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What is This?

High-Throughput Flow Cytometry–Based Assay to Identify Apoptosis-Inducing Proteins

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After sequencing the human genome, the challenge ahead is to systematically analyze the functions and disease relation of the proteins encoded. Here the authors describe the application of a flow cytometry–based high-throughput assay to screen for apoptosis-activating proteins in transiently transfected cells. The assay is based on the detection of activated caspase-3 with a specific antibody, in cells overexpressing proteins tagged C- or N-terminally with yellow fluorescent protein. Fluorescence intensities are measured using a flow cytometer integrated with a high-throughput autosampler. The applicability of this screen has been tested in a pilot screen with 200 proteins. The candidate proteins were all verified in an independent microscopy-based nuclear fragmentation assay, finally resulting in the identification of 6 apoptosis inducers. (*Journal of Biomolecular Screening* 2007:510-520)

Key words: apoptosis, caspase-3, genomics, flow cytometry, cell-based assay

INTRODUCTION

SEQUENCING OF THE HUMAN GENOME has revealed the presence of 20,000 to 25,000 protein-coding genes.[1] The function and biological role of many of the proteins encoded by these genes and, even more so, their role in diseases is unknown. The development and application of high-throughput approaches are needed for an efficient functional characterization of the human proteome. To this end, we have developed a high-throughput cell-based assay that is suitable to screen for proteins dominantly inducing apoptosis upon overexpression. The screen is based on transiently expressing yellow fluorescent protein (YFP)–tagged proteins in mammalian cells.

Apoptosis is a genetically programmed process of eliminating unwanted or abnormal cells in the body. Dysregulation of apoptosis has implications in the development of diseases such as cancer, AIDS, autoimmune, and neurodegenerative/ neurodevelopmental diseases. This diversity of diseases associated with apoptosis stresses the need to identify proteins that are involved in programmed cell death. Apoptotic cells exhibit certain morphological and biochemical features that can serve as markers for detection. Activation of the caspase cascade, and specifically caspase-3, is considered a direct and specific marker for identifying apoptosis. Caspase-3 is a major effector caspase that cleaves several key cellular substrates and eventually leads to cell death. Recent studies have shown that apart from death receptor and mitochondrial apoptosis pathways, other cellular organelles also initiate specific apoptosis pathways.[2] Although these pathways originate in an organelle-specific manner, they all converge on the central executioner, which is the activation of the caspase cascade. Therefore, we have chosen the activation of caspase-3 as a marker for detecting apoptosis.

Many of the high-throughput apoptosis screens reported to date have been applied to test chemical compounds for their activity as inhibitors or activators of apoptosis.[3,4] In these compound-screening assays, all the cells within a well are treated uniformly with the same compound. So the results from such screens can typically be detected from the whole population of cells using uniform well readout methods such as plate readers. However, transient transfections with expression constructs introduce high cell-to-cell variations in the level of protein overexpression even within the same well. Moreover, some proteins exert their effect only once a certain expression level has been reached, or high levels of protein may even induce nonspecific effects.[5] Such effects cannot be identified by plate reader–based assays, which provide ensemble average values over the whole well. Therefore, methods allowing for single-cell resolution are mandatory for data acquisition of transient transfections, in which the measured effects in each individual cell can be directly associated with the elevated levels of the protein of interest.

Antibodies that are selective for the cleaved or activated forms of caspase-3 have been developed and successfully applied in some studies to detect apoptosis.[6,7] Despite their high specificity, these antibodies have not yet been exploited for any high-throughput applications. Also, flow cytometry has not been used for data acquisition in high-throughput apoptosis assays, although it is an established detection method in apoptosis research. Combining antibodies directed against activated caspase-3 and the detection of signals via automated flow cytometry was key for the development of our high-throughput apoptosis assay. As a proof of principle, the assay was tested using cDNAs that encoded 200 previously uncharacterized proteins. Open reading frames (ORFs) had been cloned into Gateway vectors,[8,9] and plasmids were transiently transfected into HEK293T cells to allow for expression of the proteins as N- or C-terminal fusions with YFP. The activation state of caspase-3 in response to protein overexpression was detected by staining with a specific antibody against cleaved caspase-3. Apart from the specificity of detecting apoptosis, this assay offers the advantage that the activation of caspase-3 and the expression level of the YFP-tagged protein can be measured and correlated at the level of single cells. Finally, we verified the specificity of this assay by validating the candidate proteins in a classical nuclear fragmentation assay.

MATERIALS AND METHODS

Materials

HEK293T/17 cells used for the assay were purchased from ATCC (Manassas, VA; catalog no. CRL-11268) and maintained in DMEM from Invitrogen (Paisley, UK; catalog no. 41966-052). Transfections were carried out with Effectene transfection reagent from Qiagen GMBH (Hilden, Germany; catalog no. 301427). The antibody against cleaved caspase-3 was from Cell Signaling Technology (Danvers, MA; catalog no. 9661), and an allophycocyanin (APC) cross-linked antirabbit secondary antibody was obtained from Molecular Probes (Karlsruhe, Germany; catalog no. A-10931). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) for the nuclear fragmentation assay was from Sigma Aldrich (Munich, Germany; catalog no. D9542). Montage Plasmid Miniprep96 kits were obtained from Millipore (Bedford, MA; catalog no. LSKP 096 24).

