Published in final edited form as:

Methods Enzymol. 2011 ; 491: 57–71. doi:10.1016/B978-0-12-385928-0.00004-3.

Large-scale analysis of UPR-mediated apoptosis in human cells

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Abstract

The historic distinction between academic- and industry-driven drug discovery, whereby investigators at universities worked to uncover the elusive principles of basic science and drug companies advanced the identification of drug targets and probe discovery, has been blurred by an academic high throughput chemical genomic revolution. It is now common for academic labs to use biochemical or cell-based high throughput screening (HTS) to investigate the effects of thousands or even hundreds of thousands of chemical probes on one or more targets over a period of days or weeks. To support the efforts of individual investigators, many universities have established core facilities where screening can be performed collaboratively with large chemical libraries managed by highly trained HTS personnel and guided by the experience of computational, medicinal and synthetic organic chemists. The identification of large numbers of promising hits from such screens has driven the need for independent labs to scale-down secondary *in vitro* assays in the hit to lead identification process. In this chapter we will describe the use of luminescent and quantitative reverse transcription real-time PCR (qRT-PCR) technologies that permit evaluation of the expression patterns of multiple Unfolded Protein Response (UPR) and apoptosis-related genes and simultaneously evaluate proliferation and cell death in 96 or 384 well format.

1. Introduction

The ability of the Unfolded Protein Response (UPR) to modulate cell death, following an unsuccessful attempt to restore homeostatic protein folding in the ER lumen, remains an incomplete story. Recently some of the key molecular players have been identified and at the transcriptional level and it has become clear that multiple proteins interacting in the nucleus to coordinately shut off survival genes and activate pro-death genes is a common theme. The ATF4-mediated induction of CHOP, following PERK activation and eIF2 α phosphorylation, is a key event in the switch, under stress, from adaptation toward death and has received the most attention in the literature. Initial clues implicating CHOP as a participant in the UPR-mediated cell death program came to light when it was reported that overexpression of CHOP could induce cell cycle arrest and apoptosis (Barone, Crozat et al.

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1994; Matsumoto, Minami et al. 1996); and that *Chop* null mice were partially resistant to ER stress-mediated apoptosis (Zinszner, Kuroda et al. 1998; Oyadomari, Koizumi et al. 2002). Though it is clear that CHOP has an important role in ER stress-induced apoptosis a comprehensive analysis of its target (downstream of *CHOP* or *DOC*) genes has not revealed a smoking gun (Wang, Kuroda et al. 1998) (and our un-published observation), suggesting that this effect might be indirect.

Though CHOP target genes capable of directly inducing apoptosis have not been identified it can induce the expression of death receptor 5 (DR5) and *tribbles*-related protein 3 (TRB3) in a stress-dependent fashion to modulate the UPR death response. DR5 is a member of the TNFR family and can mediate cell death via the FADD signaling complex (Chaudhary, Eby et al. 1997). Thapsigargin enhanced DR5 expression was found to be mediated by CHOP in human cancer cell lines and sensitized them to TRAIL-induced cell death (Hetschko, Voss et al. 2008). Increased expression of DR5 enhanced ligand binding and led to the recruitment of adaptor proteins at the intracellular DR5 death domain and initiated a signaling cascade that culminated in the cleavage and activation of caspase 8 similar to TNFR1, Fas and DR3 and DR4. The discovery that CHOP could modulate DR5 expression linked the UPR to the “extrinsic” death receptor-mediated apoptosis pathway which, following caspase 8 cleavage, culminates in the activation of executioner caspases 3 and 7 to target substrates in the nucleus such as lamins and PARP immediately prior to DNA fragmentation. It should be noted that additional *in vitro* experiments revealed that siRNA knockdown of DR5 could interfere with the conformational change of Bax and caspase 3 activation required for apoptotic cell death following stress (Yamaguchi and Wang 2004). *Tribbles*-related protein 3 (*TRB3*) has also been identified as an ER stress-inducible target of CHOP/ATF4 signaling that can modulate UPR-dependent cell death induced by various ER stressors (Ohoka, Yoshii et al. 2005; Ord and Ord 2005; Ord, Meerits et al. 2007). Though there is a report that siRNA knockdown of TRB3 could reduce ER stress-dependent cell death in 293 cells (Ohoka, Yoshii et al. 2005) most studies have reported that TRB3 antagonizes the anti-proliferative and cytotoxic effects of the UPR by down-regulating ATF4 transcriptional activity thereby lowering the level of intracellular reactive oxygen species (ROS) (Ord, Meerits et al. 2007).

