao, 1998; Kataoka et al., 1995). Considering these limitations, monitoring of other important key factors of the intrinsic pathway, such as formation of the apoptosome and activity of caspases, are under specific attention (Würstle and Rehm, 2014; Lademann et al., 2003; Gyrd-Hansen et al., 2006). The apoptosome is a multiprotein complex comprising seven copies of Apaf-1 and seven copies of cytochrome *c* (Kim et al., 2005). In mammalian cells, Apaf-1 presents in the monomeric autoinhibited form. In response to intrinsic apoptosis-triggering stimuli, cytochrome *c* is released from mitochondria to the cytoplasm and binds to Apaf-1 monomers (Jiang and Wang, 2000). Replacement of ADP by dATP or ATP, and conformational changes in the Apaf-1 structure lead to its oligomerization that, in turn, activates caspase-9. It should be pointed out that, in the intrinsic pathway, the only way of autocatalytic activation of caspase-9 is through formation of the apoptosome. Activation of caspase-9 and subsequent cleavage of caspase-3, ultimately trigger cell death through sequential events (Li et al., 2017b; Hu et al., 2014; Shiozaki et al., 2002). Apaf-1 consists of three functional domains; the N-terminal caspase recruitment domain (CARD), the nucleotide-binding domain (NOD) and the C-terminal regulatory region, which contains 13 WD40 repeat domains that seem to keep Apaf-1 in an autoinhibitory state. Binding of cytochrome *c* to the C-terminal of WD40 domain leads to dATP/ATP-dependent Apaf-1 oligomerization (Zhou et al., 2015; Bao et al., 2005; Vaughn et al., 1999; Reubold et al., 2011).

In our previous study, we reported a novel apoptosome formation assay that is based on Apaf-1 oligomerization in response to induction of the intrinsic apoptosis pathway. The apoptosis reporter system was designed on the basis of a complementary split-luciferase assay, wherein firefly luciferase was split into two non-functional parts, i.e. the N-terminus (Nluc) and the C-terminus

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(Cluc), both of which were then fused to the Apaf-1 CARD domain (Torkzadeh-Mahani et al., 2012). Formation of the apoptosome upon induction of apoptosis brought about a reconstitution of luciferase fragments; emitted light was assessed in the presence of luciferase substrate. Costs and time required for *in vivo* reproductive and developmental toxicity studies have driven the development of *in vitro* alternatives. In a recent report, we used our split-luciferase assay to screen a library of 177 toxicants in order to identify inhibitors of apoptosome formation. Transient transfection of designed constructs was used for cell-based and cell-free assays (Noori et al., 2018). A cell-free assay indicated that pentachlorophenol (PCP) inhibits apoptosome formation and further investigation revealed a mechanism of PCP binding to cytochrome *c* (Tashakor et al., 2019). These data demonstrated the use of the cell-free assay in identifying apoptosome inhibitors.

Transient transfection is often suitable to study the impact of short-term expression of genes such as gene knockdown or silencing with inhibitory RNAs or protein production in a small scale. By contrast, stable transfection, especially precise integration, is more practical for long-term gene expression, such as large-scale protein production, long-term pharmacology studies and gene therapy (Stepanenko and Heng, 2017). Since apoptosome formation using split-luciferase complementary assay was used to, potentially, assess chemotherapeutic drugs, we used CRISPR-Cas9 technology to develop the luciferase reporter to a stable cell biosensor by integrating the split-luciferase fragments Nluc and Cluc into the *APAF1* gene of HEK293 cells, and to detect apoptosome formation following induction of apoptosis, by eliminating any defects of previous transient systems. CRISPR was considered to be the most versatile tool for gene editing (Adli, 2018; Wang et al., 2016); and HEK293 cells were used due to high yield of transfection, as well as correct folding, assembly and post-translational modification of recombinant proteins (Büssow, 2015; Schiedner et al., 2008). Maintenance of genome integrity and of transgene expression at physiological levels are crucial factors that need to be achieved, in order to create a stable cell line without pressure on the host cell (Kaufman et al., 2008).

After integration of Nluc and Cluc into Apaf-1 of HEK293 cells, we performed western blotting and activity assays to validate protein expression of functional Nluc- and Cluc-tagged Apaf-1. We found that the luminescent signal is consistent with the rate of cell death, and with caspase-9 and caspase-3 activity in a dose- and time-dependent manner in response to different chemotherapeutic drugs. Moreover, to validate whether formation of the Lumiptosome is dependent upon release of cytochrome *c*, and since overexpression of Bcl2 prevents cytochrome *c* release from mitochondria, we overexpressed Bcl2 in transgenic HEK293 cells and assessed activity of caspase-3 and the luciferase signal. Dependency of the Lumiptosome formation to release of cytochrome *c*, sensitivity to well-known Apaf-1 activators, as well as a few changes to caspase-9 and caspase-3 are advantages of our sensor when compared to the use of other assays, as its luminescence signal can be identified as the intrinsic pathway of apoptosis signature applying in parallel with the other orthogonal methodologies (Fig. 1).

RESULTS

Editing of Apaf-1 locus

Apoptosome formation in cells based on split luciferase assay has been previously investigated using transient expression of Apaf-1 fused to the split luciferase fragments (Torkzadeh-Mahani et al., 2012; Noori et al., 2018; Tashakor et al., 2019). However, in the previous studies the apoptosome was a heterogeneous mixture of endogenous native Apaf-1 and overexpressed Apaf-1

fused to the split luciferase fragments, which was ambiguous owing to the mixture of Apaf-1 forms. Since overexpression of Apaf-1 can trigger cell death (Perkins et al., 1998), we sought to fuse luciferase fragments to endogenous Apaf-1. Two plasmid donors (Nluc-Apaf-1 and Cluc-Apaf-1) were designed to target the Apaf-1 locus on chromosome 12 in the genome. Up- and downstream regions of the *APAF1* start codon were cloned into the MLM6-derived promoter-less plasmid that was deprived of selection cassettes. The cDNAs of complementary luciferase fragments, comprising the N-terminal amino acids 1–416 [Nluc-(G₄S)₃] and the C-terminal amino acids 395–550 [Cluc-(G₄S)₄], and a flexible G₄S linker sequence were positioned upstream the *APAF1*-start codon (Fig. 2A).

Three sgRNAs were designed to target *Streptococcus pyogenes* Cas9 (SpCas9) to the *APAF1*-5'-UTR, in close proximity to the start codon in exon 2 of the *APAF1* gene. To evaluate the frequency of mutations generated in the pools of transfected cells a TIDE assay was used. sgRNA1 was found to generate a higher frequency of mutations than the other two tested sgRNAs (sgRNA2 and sgRNA3) (Fig. S1).

Nluc-Apaf-1 and Cluc-Apaf-1 donors were co-transfected into HEK293. To validate whether Nluc and Cluc had accurately been inserted at the Apaf-1 locus, PCR genotyping was conducted by using specific primers (Fig. 2B; primer sequences are provided in Table S1). Unexpectedly, we found that the rate of integration for both donors was extremely high. Genotyping of clones identified 11 out of 22 (50%) and 19 out of 22 (86%) positive clones for Nluc-3'HA and Cluc-3'HA integration, respectively, without using any selection procedure (Fig. 2C). Imprecise integration was also detected for a few colonies; for example, clones 8, 13, 16, 17 and 18 were Nluc-3' HA negative, while being Apaf-1 transgene positive, and clone 1 was Cluc-3' HA negative but Apaf-1 transgene positive.

Integration assessment was repeated more than three times and the number of positive clones was approximately as high as the first time. Even though, the rate of integration and number of modified alleles were high, with some copies of *APAF1* that had remained unmodified (Fig. 2D). Since we needed a cell line with the entire pool of *APAF1* modified to Nluc-*APAF1* and Cluc-*APAF1*, another strategy was chosen. It is worth mentioning here that HEK293 cells are highly aneuploid; so, modification of all *APAF1* without any selection process was challenging.