Selection of full-length cDNAs for screening

The cDNA resources required for the apoptosis screen were provided by the German cDNA consortium.[8] Based on the availability of full-length cDNAs, we selected 200 previously uncharacterized proteins for screening in the apoptosis assay. The cDNAs had been isolated from human cDNA libraries, cloned into Gateway vectors, sequence validated, and then shuttled into mammalian expression vectors producing proteins tagged either

NH_2- or COOH-terminally with YFP. Subcellular localization of the proteins was determined as described.[9]

Automated apoptosis assay to detect activation of caspase-3

Miniprep DNA of the expression plasmids was prepared in a 96-well plate format using a MultiProbe IIex-Robot (Perkin-Elmer, Wellesley, MA) and Montage Plasmid Miniprep96 kits. DNA master plates were generated with a final concentration of 27.5 ng/μl and a total volume of 80 μl/well. HEK293T cells were cultured in four 24-well plates (to mimic a 96-well plate) at a density of 32,000 cells/well and were transfected with 200 ng DNA/well using Effectene transfection reagent. Seventy-two hours after transfection, the adherent and floating cells from the four 24-well plates were collected (by trypsinization) into a 96-U bottom deep-well plate and stained with the antibodies in suspension using the MultiProbe IIex-Robot. Briefly, the cells were fixed with 2% paraformaldehyde (PFA), permeabilized with 90% methanol, and stained with cleaved caspase-3 antibody (dilution 1:50) followed by an APC-conjugated secondary antibody (dilution 1:350). The cells were then measured with FACSCalibur (BD Biosciences, San Jose, CA) equipped with a high-throughput sampler.

Data acquisition and statistical analysis

Data from at least 10,000 cells were acquired from each well with CellQuestPro® software (version 5.2), and the data analysis was performed as described.[5] The raw data were analyzed using the statistical programming language R, which is openly available through the Bioconductor package *prada*. A measure for the effect size of a protein on activation of caspase-3 was obtained through the log-transformed odds ratio, which is defined by

$$-\log\left(\frac{TP+1}{TN+1}\cdot\frac{UTN+1}{UTP+1}\right),$$

where TP is the number of transfected, active caspase-3–positive cells; TN is the number of transfected, active caspase-3–negative cells; UTN is the number of untransfected, active caspase-3–negative cells; and UTP is the number of untransfected, active caspase-3–positive cells.

Pseudo-counts of 1 were added to each term to avoid infinite values caused by division with 0. A positive value of this score indicates activation; a negative score indicates inhibition of caspase-3 activity. A value of 0 indicates no effect at all. Because the assay was designed to detect caspase-3 activation only, negative values were disregarded in this case. A measure of significance was obtained by testing against the null hypothesis of an odds ratio of 1, by means of the Fisher exact test. Results from replicate measurements for each protein were
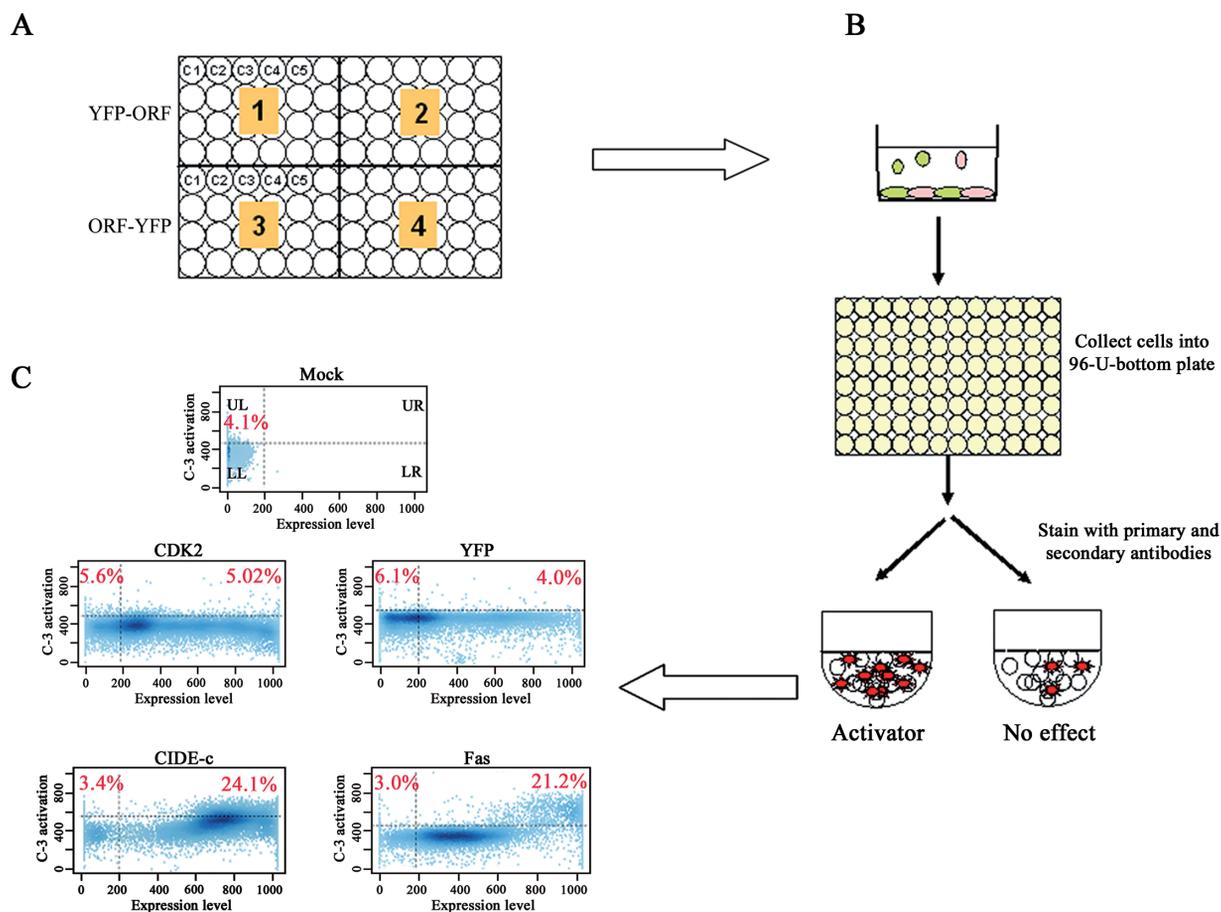
FIG. 1. Assay design and work flow. (**A**) Cells were transfected with yellow fluorescent protein–tagged open reading frames (ORFs) in four 24-well plates, and culture was continued for 72 h (controls are indicated as C1 to C5) to allow for recombinant protein expression. (**B**) The adherent and floating cells were collected, stained with the antibodies in a 96-well U-bottom plate, and measured by flow cytometry. (**C**) The scatter plots obtained from mock-transfected cells, negative controls CDK2-YFP and YFP, and positive controls CIDE-c-YFP and Fas-YFP are shown. The percentage of active caspase-3–positive cells is indicated separately for the nontransfected and transfected subpopulations (in the upper left and right corners, respectively).