The UPR utilizes the BCL2 family during the cell death process via distinct and complementary mechanisms. CHOP induction can dramatically reduce cellular levels of BCL2 to directly potentiate the release of cytochrome c and initiate the mitochondrial or “intrinsic” cell death pathway (McCullough, Martindale et al. 2001). The subset of BCL2 family members that possess only the BCL2 homology domain 3 (BH3 domain), in stark contrast to BCL2, are all known to be pro-apoptotic. In general this small group of proteins have a similar *modus operandi* in the apoptotic push toward death which is characterized by their ability to interact with BCL2 impeding its ability to keep Bax and Bak in an inactive conformation. Activation of Bax or Bak precipitates the release of cytochrome c from mitochondria and Ca^{+2} from the ER, thus setting in motion the process of apoptosis. Though currently 9 members of the BH3-only protein family have been identified only NBK/BIK, BIM, NOXA and PUMA have been closely associated with the UPR-mediated cell death (Morishima, Nakanishi et al. 2004; Fribley, Evenchik et al. 2006; Kieran, Woods et al. 2007; Shimazu, Degenhardt et al. 2007; Zou, Cao et al. 2009).

A number of molecules in addition to CHOP, ATF4, Bax/Bak, and caspase 12 are known to be involved with UPR-mediated cell death. It has been known for over a decade that thapsigargin could activate the c-Jun NH(2)-terminal kinase cascade and apoptosis in an oxidative stress-dependent fashion (Srivastava, Sollott et al. 1999). Several years later it was reported that the activation of IRE1 α led to the formation of a tripartite complex at the cell membrane with TRAF2 and ASK1 prior to the activation of the JNK cell death program

(Urano, Wang et al. 2000; Matsuzawa, Nishitoh et al. 2002; Nishitoh, Matsuzawa et al. 2002). It is clear that JNK plays an important role in UPR-mediated cell death. Since we will not describe any large scale methods focused to identify the activation of JNK signaling, further discussion has been omitted. For thorough and recent reviews of stress mediated activation of JNK signaling: [Nagai, Noguchi et al. 2007; Rincon and Davis 2009]

2. Monitoring proliferation and caspase activation following UPR activation

When cells undergo apoptosis many distinct biochemical changes occur that can be readily detected to identify early or late stages of the death process. Recent advances in fluorescent and luminescent technology has made it possible to detect these changes with very limited numbers of cells and reagents in a 96 or 384 well format in a relatively cost-effective fashion. Importantly, we will describe how to monitor cell viability/proliferation and caspase activation in parallel to clearly establish the kinetics of cell growth/death and caspase activation to determine the contribution of apoptosis in UPR-mediated cell death. Members of the caspase family of cysteine proteases are necessary for nearly all apoptotic responses. Importantly, caspase 3^{-/-}, caspase 7^{-/-} and caspase 9^{-/-} murine embryonic fibroblast were found to be resistant to thapsigargin, tunicamycin, brefeldin A and calcium ionophore-induced ER stress suggesting an essential role for mitochondria-mediated or intrinsic cell death following unresolved protein folding defect (Masud, Mohapatra et al. 2007). Although this group demonstrated that caspase 8^{-/-} MEF's were not protected from these stresses it has been reported that in murine cells caspase 12 can activate caspase 8 following UPR activation (Morishima, Nakanishi et al. 2002; Rao, Castro-Obregon et al. 2002). The caspase 12 gene in humans is inactive due to a single nucleotide polymorphism (Saleh, Vaillancourt et al. 2004).

Required materials:

Standard tissue culture facility and reagents

Electronic multi-channel pipets (Matrix)

Luminescent plate reader (M5 Molecular Devices, GloMax-Multi+ or similar)

Cell Titer-Glo Luminescent Cell Viability Assay (Promega G7570)

Caspase-Glo 3/7 Assay (Promega G8091)

Luminescent assays for simultaneous detection of proliferation and caspase 3/7 activation