Co-targeting of *APAF1* and *ATP1A1* genes to enrich *APAF1* gene modification

To enrich knock-in events, we thought cells could be subjected to a co-selection strategy as described by Agudelo et al. (2017). Thus, *ATP1A1* and *APAF1* were co-targeted with corresponding donor DNAs, followed by treating cells with the ATPase inhibitor ouabain to select for *ATP1A1* modifications. To examine whether this co-selection strategy enriches knock-in events of split luciferase cDNAs at Apaf-1 locus, PCR genotyping was carried out. Surprisingly, the rate of knock-in for both fragments was, again, remarkably high. Analyses of the PCR amplicons demonstrated that 8 out of 17 (47%) Nluc-3'HA clones and 13 out of 17 (76%) of Cluc-3'HA clones were positive. Imprecise integration was less frequent compared with *APAF1* gene editing without co-selection, i.e. amongst tested clones only clone 5 was simultaneously Cluc-3' HA negative and Apaf-1 transgene positive (Fig. 3A,B).

In addition, in clones 10, 11, 12 and 13, all *APAF1* copies were changed to Nluc-*APAF1* or Cluc-*APAF1*. Since the ratio of Nluc-*APAF1* and Cluc-*APAF1* fragments was not equivalent in these clones, *APAF1* gene editing was repeated while applying the co-selection strategy

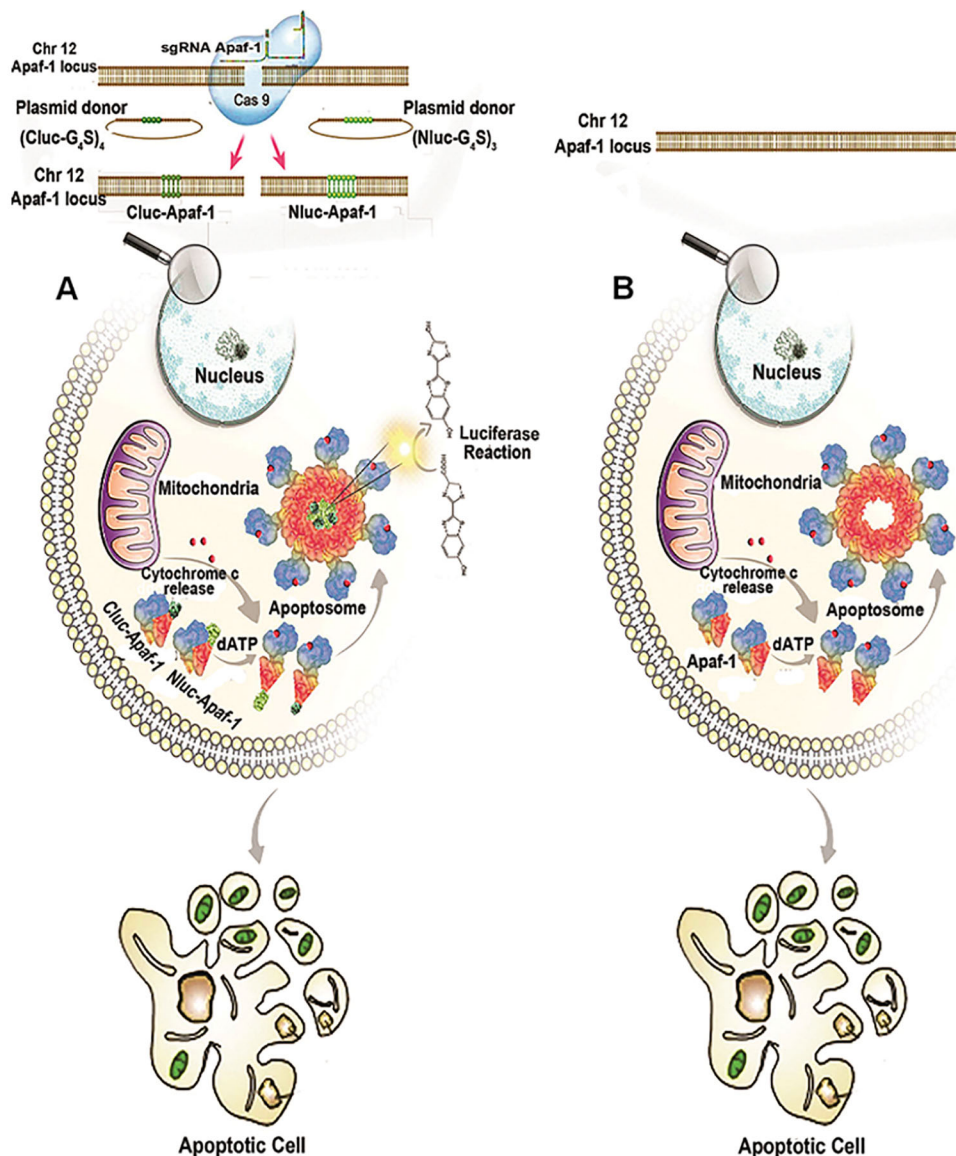


Fig. 1. Schematic illustration of Lumiptosome formation in transgenic cells and apoptosome formation in normal cells. (A) Integration of Nluc-(G₄S)₃ and Cluc-(G₄S)₄ in the Apaf-1 locus on chromosome 12 by CRISPR technology results in the formation of Nluc-Apaf-1 and Cluc-Apaf-1. Lumiptosome formation under the induction of apoptosis can be detected through split-luciferase complementary assay. (B) Apoptosome formation in normal cells in response to induction of apoptosis.

several times to find the clones with apparently equal numbers of *Nluc-APAF1* and *Cluc-APAF1* copies. Finally, two clones (clone 7 and 18) were selected for more evaluations (Fig. 3C,D).

By using Sanger sequencing analysis, junctions between donor and chromosomal sites were probed in the selected clones 7 and 18 that had indicated the precise insertion of Nluc and Cluc cDNAs within the Apaf-1 locus. Additionally, expression of split luciferase fragments fused to Apaf-1 was detected by using anti-Apaf-1-antibody. Predicted molecular masses of Nluc-Apaf-1 and Cluc-Apaf-1 are illustrated in Fig. 4A. Therefore, western blot confirmed the precise integration of split luciferase fragments at the Apaf-1 locus and absence of non-modified Apaf-1. To establish whether the fusion protein of Apaf-1 has the same intrinsic properties of Apaf-1 in the intrinsic pathway of apoptosis, cells were induced by addition of increasing concentrations (0.4, 0.6, 0.8, 1.2 μ M) of doxorubicin (DOX) for 16 h, and processing of procaspase-9 and procaspase-3 was studied by immunoblotting. As shown in Fig. 4B, cleavage in procaspase-9 resulted in p35 and p37 fragments that, in return, triggered processing of procaspase-3 and activation of caspase-3, which was detected by using antibodies against the cleaved fragments of caspase-3 (Fig. 4C).