combined using a generalized χ^2 statistic for stratified contingency tables through the Mantel-Haenszel test. The results were then visualized as color-coded, 96-well plate plots displaying various quantities of interest such as odds ratio, transfection efficiency, total cell number/well, and so forth.

Nuclear fragmentation assay

HEK293T cells seeded in 24-well plates (32,000 cells/well) were transfected with YFP-tagged ORFs; recombinant proteins were expressed for 72 h of continued culture. The floating and adherent cells were collected by trypsinization, fixed with 2% PFA, and permeabilized with 0.2% Triton X-100. The cells were then stained with 1 µg/ml of DAPI and were fixed on slides using Cytospin (Thermo Electron Corporation, Waltham,

MA). Fluorescence images were taken using a Confocal laser scanning microscope Axiovert 200M (Carl Zeiss, Thornwood, NY) through a 40 × oil immersion objective.

RESULTS

Assay design and data analysis

HEK293T cells were cultured in four 24-well plates and transfected with YFP-tagged ORFs. Both expression constructs for each ORF (i.e., proteins tagged NH_2- and COOH-terminally with YFP) were included in the same experiment (**Fig. 1A**). Mock transfected cells, negative control cDNAs of CDK2 (cyclin-dependent kinase 2) and YFP, and positive controls CIDE-C (C-terminal domain of cell death–inducing DFFA-like effector)
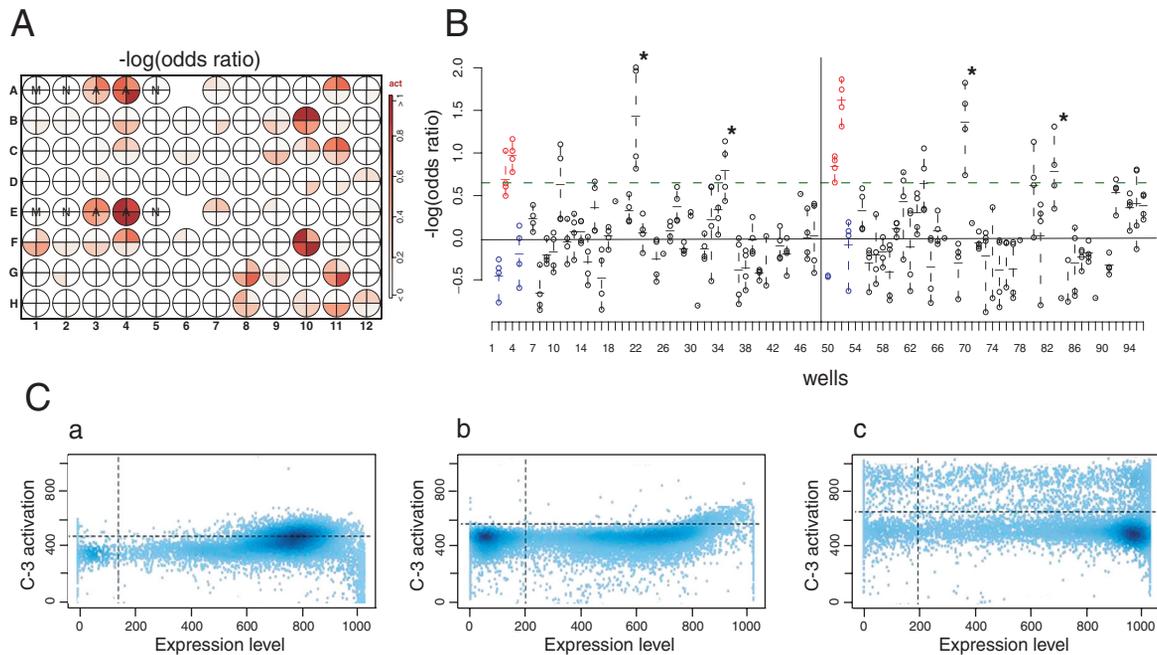
FIG. 2. Selection of potential candidates of the caspase-3 assay. (**A**) Visual representation of the odds ratio of the 4 replicates as a color-coded, 96-well plate plot. The N-terminally tagged proteins are present in the upper half of the plate, whereas the corresponding C-terminally tagged proteins are present in the lower half. The mock-transfected well is indicated by M, positive controls by A, and the negative controls by N. Expression clones that showed an activating effect in the assay are indicated in red. (**B**) The odds ratio of proteins from the 4 replicate experiments is plotted. Each vertical bar in this plot represents 1 expression clone. The positive controls are shown in red and the negative controls in blue. A dotted line is drawn at an odds ratio of 0.65 to represent the threshold value for selection of candidates. The clones that show an effect size (or –log odds ratio) of more than 0.65 (shown with an asterisk) were selected for further analysis. (**C**) The 3 patterns of scatter plots seen in the assay are shown. The 1st and the 2nd patterns are recognized as activators by the statistical analysis method, whereas the 3rd requires visual identification.

and Fas were included at fixed positions in each screening plate (C1 to C5 in **Fig. 1A**). After 72 h, the cells were stained with the antibodies and measured by flow cytometry, and the raw data were analyzed (as described in the Materials and Methods section; **Fig. 1B**). Scatter plots for all 96 wells were generated automatically.