1. 12,500 cells (~75% confluent) in 50μL of appropriate medium are plated in white opaque 96 well tissue culture plates the day before addition of ER stress-inducing (or other compound of interest) reagent. Thapsigargin (Tg) used at 250nM – 1.0μM is a good positive control for most cell types for 24 hour time course experiments. A clear observation plate should be treated in parallel to determine cell density and to visually observe the experimental wells. Measurement of caspase activation and proliferation should be performed 4, 8, 16 and 24 hours after treatment. Two plates of cells (one for 4 and 8h and one for 16 and 24h) should be plated with triplicates for each time point. A 500μM Tg stock can be prepared in DMSO; positive control culture wells should contain 250nM -1μM Tg. It is imperative that vehicle and Tg-treated controls are included for each plate.
2. Assuming ~10% evaporation of the medium after overnight plating, the controls and compound/s of interest should be added to cell cultures in a volume of 5μl. Six point dose-response assays performed in triplicate are sufficient to attain significance. Proliferation and caspase activity assays should be performed on the same plate, therefore, six wells must be treated for each time point and

concentration. If serial dilutions are performed to achieve desired concentrations, the vehicle-treated wells should contain the highest concentration of vehicle any condition is exposed to.

3. For the simultaneous measurement of proliferation and caspase 3/7 activation both luminescent reagents should be thawed in a room temperature water bath until they have equilibrated completely with room temperature. 30–50 μ l of each reagent should be added to the appropriate wells and incubated with shaking at room temperature for 10–15 minutes. When beginning this assay the plates should be read every 15 minutes over a 45 minute period to determine when the optimal signal occurs. Data may be represented graphically in a dual y-axis-fashion to compare the reduction in proliferation and activation of caspases following exacerbation of the adaptive capacity of the UPR (Figure 1).

Special note on “edge effect”. Unequal distribution of attached cells in 96 or 384 well plates, incubator heat gradient fluctuations or levelness of incubators can lead to a phenomenon known as edge effect. Edge effect is characterized by a ring or crescent-shaped pattern of adherent cells at the periphery of a well and can have dramatic effects on inter-well reproducibility. Several solutions including not using the outermost wells of a plate have been proposed. Another simple technique has been described whereby plates are allowed to sit in the tissue culture hood at room temperature for 1 hour before placing cultures in an incubator (Lundholt, Scudder et al. 2003).

3. Monitoring the expression of UPR and cell death target genes

The use of 96 or 384 well thermocyclers for qRT-PCR has moved academic laboratories' ability to analyze gene expression light years beyond the Northern blot; however, most protocols still rely on the use of cumbersome and time consuming phenol-based extractions from relatively large numbers of cells for RNA isolation. The recent introduction of Cells to CT from (Applied Biosystems/Ambion) has provided a phenol-free system that we have found can provide enough high quality RNA template for the production of cDNA from as few as 500 cells less in than 10 minutes. In this section we will describe a slight protocol modification that increases by 2-fold the number of cDNA reactions that can be performed from the manufacturer's indication. We will then describe how the cDNA can be diluted and interrogated with TaqMan (Applied Biosystems) primer probe sets to evaluate the expression of UPR and apoptosis genes in stressed cells.

Required materials:

- Standard tissue culture facility and reagents
- Electronic multi-channel pipets (Matrix)
- Cells to CTTM (Applied Biosystems/Ambion)
- TaqMan Gene Expression Assay (Applied Biosystems)
- Quantitative Real Time PCR thermocycler

3.1 Cell Culture

1. 1.25×10^4 cells (75–90% confluent is fine) are plated in a 96-well tissue culture plate 16–24 hours prior to stress induction. If working with an experimental system that requires the use of very few cells, we have successfully performed this protocol with 500 cells. Note: reduced numbers of cells may require a lower (e.g.

1:10 or 1:25) dilution of cDNA before measurement of gene expression as described in section 3.2.3.

2. ER stress and apoptosis can be induced by treating cultures with 1.0–2.5 µg/ml tunicamycin (Tm) or 0.25–1.0 µM thapsigargin (Tg) for 6–24 hours.

3.2 Cells-to-CT™ (Applied Biosystems/Ambion)

3.2.1 Cell Lysis

1. Aspirate and discard culture medium (1–6 hours after treatment for the measurement of UPR and early apoptosis genes) from the 96-well plate.
2. Wash cells briefly with 50 µl cold (4°C) 1X PBS.
3. Aspirate PBS from the wells and add 25 µl of Lysis Solution containing 1:100 DNase I to remove genomic DNA.
4. Mix well by pipetting up and down 5 times; incubate the lysis reactions for 5 minutes at room temperature (19–25°C).
5. Add 2.5 µl (1:10) Stop Solution to each lysis reaction and mix well by pipetting up and down 5 times; incubate for 2 minutes at room temperature.
6. Lysate may be used immediately for cDNA synthesis or stored promptly at –20°C.