Induction of apoptosis in transgenic cells

To determine the practicability of Lumiptosome formation assay on the basis of the bioluminescent signal, a dose-response assay was performed using three apoptosis inducers. At the first step, live-dead analysis of the cell population was carried out by nuclear staining differential assay using two fluorescent DNA intercalators, Hoechst 33342 dye and propidium iodide (PI), in transgenic and normal HEK293 cells (Fig. S2). Comparisons of normal and transgenic HEK293 cells (Fig. 5) shows that a higher death rate was detected after increasing the concentration of apoptosis inducers (DOX: $P=0.39$, etoposide: $P=0.46$, Nocodazole: $P=0.51$). At lower concentrations, the luciferase signal was measurable; however, at higher concentrations – because cells died quickly – it was impossible to detect the signal and luciferase activity dropped. Caspase-9 and caspase-3 activities were entirely consistent with results of the apoptosome formation assay. At lower concentrations of drugs, lower caspase-3 activity but higher caspase-9 and luciferase activities were detected because activation of caspase-9 is a prerequisite for activation of procaspase-3. However, at higher concentrations of drugs, caspase-9 and luciferase signals were at a plateau or a lower level of activity but caspase-3 activity was raised. Therefore, treated

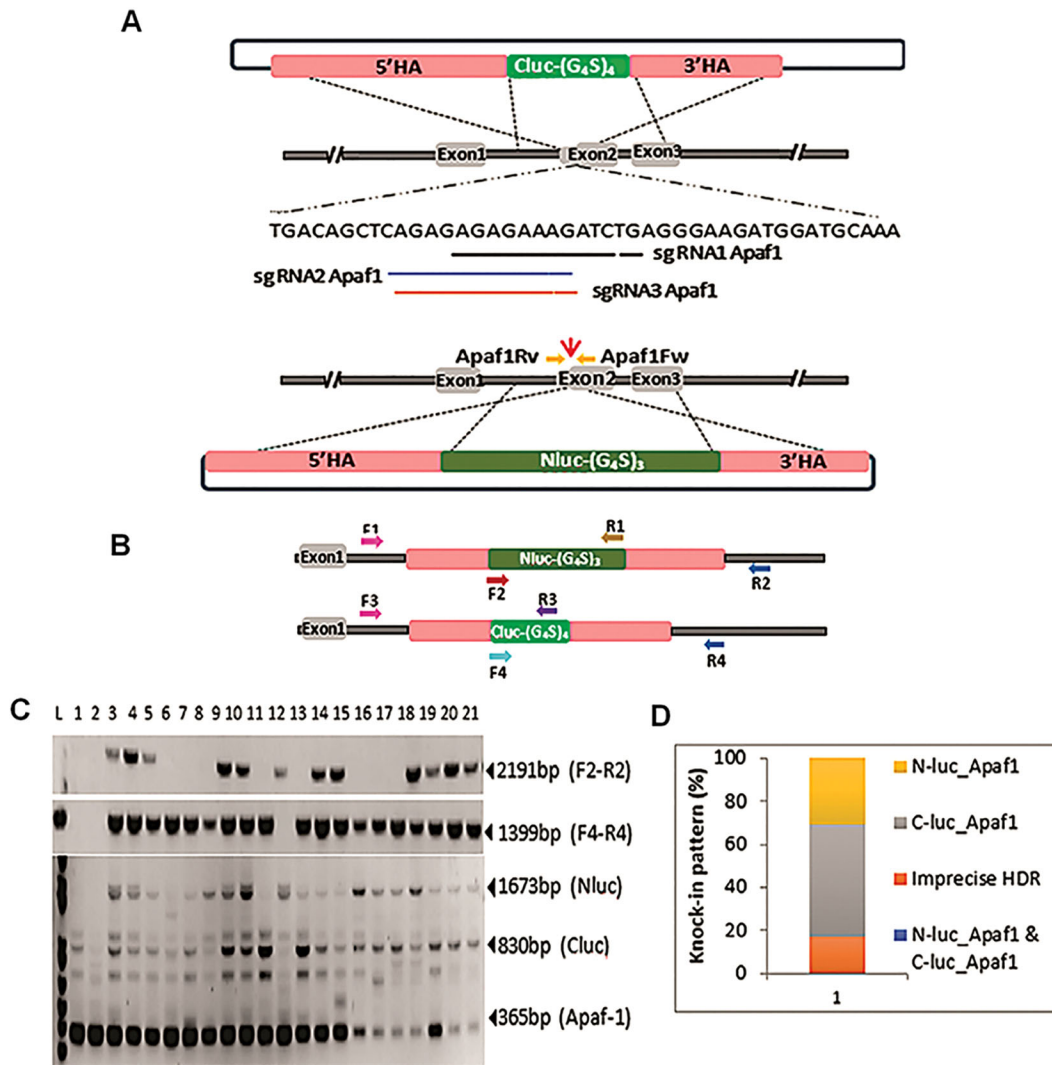


Fig. 2. Gene editing at the Apaf-1 locus in HEK293 cells by CRISPR-Cas9. (A) The start of the coding sequence of *APAF1* (Exon2) is triggered by two plasmid donors, indicated by Cluc-(G₄S)₄ and Nluc-(G₄S)₃ with 5' and 3' homology arms (HA). sgRNAs 1, 2 and 3 are indicated with their PAM motif underlined in black, blue and red, respectively. Primers Apaf1Rv and Apaf1Fw (indicated as yellow arrows) were used for PCR of the Apaf-1 transgene region. (B) Nluc 5'-junction PCR (F1-R1), Nluc 3'-junction PCR (F2-R2), Cluc 5'-junction PCR (F3-R3), Cluc 3'-junction PCR (F4-R4). (C) Agarose gel electrophoresis to analyze genotyping of the Apaf-1 locus by using 3'-junction PCR of Nluc (2191 bp), Cluc (1399 bp) and the transgene region of Apaf-1 [Nluc (1673 bp)], Apaf-1 [Cluc (830 bp)] and endogenous Apaf-1 (365 bp) by PCR. Labeling of lanes: L, molecular mass ladder; 1–21, number of clone. (D) Analysis of Nluc and Cluc knock-in events at the Apaf-1 locus. Imprecise homology-directed repair (HDR) indicates clones that are positive for the PCR of the transgene region but negative for 3'-junction PCR, i.e. clones 1, 7, 8, 16, 17 and 18.

transgenic cells triggered caspase-dependent apoptosis. Normal HEK293 cells showed the same trend of caspase-9 and caspase-3 functions, at the same concentrations of inducers. Neither apoptotic responses nor luminescence signals were detected in cell lines expressing only Nluc-Apaf-1 or Cluc-Apaf-1 as negative controls. Furthermore, to determine whether the observed apoptosis in transgenic cells was entirely dependent on Apaf-1 oligomerization, the apoptotic response of Apaf-1-deficient HEK293 cells (Apaf-1^{-/-}) was evaluated. Lower levels of cell death and no caspase-3 activity were detected after treatment with DOX (0.1, 0.3, 0.6, 0.9, 1.2 μ M) for 16 h, etoposide (1, 5, 10, 20, 30 μ M) for 16 h or Nocodazole (10, 15, 30, 60, 100 nM) for 12 h (Fig. S3).

In the following, time-response curves were determined at higher concentrations of DOX, etoposide or Nocodazole (0.8 μ M, 20 μ M or 60 nM, respectively), by using split luciferase complementary assay and analyzing caspase-3 activity. Time-response curves

revealed that luminescence intensity and caspase-3 activity reached a maximum response in cells after treatment with DOX (16 h), etoposide (14 h), and Nocodazole (10 h). At later time points, luciferase activity decreased, whereas that of caspase-3 was still high; this could be related to the process order (Fig. 6). It should be pointed out that apoptosome complexes comprising either native Apaf-1 or modified Apaf-1 with Nluc and Cluc have quite similar retention times when eluted from a gel filtration column.