Mock-transfected cells were taken as standard to define the level of autofluorescence of cells as well as the background level of activated caspase-3. Based on the distribution of cells from the mock-transfected sample, quadrants were drawn on the scatter plot separating the 4 different populations expected in cells expressing the YFP-tagged proteins (**Fig. 1C**). Untransfected, nonapoptotic cells are in the lower left quadrant; untransfected, apoptotic cells in upper left; transfected, nonapoptotic cells in lower right; and transfected, apoptotic cells in the upper right quadrant. The same quadrants as those of the mock-transfected sample were applied to analyze the effects induced by the overexpressed proteins. A protein was considered as an activator when transfected cells showed a significantly higher level of active caspase-3 compared to the untransfected cells of the same well. As shown in **Figure 1C**, transfections with the negative controls CDK2 and YFP resulted in only 5.02% and 4.0% of

transfected caspase-3 positive cells, respectively, whereas cells transfected with the positive controls CIDE-c and Fas showed 24.1% and 21.2% active caspase-3 positive cells, respectively.

Screening for proapoptotic proteins

As a proof of principle, we selected 200 previously uncharacterized proteins and screened them for their potential to induce apoptosis on overexpression. A total of 400 expression clones (200 proteins that were tagged NH_2- or COOH-terminally with YFP) were screened in at least 4 independent replicate experiments, finally resulting in data from 2304 wells, including controls.

Selection of candidates from the caspase-3 assay

The procedure of candidate selection is shown in **Figure 2**, taking one 96-well screening plate as an example. To obtain an overview of protein effects across a 96-well plate in different replicate experiments, the odds ratios were visualized for every protein as color-coded, 96-well plate plots (**Fig. 2A**). Every well in this plate was divided into 4 parts, with each part representing 1 experimental replicate. Proteins and/or replicates showing an

Table 1. Validation of Candidates in the Nuclear Fragmentation Assay

No.	Gene ID	*p-Value of Fisher Test (Caspase-3 Assay)*	*Localization*		*Nuclear Fragmentation*	
			ORF-YFP	*YFP-ORF*	*ORF-YFP*	*YFP-ORF*
1	FUNDC2	0.000152	Mitochondria	Mitochondria	+++	+++
2	IFI27	6.4e-07	Endoplasmic reticulum and Golgi apparatus	Unknown	+++	++
3	SLC25A31	0.00195	Mitochondria	Cytoplasm and mitochondria	+++	+++
4	BRP44	0.00043	Mitochondria	Mitochondria	−	+++
5	C11orf56	2.5e-05	Cytoplasm and nucleus	Cytoplasm and nucleus	+	++
6	SLC30A8	0.00057	Endoplasmic reticulum	Endoplasmic reticulum	+	+
7	RTN4R	0.00127	Endoplasmic reticulum	Cytoplasm and endoplasmic reticulum	+	+
8	VMP1	2.39e-52	Plasma membrane	Plasma membrane	++	++

Proteins 1 through 5 were selected on the basis of –log odds ratio of ≥0.65 and a *p*-value of ≤0.001 in addition to a correct subcellular localization as described in LIFEdb.[12,13] Proteins 6 through 8 were selected on the basis of scatter pattern 3 as depicted in **Figure 2C** and a correct subcellular localization according to LIFEdb. The subcellular localization of these proteins was then verified for HEK293T cells in this study. Depending on the strength of nuclear fragmentation, the proteins were graded from – to +++, with – indicating no fragmentation and +++ indicating strong fragmentation.

activating effect in the assay were indicated in red, with the color intensity being proportional to the strength of the effect. For example, in the plate shown in **Figure 2A**, both tag orientations and all 4 replicates of the wells transfected with the positive controls (indicated with A) showed an activating effect, whereas the mock and neutral controls (indicated with M and N, respectively) did not induce caspase-3 activation. Some of the proteins in the plate induced caspase-3 activation for both tag orientations (e.g., wells B10 and F10 and wells C11 and G11). However, some proteins induced apoptosis only when YFP was fused to the N-terminus of the protein (wells F1, G8, H8, etc.). This shows that orientation of the tag has an effect on the assay outcome of some fusion proteins, likely due to mislocalization of the protein caused by masking of the localization signal and/or the functional domains by the YFP tag.[9-11]

Effects from the 4 replicate experiments for this plate were then plotted to facilitate candidate selection (**Fig. 2B**). Each segment on the x-axis in this plot represents 1 expression clone. The log odds ratios obtained for each of the expression clones from all 4 independent replicates was plotted (shown as circles), and the mean odds ratio calculated from these (indicated by the horizontal bars) was considered for selection of candidates. A –log odds ratio of 0.65 (horizontal dashed line), which is 1 standard deviation below the average value obtained for positive controls in all the plates, was chosen as the threshold for selection of candidates. Although the effect of some proteins in the assay was observed to vary with orientation of the tag, initially all the expression clones with a –log odds ratio of ≥ 0.65 (e.g., expression clones shown with asterisks in **Fig. 2B**) and a *p*-value of ≤0.001 were selected. Six proteins were selected based on these 1st selection criteria. The subcellular localization of these fusion proteins was determined and then

compared to that described in LIFEdb,[12,13] a freely available online database for functional profiling. Only 5 of these fusion proteins that showed a subcellular localization similar to that described in the database were considered as candidates for further validation (**Tables 1** and **2**, no. 1-5).