3.2.2 Reverse Transcription

1. Preparation of Master Mix:

Component	Per Rxn (µl)	96 Rxns [‡] (µl)	384 Rxns [‡] (µl)
2X RT Buffer	12.5	1320	5280
20X RT Enzyme Mix	1.25	132	528
Nuclease-free Water	1.25	132	528
Final Volume	15	1580	6340

[‡]96 and 384 Rxn amounts reflect 10% overage.

2. Add 10 µl of each Cells to CT lysate to a 15 µl aliquot of RT Master Mix and mix gently.
3. IMPORTANT: Centrifuge tubes or plates prior to thermocycling to assure mixing.
4. Thermocycling:

	Stage	Repeats	(°C)	Time
Reverse transcription	1	1	37	60 min
RT inactivation	2	1	95	5 min
Hold	3	1	4	∞

3.2.3 Quantitative reverse-transcription real time PCR (qRT-PCR)—The TaqMan Gene Expression Assay and Master Mix cocktail and cDNA templates are added to 96 or 384 well plates separately, as described:

Reaction summary

Component	Per Rxn (μl)
20X TaqMan Gene Expression Assay	0.25
2X TaqMan Gene Expression Master Mix	2.5
cDNA template (diluted 1:50)	2.25
Total Volume	5 *

* 5 μl reaction volumes will dramatically reduce reagent use.

A. Preparation of TaqMan Gene Expression Assay/Master Mix cocktail and plate set up: Calculate the volume of TaqMan Gene Expression Assay and Master Mix cocktail required to measure each cDNA in triplicate. For example: To measure the expression of *18S* (housekeeping gene) in 8 cDNA samples would require TaqMan Gene Expression Assay and Master Mix cocktail for 24 wells; (Figure 2, gray shaded wells). **Calculations include a 10% overage to account for errors in pipetting.**

TaqMan Gene Expression Assay/Master Mix cocktail for 8 samples (24 Rxns)

Component	Per Rxn (μl)	24 Rxns (μl)
20X TaqMan Gene Expression Assay	0.25	6.6
2X TaqMan Gene Expression Master Mix	2.5	66
Final Volume	2.75	72.6

* For UPR, oxidative stress and cell death gene expression assay commonly used by our lab, see Table 1.

B. Dilution of cDNA: Each cDNA should initially be diluted 1:50 in nuclease-free water; calculate the volume of diluted cDNA needed for triplicate samples (Figure 2, black shaded wells). **Calculations include a 10% overage to account for errors in pipetting.**

Diluted cDNA Template:

Component	Per Rxn (μl)	12 Rxns (μl)
cDNA Template (1:50)	2.25	29.7

C. Assay plate set up

1. Transfer 2.75μl of the TaqMan Gene Expression Assay/Master Mix cocktail from 3.2.3A to the bottom of the appropriate wells of a 96 or 384 well plate.
2. Transfer 2.25μl of the Diluted cDNA Template made in 3.2.3B to the bottom of the appropriate wells of the 384-well plate.
3. Seal the plate with the appropriate cover and IMPORTANT: centrifuge briefly.

D. Thermocycling

	Stage	Step	Repeats	°C	Time
Hold	1	1	1	50	2 min
Hold	2	1	1	95	10 min
Cycle	3	1	40	95	15 sec
		2		60*	1 min

*TaqMan primer/probes are optimized for annealing at 60°C

E. Data analysis: Methods of calculating changes in gene expression vary by investigator and instrument and are therefore, omitted.

3.3 Semi-quantitative PCR analysis of *XBPIu* and *XBPIs* with Cells to CT-derived cDNA

For semi-quantitative PCR analysis of *XBPI* unspliced (*XBPIu*) and spliced (*XBPIs*) with Cells to CTTM-derived cDNA the Taq PCR Core Kit (Qiagen) is routinely utilized, according to the manufacture's protocol.