To validate dependency of Lumiptosome formation on cytochrome *c* release, the apoptosis regulator Bcl2 was overexpressed in transgenic and normal HEK293 cells (Fig. S4). After 18 h, cell death rates of both transgenic and normal cells overexpressing Bcl2 were significantly lowered ($P=0.041$) in response to treatment with DOX (0.8 μ M) (Fig. 7A). However, death rates of transgenic and normal cells overexpressing Bcl2 or empty plasmid (pBABE puro) as a control, were significantly higher

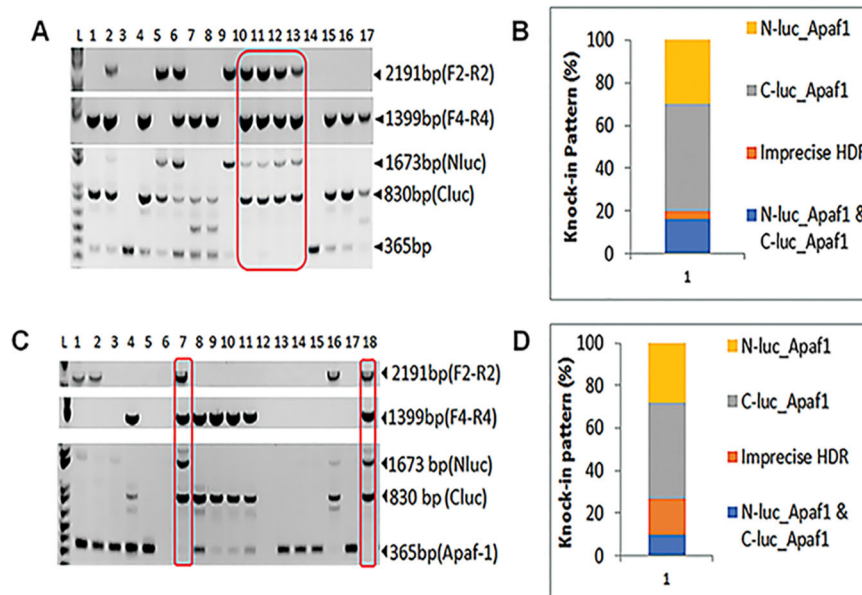


Fig. 3. Enrichment of knock-in events by co-selection targeting of two loci, Apaf-1 and ATP1A1. (A) Agarose gel electrophoresis showing 3'-junction PCR of Nluc (2191 bp), Cluc (1399 bp) and transgene region of Nluc-Apaf-1 (1673 bp), Cluc-Apaf-1 (830 bp) and endogenous Apaf-1 (365 bp). Labeling of lanes: L, molecular mass ladder; 1–17, number of clone. Encircled lanes indicate positive clones with all *APAF1* copies modified to *Nluc-APAF1* and *Cluc-APAF1*. (B) Analysis of the Nluc and Cluc knock-in events profile of the Apaf-1 locus. Imprecise homology-directed repair (HDR) indicates clones that are positive for the transgene region PCR but negative for 3'-junction PCR (i.e. clone 5). (C) Agarose gel electrophoresis showing the repeat of 3'-junction PCR of Nluc (2191 bp), Cluc (1399 bp) and the transgene region of Nluc-Apaf-1 (1673 bp), Cluc-Apaf-1 (830 bp) and endogenous Apaf-1 (365 bp) to acquire equal ratio between Nluc and Cluc. Labeling of lanes: L, molecular mass ladder; 1–18, number of clone. Encircled lanes indicate positive clones with all *APAF1* copies modified to *Nluc-APAF1* and *Cluc-APAF1*, with equal ratio. (D) Nluc and Cluc knock-in events profile of the Apaf-1 locus in the repeated PCR. Imprecise HDR indicates clones that are positive for the transgene region PCR but negative for 3'-junction PCR, i.e. clones 16, 19 and 23.

($P=0.019$) compared with those of untransfected cells ($P=0.030$), which could be related to transfection toxicity. In contrast, upon treatment with DOX, the death rates of Bcl2-overexpressing transgenic and normal cells were significantly lower ($P=0.001$) than those of pBABE puro-overexpressing cells (Fig. 7A).

In response to treatment with DOX, levels of luciferase activity in untransfected and pBABE puro-overexpressing transgenic cells was significantly higher ($P=0.04$) compared with those overexpressing Bcl2 (Fig. 7B). Levels of caspase-9 activity in transgenic and normal cells overexpressing pBABE puro or Bcl2, were significantly higher ($P=0.001$ and $P=0.004$, respectively) compared with those of untransfected cells (Fig. 7C). Moreover, upon treatment with DOX, levels of caspase-9 activity in transgenic and normal cells overexpressing pBABE puro or not was significantly higher ($P=0.001$, $P=0.002$, respectively) compared with those overexpressing Bcl2 (Fig. 7B). The levels of caspase-3 activity assessed by pBABE puro- or Bcl2-overexpressing transgenic and normal cells were significantly higher ($P=0.009$ and $P=0.006$, respectively) than in untransfected cells. In response to DOX treatment, levels of caspase-3 in transgenic and normal cells overexpressing pBABE puro or not (untransfected) were significantly higher ($P=0.004$ and $P=0.008$, respectively) compared with those cells overexpressing Bcl2 (Fig. 7D).

Overexpression of Bcl2, therefore, seems to hamper cytochrome *c* release and abrogate the intrinsic pathway of apoptosis. However, a low level of cell death could be detected, which might be due to overexpression of pBABE puro and Bcl2 in transgenic and normal cells, leading to activation of caspase-9 and caspase-3.

DISCUSSION

Apaf-1 has long been studied for its role in the intrinsic pathway of apoptosis (Yoshida et al., 1998) and a model for apoptosome

structure based on single-particle, cryo-electron microscopy has been reported recently (Zhou et al., 2015). In this study, split luciferase fragments were tagged to Apaf-1, yielding a construct that we termed 'Lumiposome' – a luminescent form of the apoptosome, which can indicate the oligomerization of modified Apaf-1 upon induction of apoptosis in response to apoptosis inducers. Since Nluc and Cluc parts of the split luciferase integrated accurately at the Apaf-1 locus, and non-modified Apaf-1 was no longer present, physiological level of expression occurred in target cells leaving no other possibility for functional apoptosome formation. Thus, despite our previous report on transient expression of recombinant Apaf-1 followed by formation of heterogeneous apoptosomes of native and recombinant Apaf-1, we got rid of any intervention from free and endogenous Apaf-1 in our current study. Here, we measured the innate behavior of cells under intrinsic pathway of apoptosis detected by luciferase complementary assay. Various biosensors have been established based on the split luciferase strategy due to the production of detectable signal and high quantum yield (Azad et al., 2014; Chen et al., 2017; Leng et al., 2013; Li et al., 2017a; Mohammadi et al., 2017). Specificity and accuracy of measuring is crucial to study a biological process. Although several modalities have been applied to assess apoptosis, including DNA fragmentation, caspase processing assays, membrane alterations and mitochondrial assays, the most significant issue regarding apoptosome formation assay is due to the specificity of this complex compared with other events of the intrinsic pathway of apoptosis (Papaliagkas et al., 2007). Stable cell lines are able to express exogenous genes permanently. To locate a gene of interest under the control of the endogenous promoter in order to obtain locus-specific integration and physiological expression can be achieved by using CRISPR-Cas9 technology. In spite of knock-in experiments with low level of integration and associated limitations