Upon examining the scatter plots from all expression constructs tested in the assay, 3 different patterns of scatter plots for activators were observed (**Fig. 2C**). The 1st pattern indicated a strong apoptosis induction in transfected cells, whereas most of the untransfected cells were negative for active caspase-3 (**Fig. 2Ca**). In the 2nd pattern, apoptosis was induced in cells only when the protein of interest was overexpressed at high levels (**Fig. 2Cb**). Any protein that exhibited either of these patterns was identified as an apoptosis inducer by the automated analysis method because of high odds ratios (**Table 1**, no. 1-5). In the 3rd kind of pattern, even untransfected cells were found to undergo apoptosis along with the transfected cells (**Fig. 2Cc**). In this case, the odds ratio was small, and these proteins were not recognized as activators by the automated analysis method that we applied. Nevertheless, proteins that show such a pattern might indeed represent death-inducing ligands, secreted proteins, or other proteins that induce apoptosis also in neighboring untransfected cells. In consequence, 3 proteins that exhibited such a pattern were also considered for further evaluation (**Table 1**, no. 6-8). Thus, a total of 8 proteins were selected as candidates for a validation screen, in which the apoptosis-inducing effect was tested in a nuclear fragmentation assay (**Table 1**).

Validation of the high-throughput caspase-3 assay

A nuclear fragmentation assay was performed to validate the specificity of the caspase-3 assay. YFP-tagged proteins of

Table 2. Summary of the 6 Candidate Proteins

| No.	Gene ID	Protein Name	Genbank Accession No.	Annotation (SOURCE, InterPro)
1	FUNDC2	FUN14 domain containing 2	BC000255	FUN14 domain containing 2 (hepatitis C virus core-binding protein 6, Cervical cancer oncogene 3); IPR007014 FUN14
2	IFI27	Interferon α–inducible protein 27	BC015492	Interferon α–inducible protein 27; IPR002091 aromatic amino acid permease; IPR009311 interferon-induced 6-16
3	SLC25A31	Solute carrier family 25, member 31	AL136857	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31; IPR001993 mitochondrial substrate carrier; IPR002113 adenine nucleotide translocator 1; IPR002167 Graves disease carrier protein
4	BRP44	Brain protein 44	AL110297	Brain protein 44; IPR005336 protein of unknown function
5	C11orf56	Chromosome11 open reading frame 56	AL136723	Identical to *Caenorhabditis elegans* hypothetical protein C05D11.8; no InterPro hits
6	VMP1	Vacuole membrane protein 1	AL136711	Likely homologue of rat vacuole membrane protein 1 (Vmp1); no InterPro hits

Functional annotation of the 6 candidate proteins is provided with the help of the SOURCE and InterPro databases.
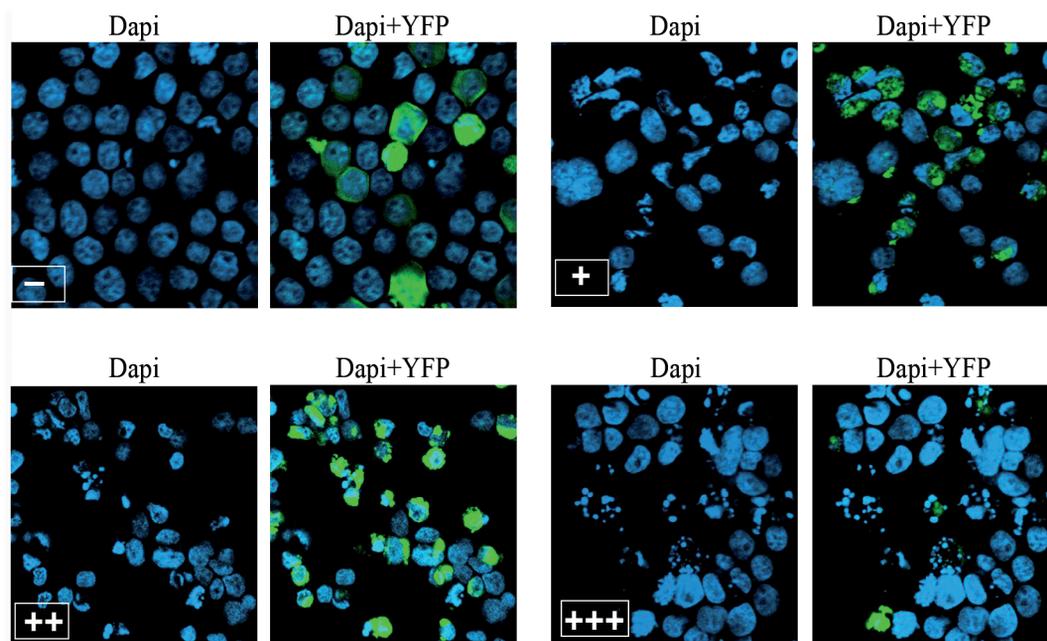
FIG. 3. Patterns of nuclear fragmentation. Shown are the example images of different patterns of nuclear fragmentation. Nuclei with no evident nuclear changes are indicated with –, condensed or deformed nuclei with +, both condensed and fragmented nuclei with ++, and strong fragmentation with +++.