1. Human primers for *XBPIu* and *XBPIs* (Park, Woo et al. 2007):

hXBPI Forward: CCTTG TAGTTGAGAACCAGG

hXBPI Reverse: GGGGCTTGGTATATATGTGG

2. Preparation of Master Mix

Component	Volume/Rxn (μl)	Conc./Rxn
Forward Primer	1 (10 pmol/μl)	200 nM
Reverse Primer	1	200 nM
dNTP mix	1	200 μM each dNTP
10X Buffer (w/15mM MgCl₂)	5	1X
Taq DNA Polymerase	0.25	2.5 U
Nuclease-free Water	36.75	
Final Volume Master Mix	45	

3. Mix PCR Master Mix thoroughly and add 45μL to PCR tubes or plates.
4. Add 5μL of UNDILUTED cDNA from 3.2.2 to the tubes/plates containing the Master Mix and centrifuge briefly.
5. Thermocycling

	Stage	Step	Repeats	(°C)	Time
Initial Denaturation	1	1	1	94	3 min
Denaturation	2	1		94	30 sec
Annealing		2	30	54	30 sec
Extension		3		72	1 min

	Stage	Step	Repeats	(°C)	Time
Final Extension	3	1		72	10 min
Hold	4	1	40	4	∞

6. Amplicons can be resolved electrophoretically with a 1.8% agarose gel: XBP1u is 442 bp and XBP1s is 416 bp.

Note: We have improved throughput of hXBP1 amplicon analysis with the QIAxcel System (Qiagen). For amplicon analysis with this machine, the appropriate cartridge is the QIAxcel DNA High Resolution Gel Cartridge (number); and the analysis can be performed using the OM500 method.

4. DNA fragmentation analysis

The hallmark of late-stage apoptosis has come to be the endonucleolytic cleavage (laddering) of DNA between nucleosomes into different sized fragments (Schwartzman and Cidlowski 1993).

Required materials

DNA lysis buffer prepared by mixing:

0.5ml 1M Tris-HCL (pH 7.4)

0.1 ml 5M NaCl, 1.0 ml 0.5M EDTA (pH 8.0)

2.5ml 10% sodium dodecyl sulfate (or sodium laurel sarkosinate)

45.9ml ddH₂O.

Proteinase K (cat# P6656, Sigma)

Standard agarose gel electrophoresis equipment and reagents

1. Approximately 2×10^6 cells are plated the day before the addition of an apoptotic stimulus in 10 cm tissue culture dishes. This assay can be performed with fewer cells if necessary however, the reduction of cells from death and extensive organic extractions will reduce the yield of fragmented genomic DNA considerably.
2. Following treatment (16–24 hours), the attached and floating cells are pooled by scraping and gently pelleted at 1200–1400 rpm in a table-top swinging bucket centrifuge.
3. The cell pellet is solubilized in 0.5 ml DNA lysis buffer. Histone proteins need to be digested from the genomic DNA by supplementing the lysis buffer at the time of use with 100µg/ml of proteinaseK. The reaction is incubated for 2 hours in a 50°C water bath. Note: for this and all subsequent steps the use of blunt ended pipet tips is recommended to avoid shearing of genomic DNA; alternatively scissors may be used to trim a few millimeters off the ends of conventional tips.
4. DNA is extracted twice with phenol-chloroformmixed 1:1, and twice with chloroform alone. Following the final chloroform extraction the aqueous phase is transferred to a clean Eppendorf tube and a 1/10 volume of 3M sodium acetate (pH 5.2) is added along with either a 0.8 volume of isopropanol OR 2.5 volumes of 100% ethanol to precipitate the DNA.
5. Precipitated DNA is washed and more completely pelleted by the addition of 0.5ml of 70% ethanol, centrifuging at high speed for 10–15 minutes, and allowed to dry

briefly at room temperature. The washed pellet is re-suspended in 20–30µl of TE (pH 8) supplemented with 0.25mg/ml RNase A (AM2270, Applied Biosystems). Fifteen to thirty micrograms of DNA (A260 determined) is resolved on a 1.5% agarose gel (Figure 3).

Note: A ladder appearance of the DNA is indicative of apoptosis; a smeared appearance suggests the observed cell death is the result of some other form cell death such as necrosis.

Acknowledgments

The authors appreciate the critical review of this manuscript by Dr. Harmeet Malhi. Portions of this work were supported by NIH grants DK042394, HL052173, and HL057346, as well as a MH084182 and MH089782 (RJK). Additionally, AMF is supported by DE019678.

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Figure 1.

Figure 2.

Figure 3.