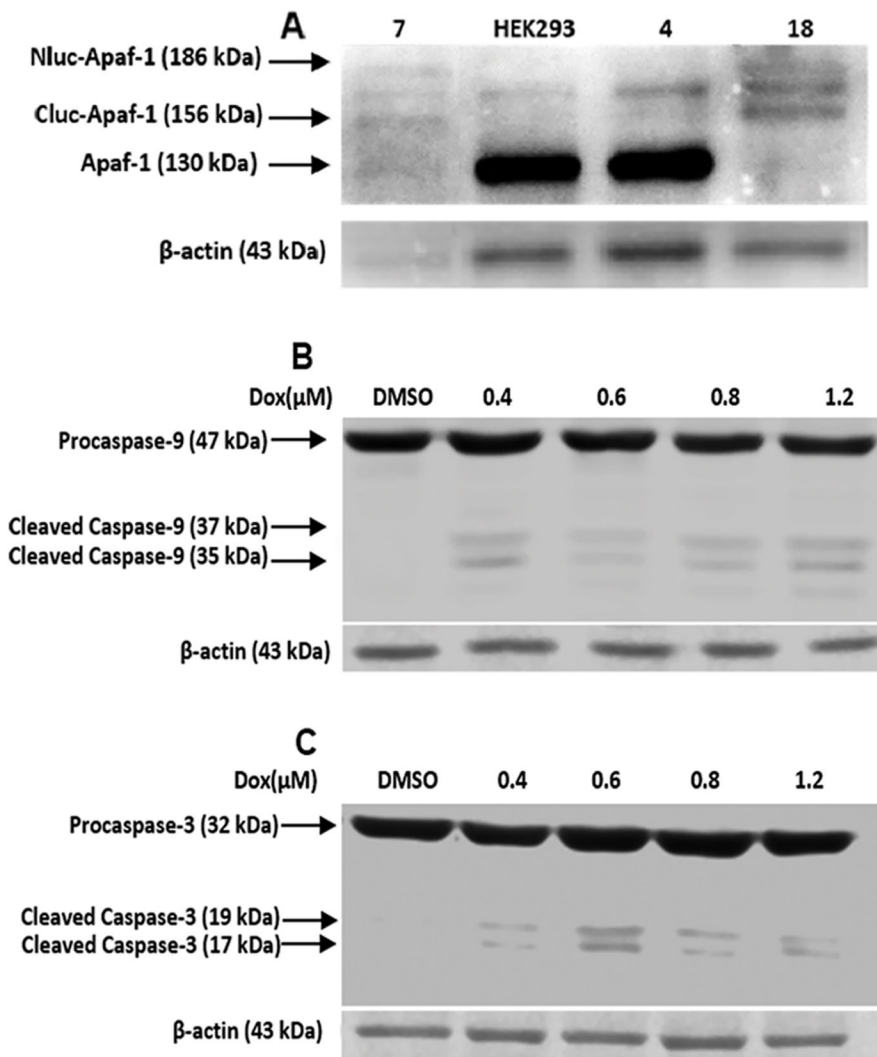


Fig. 4. Protein expression validation by immunoblotting. (A) Western blotting of clones 7, 4 and 18. Clones 7 and 18 indicate Nluc and Cluc integration at the Apaf-1 locus without unmodified Apaf-1 copies; Clone 4 shows Cluc integration and unmodified copies of Apaf-1. (B,C) Western blotting of HEK293 cell extracts. Transgenic HEK293 cells treated with DOX at concentrations as indicated for 16 h, and caspase-9 and caspase-3 processing was detected by immunoblotting. Extracts of cells treated with DMSO were used as a control, β-actin was used as a loading control.

(Xu et al., 2018; Jang et al., 2018; Park et al., 2017), our current results illustrate that the Apaf-1 locus possesses a potential for higher rate of integration. The co-selection strategy also helped us to enhance the knock-in event and obtain clones with modification of all *APAF1* gene copies to fusion of *Nluc-APAF1* or *Cluc-APAF1*, because robust gene editing without exogenous selection markers is the main advantage of this method. It seems that biased knock-in events occurred regarding Cluc compared with Nluc, as we obtained more Cluc-positive colonies; this could be because Cluc is three times smaller than Nluc. We also conducted several experiments to confirm that the measured reconstituted luciferase activity directly related to oligomerization of Nluc-Apaf-1 and Cluc-Apaf-1. Various inducers trigger apoptosis through different mechanisms. The proapoptotic and chemotherapeutic agents Doxorubicin (DOX, also known as Adriamycin; Patel and Kaufmann, 2012), etoposide (Montecucco et al., 2015) and Nocodazole (Xu et al., 2002) were selected to trigger apoptosis in cells. DOX and etoposide halt topoisomerase II activity through binding to the cellular DNA, whereas Nocodazole attaches to the tubulin dimers and changes their direction of movement. Transgenic and normal cells were treated with DOX, etoposide and Nocodazole, and the percentage of living and dead cells was determined as described by Lema et al. (2011). To confirm that the Lumipptosome signal can be used to measure apoptosome

formation regardless of the mechanism of apoptosis induction, dose-response curves of three different proapoptotic compounds were investigated. We concluded that an increased concentration of inducers increased the death rate of cells and the activity of caspases, which was in accordance with the trend of luciferase complementary activity. At higher doses of DOX, etoposide and Nocodazole, most of the cell population was at the stage of late apoptosis, because caspase-3 activity was increased while the detected luciferase signal was decreased. In addition, apoptosis time-course studies of transgenic and normal cells revealed that luminescence signals dropped at longer times of treatment with apoptosis inducers, whereas caspase-3 activity reached the maximum response, which might indicate the order of the intrinsic apoptosis steps. Compared with normal HEK293 cells, no significant differences were observed in transgenic HEK293 cells, regarding caspases activity and cell sensitivity in response to apoptosis inducers after cell modification of Apaf-1. As a result, at a higher concentration of inducers, oligomerization of Apaf-1 was detectable earlier. Luciferase complementation, together with the active forms of caspase-3 and/or caspase-9, suggests correct juxtapositioning of Apaf-1 and apoptosome formation despite modification of the Apaf-1 N-terminal. If it had not been for low expression of innate Apaf-1, much higher luciferase activity upon Apaf-1 oligomerization would have been obtained. However,