the 8 candidates were expressed in HEK293T cells and stained with DAPI, and the proteins were scored according to the extent of nuclear fragmentation (**Table 1**). Proteins that induced no evident nuclear changes were indicated with –, those with only deformed but no fragmented nuclei were indicated with +, nuclei that are both condensed and fragmented were indicated by ++, and nuclei with clear fragmentation were scored +++ (examples shown in **Fig. 3**). A total of 200 cells were analyzed per sample, and the phenotypic classification was based on the finding that at least 50% of the transfected and less than 2% of the nontransfected cell populations are positive for the respective phenotype. All 8 candidates were confirmed in the nuclear fragmentation assay, but only 6 proteins that showed medium (++) to strong fragmentation (+++) were selected as final candidates. The protein domain classifications from SOURCE[14] and Interpro[15] provided the functional annotation of these candidate proteins (**Table 2**). **Figure 4** shows the results from this assay for the 6 candidate proteins, YFP (negative
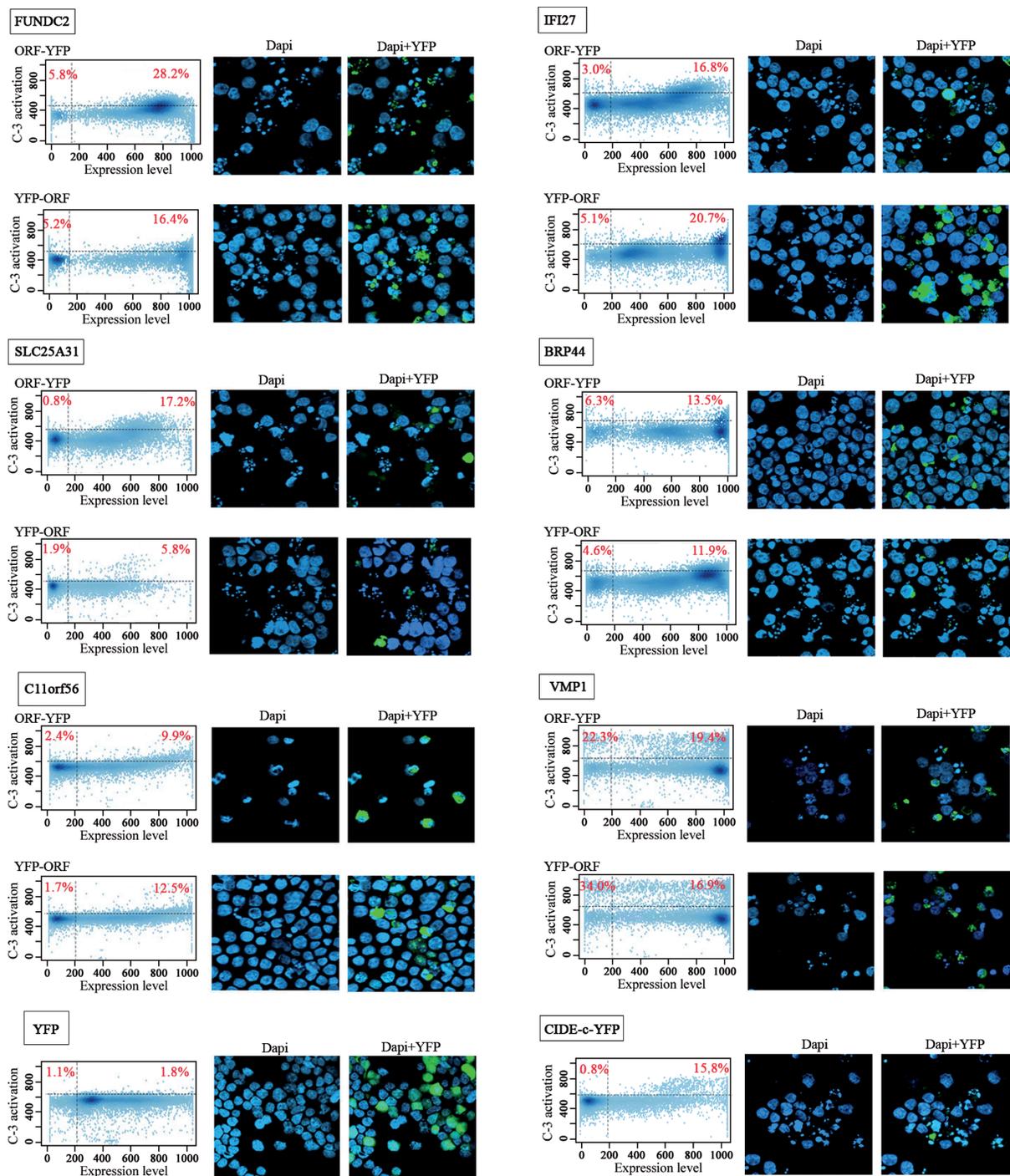
FIG. 4. Confirmation of the candidates in a nuclear fragmentation assay. Shown are the scatter plots of caspase-3 activation and images of DAPI-stained nuclei of the 6 candidate proteins, with YFP as the negative control. In each scatter plot, the percentage of untransfected apoptotic cells is shown in the left corner and the percentage of transfected apoptotic cells in the right corner.

control), and CIDE-c (positive control) in conjunction with the scatter plots for the same proteins from the high-throughput caspase-3 assay. The nuclei of all cells were stained by DAPI, although only transfected cells also show the YFP signal.

Examination of scatter plots and the images of DAPI-stained nuclei indicate that the strength of signal in the caspase-3 assay strongly correlates with the nuclear fragmentation results of the corresponding expression clone (i.e., the proteins that induced

a strong caspase-3 activation also showed a clear nuclear fragmentation). However, 1 exception seems to be the candidate BRP44. Overexpression of both C- and N-terminally tagged protein resulted in caspase-3 activation in 13.5% and 11.9% of transfected cell population, respectively. But nuclear fragmentation was observed only in cells transfected with the N-terminal fusion protein, although both fusion proteins apparently localized to the same subcellular compartment (**Fig. 4** and **Table 1**).