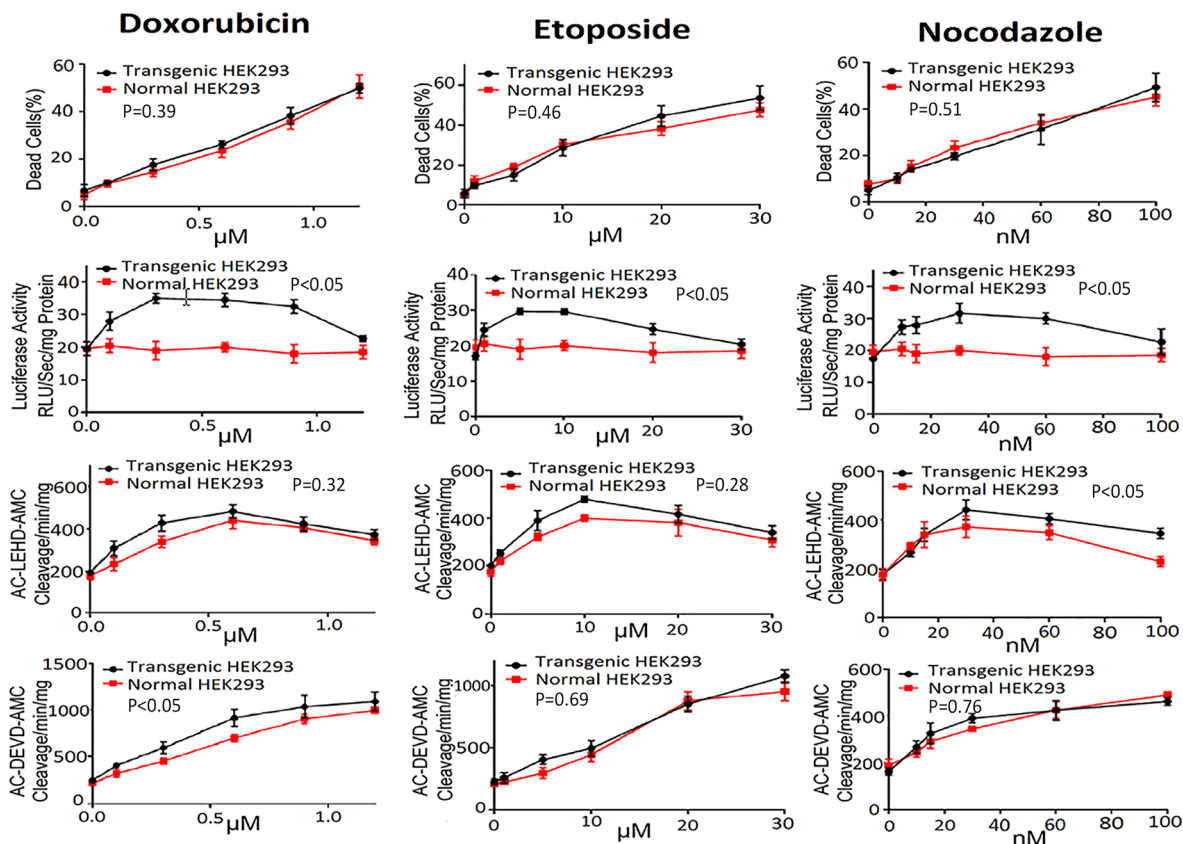


Fig. 5. Statistical trend analysis on the basis of dose-dependent apoptosis activation upon apoptosome formation in transgenic and normal HEK293 cells. Dose-response curves of DOX-, etoposide- or Nocodazole-induced apoptosis of transgenic and normal HEK293 cells. Cell death rates were evaluated after Hoechst and PI nucleus staining, apoptosome formation deduced from split-luciferase complementary signals, caspase-9 activation based on AC-LEHD-AMC cleavage and caspase-3 activity based on AC-DEVD-AMC cleavage. All values are mean $\pm$ s.d. of three separate experiments ($n=3$).

according to our results, even a small fraction of Apaf-1 (Fig. 4) seems to be sufficient to activate the caspase cascade in apoptotic cells. This is similar to the findings in a previous report on dATP-activated cell lysates, where all Apaf-1 oligomerized (Cain, 2003).

Other validation data achieved from the loss of apoptosome function through overexpression of Bcl2 in transgenic and normal cells to suppress cytochrome *c* release from mitochondria. Attachment of cytochrome *c* to the WD40 domain of Nluc-Apaf-1

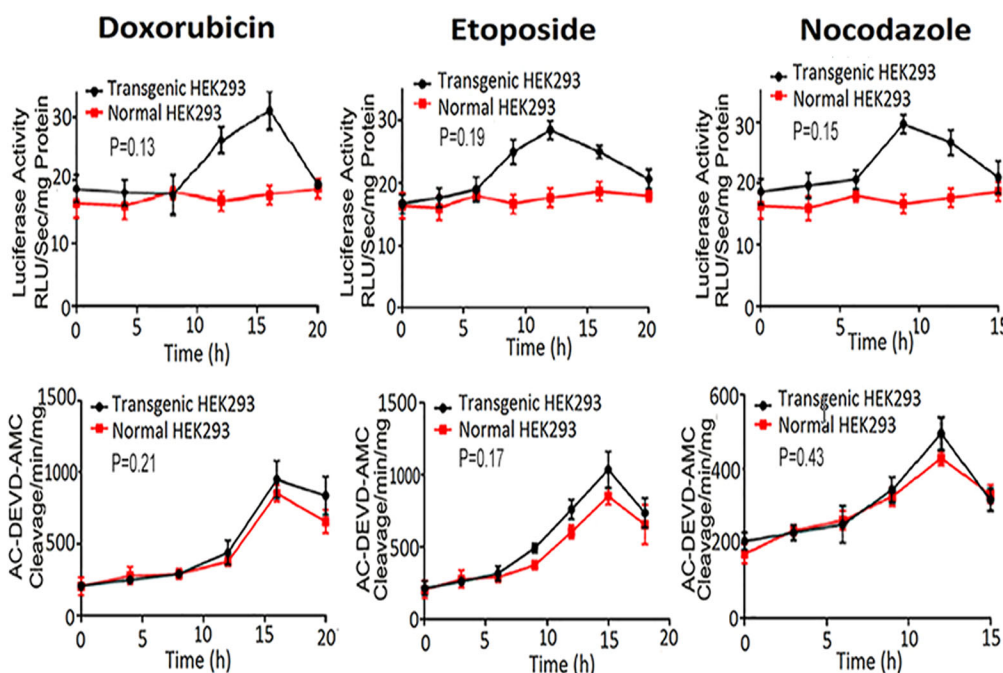


Fig. 6. Time-dependent apoptosis activation upon apoptosome formation in transgenic and normal HEK293 cells. Time-course assessment of DOX-, etoposide- and Nocodazole-induced apoptosis determined by apoptosome formation based on split-luciferase complementary signal and caspase-3 activity through AC-DEVD-AMC cleavage for transgenic and normal HEK293 cells. Values are mean $\pm$ s.d. of three separate experiments ($n=3$).

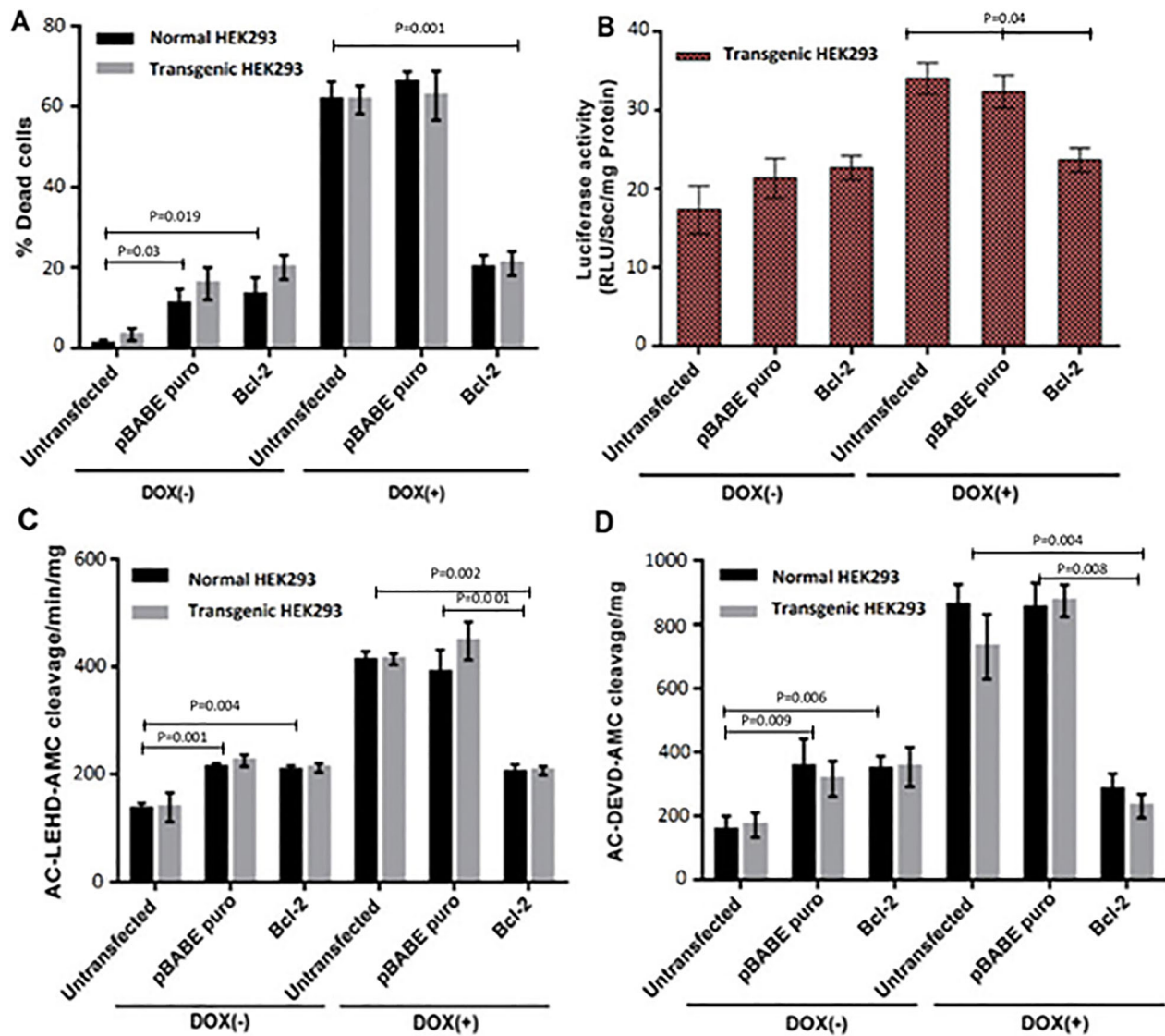


Fig. 7. Bcl2 overexpression in transgenic and normal HEK293 cells to arrest cytochrome c release. Transgenic and normal HEK293 were left either untransfected or were transfected with control plasmid (pBABE puro) or with Bcl2 (Bcl-2). Induction of apoptosis was achieved by treatment with (+) or without (-) Dox (0.8 μ M). (A) After 18 h death rates (i.e. 20%) were evaluated using Hoechst 33342 and or PI nucleus staining, showing that overexpression of Bcl2 resulted in the inhibition of cytochrome c release and lower levels of apoptosis compared to untransfected cells or cells transfected with pBABE puro plasmid. (B) Apoptosome formation based on the split-luciferase complementary signal, showing decreased levels of luciferase signal because overexpression of Bcl2 prevented the formation of the apoptosome compared to cells transfected with pBABE puro or untransfected cells, both of which did show apoptosome formation. (C,D) Caspase-9 activation measured using AC-LEHD-AMC cleavage (C) and caspase-3 activity measured using AC-DEVD-AMC cleavage (D), showing decreased levels of caspase-9 and caspase-3 because apoptosome formation was inhibited in cells overexpressing Bcl2 compare to cells, transfected with pBABE puro or untransfected cells. Values are mean $\pm$ s.d. of three separate experiments ($n=3$). P -value labels relate to the bars below.

and Cluc-Apaf-1 causes conformational change in Apaf-1 and, consequently, replacement of ADP with dATP or ATP triggers formation of the Lumiposome. Reconstituted luciferase and caspase-3 activities decreased dramatically 48 h after overexpression of Bcl2 in response to induction of apoptosis by Dox (0.8 μ M). Therefore, this stable HEK293 cell line harboring the split luciferase apoptosis reporter can be readily used to screen cancer drugs. Moreover, Apaf-1 oligomerization can be detected in various cell lines by using this split luciferase reporter strategy.

It should also be emphasized that another basic aspect of the current work is that modification of the Apaf-1 N-terminus did not affect oligomerization of Apaf-1 and formation of apoptosome. This is intriguing because, based on previous reports, high concentration of cytochrome c was required to induce significant

caspase-3 activity in extracts from cells expressing Apaf-1_{K160R} (Hu et al., 1999). Consistent with this finding, the Apaf-1 NOD p-loop mutant (K160R), which is partially resistant to oligomerization (Hu et al., 1999), was also less efficient at stimulating split-luciferase activity. In contrast to this mutant, expression of modified forms of Apaf-1 in cell extract without any endogenous Apaf-1 did not stop CARD-CARD interactions in these modified forms of Apaf-1 and consequent complementary luciferase activity.

Based on the results presented in this article, we conclude that a genetically modified apoptosome – i.e. the Lumiposome – like the native apoptosome, can induce cell death through the recruitment of procaspase-9 and the consequent activation of caspase-3. Moreover, higher rate of integration at upstream of the *APAF1* locus indicates a region that is prone to mutations by CRISPR-Cas9. The reporter

constructs obtained by us might have the potential for high-throughput screening of chemical libraries against proteins involved in apoptosis.

MATERIALS AND METHODS

sgRNA design and plasmid construction

Three single guide RNAs (sgRNAs) targeting the start codon region of the human *APAF1* gene on chromosome 12 (q23.1) were designed using the CRISPOR web tool (<http://crispor.tefor.net/>). sgRNA1 (5'-GAGAAAGATCTGAGGGAAGA-3') by using the protospacer adjacent motif (PAM) TGG at the beginning of the start codon; sgRNA2 (5'-CTCAGAGAGAGAAAGATCTG-3') by using the PAM AGG four nucleotides upstream of the start codon; and sgRNA3 (5'-TCAGAGAGAGAAAGATCTGA-3') by using the PAM GGG three nucleotides upstream of the start codon. sgRNA oligos were cloned in an MLM3636-derived vector (Addgene #43860, a kind gift of the Joung lab). The Cas9-expression vector was Addgene #41815 from the Church lab. ATP1A1 G3 sgRNA (5'-GAGTTCTGTAATTCAGCATA-3'). Both were used together with a eSpCas9-expression vector targeting the *ATP1A1* intron that is located adjacent to the exon encoding the first extracellular loop of the ATP1A1 pump (Agudelo et al., 2017).

Donor plasmid construction

The luciferase sequence was divided into two parts: the N-terminal fragment (Nluc; aa 1–416) and the C-terminal fragment (Cluc; aa395–550; Ozawa, 2006; Paulmurugan and Gambhir, 2003). Accordingly, Nluc and Cluc donor plasmids, containing N- (amino acids 1194–1650) and C-terminal fragments (amino acids 1194–1650) of the luciferase sequence from *Photinus pyralis* were derived using the pGL3-Control Vector (Promega) (Misawa et al., 2010) and amplified by PCR using the primers Nluc_Forward (5'-CTAGCTAGCATGGAAGACGCCAAAAAC-3'), Nluc_Reverse (5'-CTATGTCGACTCCGCCTCTCCAGATCCGCCTC-CACCTGACCCGCCACCTCCACTATCCTTGTCATCAAGCGCTTG-GTC-3'), Cluc_Forward (5'-GTAGCTAGCATGGCTCCTATGATTATGTCGGTTATGT-3') and Cluc_Reverse (5'-ACTATGTCGACTCCG-CCTCCACCTGATCCGCCTCTCCAGATCCGCCTCCACCTGACCC-GCCACCTCCACTCAGGCGATCTTCCGCCCTTC-3'). A flexible Gly-Ser linker between fragments of the split luciferase and *APAF1* genes, and a Kozak consensus sequence initiating with the ATG codon for an efficient translation were joined in the forward primers of Nluc and Cluc fragments (Chumakov et al., 2012; Kato and Jones, 2010). The PCR products were cloned into a MLM6-derived vector that had previously been cloned to obtain the (5' HA-1100 bp and 3' HA-800 bp) homology arms of the target site within the Apaf-1 locus. It is important to note that this donor vector is deprived of any selection cassettes. The single-stranded oligonucleotide donor (RD ssODN), giving rise to the double replacements of Q118R and N129D (RD) in the ATP1A1 alleles conferring ouabain resistance, was used as previously described (Agudelo et al., 2017).

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C under 5% CO₂. HEK293 cells (7×10⁵) were co-transfected with 2 µg of Cas9 expression plasmid together with 2 µg of Apaf-1-sgRNA 1, 2 or 3, using Lipofectamine 3000. Activity of sgRNA was analyzed by TIDE assay as described below; sgRNA1 was found to be the most efficient. Following selection of the most-efficient sgRNA, 10⁶ HEK293 cells were co-transfected with 1 µg of Cas9/G3 expression plasmid and 0.4 µg of ssODN Q118R N129D (RD) together with 1 µg of Apaf-1-sgRNA1 expression plasmid, 3 µg of Nluc and 3 µg of Cluc donors by using V solution (Lonza) and the Amaxa D-023 program (AMAXA electroporation system). When indicated, selection of ouabain-resistant cells was initiated 48 h after transfection by the addition of the ATPase inhibitor ouabain (1 mM). 10 days after treatment, cell sorting was carried out to isolate single cell colonies.