DISCUSSION

Along with uncontrolled proliferation, the survival of tumor cells is also due to their inability to activate cell death. Although apoptosis research is progressing at a rapid pace, the list of proteins in the apoptosis network is far from complete. Therefore, it is necessary to identify the unknown players in this large network prior to a systems biological analysis of the relations and interrelations of the individual proteins. In an effort toward this, we have established a flow cytometry–based, high-throughput, cell-based apoptosis assay and screened for dominant apoptosis-inducing proteins. Other currently available flow cytometry–based apoptosis assays are either difficult to automate (TUNEL) and/or not very specific for apoptosis (detection of sub-G1 population). A fluorescent-activated cell sorting (FACS)–based Annexin V assay is suitable only for cell types that grow in suspension because the procedures applied to detach adherent cells from the surface prior to measurement leads to structural changes of the plasma membrane, resulting in the exposure of phosphatidylserine even in nonapoptotic cells. However, most suspension cell lines have a low transfection efficiency, making them unsuitable for experiments based on transient transfections. These difficulties have hindered the development of reliable high-throughput screens to identify proteins inducing apoptosis on transient transfection. The screen described here is a specific and sensitive approach for high-throughput detection of apoptosis in transiently transfected cells.

We followed an overexpression strategy in which cells were transiently transfected with YFP-tagged ORFs and stained with a polyclonal antibody detecting activated caspase-3. HEK293T cells were selected for screening after validating them with known apoptosis inducers for the presence of intact apoptosis machinery (**Fig. 1C**). This cell line also showed a high transfection efficiency (70%-100%), even with miniprep DNA, which resulted in clear and statistically significant effects in the assay. Using a fluorescent tag (such as YFP) is a very convenient approach to identify cells overexpressing the protein of interest. But one frequent problem associated with the use of such a large tag (the molecular weight of YFP is ~25 kDa) is the masking of the localization signals, resulting in the wrong subcellular localization of the protein.[9,10] Moreover, localization to the correct cellular compartment may not always correspond to the correct function of the fusion protein. This is possibly due to the YFP tag's masking the domains present at one or the other terminus of the protein that is necessary for the protein function. To cope with this, ORFs with both tag orientations were screened. Each experiment was also repeated 4 times to obtain statistically significant effects. A total of 200 full-length proteins have been screened in the assay, of which 8 proteins that showed strong to medium activation of caspase-3 were selected as candidates for further validation (**Table 1**). After validating these 8 proteins in a nuclear fragmentation assay, 6 were selected as final candidates. Proteins that showed a clear activating effect in the caspase-3 assay also induced a strong nuclear fragmentation (**Fig. 4**), thus confirming the specificity of our high-throughput assay.

For one of the candidate proteins, BRP44, nuclear fragmentation was detected only with YFP-ORF, though both tag orientations of the protein resulted in a similar level of caspase-3 activation and also localized to the same subcellular compartment (i.e., mitochondria). The FACS dot plots of BRP44 indicate that transfection with YFP-ORF resulted in a gradual increase in caspase-3 activation with an increase in the protein expression level, whereas ORF-YFP produced a threshold effect (i.e., only cells expressing a very high level of protein were apoptotic). Because the protein expression level was not quantified in the microscopy-based nuclear fragmentation assay, it is very well possible that the few cells with a very high protein expression were masked/dominated by the higher number of cells with a medium expression level, resulting in the underestimation of the total number of fragmented nuclei. However, we cannot exclude the possibility that BRP44 is an example of a false positive that could not be confirmed in the validation assay.

Below, 6 final candidates of the apoptosis screen are described, together with their potential association with apoptosis.

FUN14 domain containing 2 (FUNDC2)

This protein had initially been identified in a yeast 2-hybrid screen to interact with hepatitis C core protein.[16] Besides its essential role in viral replication, the core protein is involved in a variety of functions such as signal transduction, cell cycle regulation, changes in gene transcription, and apoptosis. Previous studies regarding the role of the core protein in apoptosis demonstrated controversial results. There are several studies indicating that the core protein suppresses apoptosis,[17,18] whereas other studies showed that it promotes apoptosis.[19,20] Although the differences in activity could be cell-type specific, it is clear that the core protein regulates apoptosis. But the precise mechanism by which this is done is not fully understood. The core protein was described to suppress the activation of caspase-3,[21] although we show here that overexpression of FUNDC2 leads to the activation of caspase-3. Therefore, it is well possible that the core protein suppresses the apoptosis-activating effect of the protein encoded by FUNDC2 by interacting with it.

Interferon α–inducible protein 27 (IFI 27) or ISG12(a) protein

The gene belongs to the interferon stimulated genes 12 (ISG12) gene family. The ISG proteins participate in a variety of biological functions involving antiviral, apoptotic, antiproliferative, antitumor, and immunomodulatory activities. The protein sequence of ISG12 has 33% overall sequence identity to the product of the 6-16 gene (also an interferon-stimulated gene), but the functions of 6-16 as well as ISG12 have not been identified yet. Moreover, similar induction patterns have been found for 6-16 and ISG12 in various cell lines,[22] suggesting the possibility that these proteins might play related roles in the interferon system. Increased expression of 6-16 was observed during cell growth arrest and in senescent cells,[23] indicating that 6-16 is involved in growth inhibitory processes. Overexpression of ISG12(a) induced activation of caspase-3 and subsequently apoptosis, which is consistent with the growth-inhibitory property of 6-16.