TIDE assay and analysis of indel rate

TIDE assay was performed to analyze the rate of indels created by the designed sgRNAs at the targeted locus. 72 h after Cas9/sgRNAs transfection, genomic DNA (of ~7×10⁵ cells) was extracted using a DNA

mini kit (EZNA tissue DNA kit, Omega Biotech). The Apaf-1 target sequence was amplified by PCR using Apaf-1_Fw and Apaf-1_Rv primers (Table S1). PCR settings were: 98°C for 5 min, followed by 30 s at 96°C, 30 s at 67°C and 1 min at 72°C (30×), 10 min at 72°C (1×) and finishing with 1 min incubation at 4°C (1×). Purified PCR products (~30 ng) were prepared for sequencing using 5 pM primer in a final volume of 20 µl. TIDE analysis was carried out using a cut-off significance value of *P*<0.005 for decomposition with the TIDE webtool (<http://tide.nki.nl>) and default parameters for the analysis.

Determination of NHEJ-mediated and HDR-mediated knock-in by PCR genotyping and Sanger sequencing

Cells grown in 96 wells were transferred into 24-well plates 14 days after sorting. Two or three days later, some of the cells were harvested for DNA extraction using a Genomic DNA Extraction Kit (Qiagen); remaining cells were kept in culture. To analyze precise integration of Nluc and Cluc at the Apaf-1 locus, Nluc 5'-3' junction PCR and Cluc 5'-3' junction PCR were conducted using the proposed primers (Table S1) for the target site. Isolated genomic DNA was subjected to 30 cycles of PCR at the following settings: 98°C for 5 min, followed by 98°C for 5 s, 68°C for 1 min and 1 min at 72°C for 30 cycles, 10 min at 72°C (1×). Amplicons were loaded on agarose gels 1% and sequenced by Sanger method. The sequencing data at both ends of Nluc and Cluc were aligned with the expected HDR knock-in and donor plasmid sequences by BLAST.

Western blotting

Cells were resuspended in lysis buffer, HEPES 50 mM (pH 7.2), EGTA 5 mM, KCl 10 mM, MgCl₂ 2 mM, DTT 2 mM, CHAPS 0.1%, CLAP protease inhibitors (1:1000 dilution), centrifuged at 12,000 *g* for 5 min at 4°C and supernatants were used. 100 µg of the cell extract was loaded onto an 8% polyacrylamide gel together with separation using electrophoresis followed by transfer to the nitrocellulose membranes. Membranes were then blocked with BSA 5% in TBS-T (Tris 24 mM, NaCl 137 mM, KCl 2.68 mM, and Tween-20 0.1%) at room temperature for 2 h. This was followed by probing with primary antibodies against Apaf-1 (AdipoGen, AG-20T-0134-c100), caspase-9 (Cell Signaling Technology, 9502) or caspase-3 (Cell Signaling Technology, 9662) or with polyclonal antibody against actin (ProteinTech, 66009-1-Ig), all diluted at 1:1000 for incubation at 4°C overnight. Incubation with secondary antibody (diluted 1:10,000) was performed at room temperature (Goat 131 anti-rat; Li-cor, 925-32219), goat anti-rabbit (Li-cor, 926-32211) and goat anti mouse (Li-cor, 132 926-68020) in PBST. Membranes were then scanned using the LICOR system.

Cell death rate, bioluminescence assay of reconstituted reporter, caspase-9 and caspase-3 activities

The transgenic and normal HEK293 cells were treated with various concentrations of etoposide (1, 5, 10, 20, 30 µM), DOX (0.1, 0.3, 0.6, 0.9, 1.2 µM) or Nocodazole (10, 15, 30, 60, 100 nM). At the first step, live-dead analysis of the cell population was carried out by using nuclear staining differential assay with two fluorescent DNA intercalators, Hoechst 33342 dye (Hoechst; Invitrogen, Eugene, OR) and propidium iodide (PI; MP Biomedicals, Solon, OH) in transgenic and normal HEK293 cells upon DOX, etoposide or Nocodazole treatment. Hoechst 33342; and PI stock solutions were diluted in cell culture medium and added to each experimental well at a final concentration of 1 µg ml⁻¹ for each dye. Images were taken by an automated microscope (Perkin Elmer Operetta) using a 20× objective and analyzed using the Harmony software (Perkin Elmer). Nuclei were identified (Harmony Method C) and cytoplasm was defined on the basis of nuclei (Harmony Method C). Wells were screened in triplicates and compared with control wells. For quantification, at least 1000 cells were analyzed under each condition.

After treatment with increasing doses of DOX, etoposide or Nocodazole, transgenic and normal cells (5×10⁵) were lysed after 16 h, 16 h or 12 h, respectively, using 100 µl of ice cold CCLR buffer. After 20 min, cell debris was pelleted by centrifugation at 4°C, 12,000 *g* for 30 s. The activity was immediately monitored as a luminescence signal produced upon addition of

60 µl of One-Glo luciferase assay system (Promega) to 20 µl of cell lysate using a Victor Plate reader at 25°C, over 15 min. Moreover, time-point curves of these three apoptosis inducers were examined. To validate dependency between luminescence signal and apoptosome formation, caspase-9 and caspase-3 activities were assessed, and measured by using the fluorogenic substrates AC-LEHD-AMC (a fluorogenic substrate for caspase-9) and AC-DEVD-AMC, respectively, to continuously measure their activities in cell extracts using a fluorometer (as previously explained by Cain et al., 2000). Briefly, 15 µl of lysed cells were mixed with 185 µl of assay buffer containing HEPES 20 mM, 10 µM substrate and 1 mM DTT. Fluorescence intensity was measured using a fluorescence microplate reader and normalized against the protein concentration.

Apoptosome formation blockage by overexpression of Bcl2

HEK293 cells (7×10^5) were transfected with a plasmid-expressing Bcl2 and empty plasmid as a control (pBabe puro) by lipofectamine 3000. After 24 h and 48 h, expression levels were detected by western blotting with anti-Bcl2. Cell death rate was analyzed using PI and Hoechst 33342 nucleus staining, luminescence assay determined apoptosome formation and caspase-3 activity was assessed by using Ac-DEVD-AMC.

Statistical analysis

The statistically significant differences between the groups were calculated by one-way ANOVA and two-sample *t*-test for continuous variables/Chi-square test for categorical variables. Trend analysis was used to assess activation of dose-dependent and time-dependent apoptosis upon formation of apoptosomes in transgenic and normal HEK293 cells. $P < 0.05$ was considered statistically significant.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.-P.C., S.H.; Methodology: E.S.H., M.N., H.O.F., J.-P.C., S.H.; Software: E.S.H., J.-P.C.; Validation: E.S.H., A.A.H., J.-P.C., S.H.; Formal analysis: E.S.H., M.N., H.O.F., J.-P.C., S.H.; Investigation: A.A.H., H.O.F., S.H.; Resources: S.H.; Data curation: E.S.H., M.N., H.O.F., J.-P.C.; Writing - original draft: E.S.H.; Writing - review & editing: S.H.; Visualization: E.S.H., A.A.H., J.-P.C.; Supervision: M.N., J.-P.C., S.H.; Project administration: S.H.; Funding acquisition: H.O.F., S.H.

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Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/doi/10.1242/jcs.242636.supplemental>

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