Solute carrier family 25, member 31 (SLC25A31)

SLC25A31 is a novel member of the mitochondrial solute carrier family 25. Three isoforms of this protein family (adenine nucleotide translocator [ANT] 1, 2, and 3) with tissue-specific expression patterns have been described in humans. SLC25A31 is the 4th isoform and shares 66% to 68% amino acid identity with the other 3 isoforms.[24] Overexpression of ANT1, a well-known member of this family, has also been demonstrated to induce apoptosis.[25]

Brain protein 44 (BRP44)

BRP44 localizes to the mitochondria and belongs to a family of proteins of unknown function. No similarities to known proteins or domains have been found. However, homologues have been found in dog, mouse, zebra fish, fruit fly, and worm, indicating that the function of the protein might be important in the cell. A similar protein called brain protein 44–like (from *Rattus norvegicus*) is predicted to be localized to the inner mitochondrial membrane, and it might be involved in the apoptosis of neuronal cells (SOURCE).[14] This is consistent with the mitochondrial localization and the apoptosis-inducing effect of brain protein 44 we observed here.

C11orf56

This protein localizes to the cytoplasm and nucleus. No functional data or association with apoptosis-related processes are available for the candidate C11orf56.

Vacuole membrane protein 1 (VMP1)

Vmp1 has no known domains or similarities to known proteins. Its homologue, rat *Vmp1*, was shown to be highly expressed in acinar cells of rats with acute pancreatitis and in ischemic rat kidney,[26] conditions in which cells die by apoptosis as well as necrosis. Therefore, *Vmp1* was described as a stress-induced gene that might be activated as an emergency program against pancreatitis and kidney ischemia. Overexpressed rat *Vmp1* was shown to promote intracellular vacuole formation and apoptosis, which is consistent with our results.

SUMMARY

Interestingly, 3 of the proteins described above are mitochondrial, as determined in the subcellular localization screen. This is in line with the fact that mitochondria are frequently involved in apoptosis processes.[27,28]

The selection and establishment of a suitable assay was one of the most critical parameters in the implementation of the apoptosis screen. We aimed at selecting an assay that would detect changes in individual cells. This assay was designed to detect even minute changes in the parameters being assessed (i.e., the protein expression and caspase-3 activation levels) and at the same time was robust enough to be automated for high-throughput screening. Although plate reader–based assays offer the advantage that the data acquisition times are very short, they do not allow for a single-cell resolution. Recently, automated microscopes have been used for high-throughput applications to monitor changes at the level of individual cells.[11,29] However, apoptotic cells tend to lose their adherence and detach from the surface of the well. This could result in underestimation of the total number of apoptotic cells, thus leading to a misinterpretation of the assay outcome. Consequently, we did not consider microscopy-based methods for high-throughput detection of apoptosis.

The flow cytometry–based method described here offers the advantage that several parameters of individual cells can be measured simultaneously while it facilitates the measurement of adherent as well as of floating (apoptotic) cells. When cells are transiently transfected with YFP-tagged ORFs, the transfected cells can be distinguished from untransfected cells, thus allowing us to relate the level of protein expression to the strength of the induced effect. Moreover, death ligands, secreted proteins, and other proteins that induce apoptosis also in the untransfected cells of the same well can be easily identified from the pattern of scatter plots (see **Fig. 4**). Such trans effects should be difficult to analyze with reverse-phase cell arrays,[30] which also typically acquire data from fewer than 500 transfected cells. With our assay, data from more than 10,000 cells/well have been analyzed, which resulted in statistically significant results. Any transfection of cells induces apoptosis in a fraction of the population and necessitates a distinction between noise and real signal. High cell numbers make this distinction possible and help to enlarge the dynamic range that is required to also identify mild inducers of apoptosis (e.g., C11orf56).

Despite these advantages, we also identified certain limitations of our assay. Although activation of caspase-3 is a central event in apoptosis, increasing evidence shows that apoptosis can also occur in the absence of caspase-3 activation. Proteins inducing apoptosis through such pathways cannot be identified in this assay. But the assay described here is versatile enough and can be adapted to identify any pathway by simply interchanging the antibody. An ideal high-throughput assay is one that can be performed in a single well with no other manipulation other than addition of the sample to be tested. Although it is easy to automate such methods, the specificity and/or the sensitivity of the assay will be compromised. The caspase-3 assay described here reached a reasonable compromise between sensitivity and the level of automation. Detection of activated caspase-3 by flow cytometry is a direct, specific, and sensitive approach for detecting apoptosis. Although the process of transfection and the many steps in the antibody-labeling procedure (i.e., fixation, permeabilization, antibody labeling, washes, etc.) are automated for this assay in a 96-well format, the staining procedure involves some centrifugation steps that require manual intervention. The process of aspirating the buffers from the 96-well plates resulted in loss of cells in every step. This restricts the use of this assay for ultra-high-throughput applications (in 384-well plates) that typically start with very few cells per well. The current screen was performed in quadruplicate to evaluate the between-well reproducibility and robustness of the assay. Selection of candidates based on duplicate experiments might result in an increased number of false positives or false negatives (as seen in **Fig. 2A**), and therefore, we consider at least 3 replicates necessary for any high-throughput screen.

CONCLUSION

We developed and applied a high-throughput assay to identify proteins that dominantly induce apoptosis upon overexpression. This assay makes it possible to identify those cells that are transfected with the ORF of interest and to correlate the observed effect with the expression level of the protein. A pilot screen with 200 previously uncharacterized proteins led to the identification of 6 apoptosis inducers and enabled us to test the functionality and reliability of the assay. This screen has been done in a kidney cell system, and follow-up experiments are under way to validate these candidates in a non-YFP approach also in other cell types. Extending our approach to large sets of proteins in the future will contribute to a better understanding of the regulation of apoptosis and toward the development of novel strategies to combat cancer.

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