CHAPTER ELEVEN

# Multidimensional Profiling in the Investigation of Small-Molecule-Induced Cell Death

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## Abstract

Numerous morphological variations of cell death have been described. These processes depend on a complex and overlapping cellular signaling network, making molecular definition of the pathways challenging. This review describes one solution to this problem for small-molecule-induced death, the creation of high-dimensionality profiles for compounds that can be used to define and compare pathways. Such profiles have been assembled from gene expression measurements, protein quantification, chemical–genetic interactions, chemical combination interactions, cancer cell line sensitivity profiling, quantitative imaging, and modulatory profiling. We discuss the advantages and limitations of these techniques in the study of cell death.

## 1. INTRODUCTION

While descriptions of active cell death processes can be traced as far back as the nineteenth century (Virchow & Chance, 1860), the modern era of cell death research was firmly established by the description and coining of apoptosis in 1972 (Kerr, Wyllie, & Currie, 1972). The authors described the consistent nuclear, cytoplasmic, and organellar changes in cells dying in a variety of physiological and pathological settings. Their description was entirely morphological for obvious reasons: the molecular tools to further characterize the phenomena were not available.

Such tools began to be developed in the late 1980s and early 1990s. For example, Robert Horvitz and colleagues uncovered the genetic basis of apoptosis in *Caenorhabiditis elegans* and showed that these pathways were largely conserved in mammalian cells (Ellis & Horvitz, 1986; Hengartner, Ellis, & Horvitz, 1992; Hengartner & Horvitz, 1994; Miura, Zhu, Rotello, Hartwieg, & Yuan, 1993; Yuan & Horvitz, 1990; Yuan, Shaham, Ledoux, Ellis, & Horvitz, 1993). These and subsequent studies allowed a transition from purely morphological descriptions of cell death processes to biochemical descriptions. However, this transition has been incomplete, and morphological descriptors remain prominent, if not predominant, in the study of cell death. There is a growing push to move away from morphological characterizations, however, given their dependence on subjective criteria and the recognition that morphology is not always a marker of

unique underlying biochemistry. A panel of cell death experts recently published formal recommendations to transition to fully biochemical descriptions of cell death and provided recommended biochemical descriptors of a number of cell death processes (Galluzzi et al., 2012).

In the past decade, however, not only have the identified morphological varieties of cell death expanded significantly (Fig. 11.1), but the biochemical pathways underlying these processes have been shown to be complex and interconnected. Calling a form of cell death "caspase dependent," for example, does little to clarify if the signaling was conducted through the intrinsic, extrinsic, or granzyme-mediated pathway (Taylor, Cullen, & Martin, 2008), or even if the resultant morphology is consistent with apoptosis or with pyroptosis, an inflammatory form of cell death dependent on the activity of caspase 1 (Fernandes-Alnemri et al., 2007). Necroptosis, a well-accepted form of regulated necrosis that involves signaling through the RIP family proteins, can be activated by binding of the same death receptor ligands that can initiate extrinsic pathway apoptosis (Degterev et al., 2008, 2005). Other forms of caspase-independent death can be initiated via mitochondrial outer membrane permeabilization (MOMP), the stimulus that typically initiates intrinsic pathway apoptosis (Colell et al., 2007).

How can we fully characterize, and distinguish between, complex, interconnected processes that can be difficult to distinguish either morphologically or biochemically? One solution is to vastly increase the dimensionality of

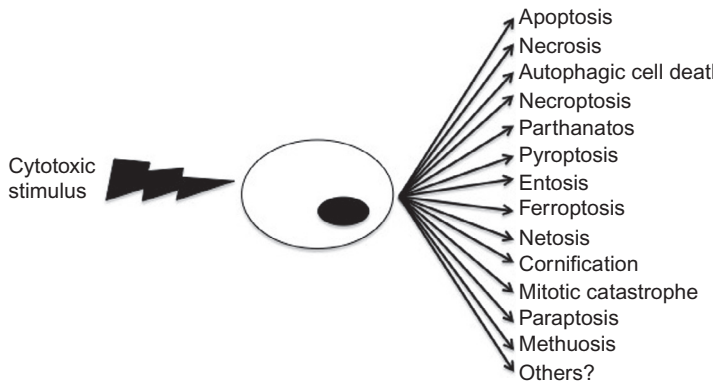


**Figure 11.1** Diversity of cell death pathways. The number of characterized cell death pathways has expanded significantly in the past decade. In a given context, the death pathway is determined by the cytotoxic stimulus, the cell type, the microenvironment, and the presence of cotreatments, among others. The total number of death pathways accessible to cells remains unknown and an important question for investigation.

the measurements taken. These high-dimensionality profiles can then be compared to each other in order to relate and distinguish between lethal processes.

Such systems have been developed and primarily implemented in the study of the bioactivity of small molecules. Small molecules are versatile tools for studying a range of biological processes (Stockwell, 2004) and are particularly useful in the study of cell death. They can easily be applied to different cellular contexts and a variety of organisms and potentially translated into *in vivo* studies. Concentrations can be varied to investigate the thresholds for processes and intermediate effects of inhibiting protein function. Compounds can be applied and removed with precise temporal control, allowing for the investigation of the kinetics of events. Small molecules can inhibit single functions of multifunctional enzymes, allowing for more detailed investigation of processes. The utility of small molecules in cell death is demonstrated by the widespread use of small-molecule-induced cell death as a model for studying apoptosis. More recently, small-molecule screens have identified compounds that are essential for defining alternative cell death processes (Degterev et al., 2005; Dixon et al., 2012).

This review summarizes a number of the systems that have been developed to create high-dimensionality profiles for small molecules (see Fig. 11.2 and Table 11.1) and focuses on their utilization or potential utilization in the study of cell death.

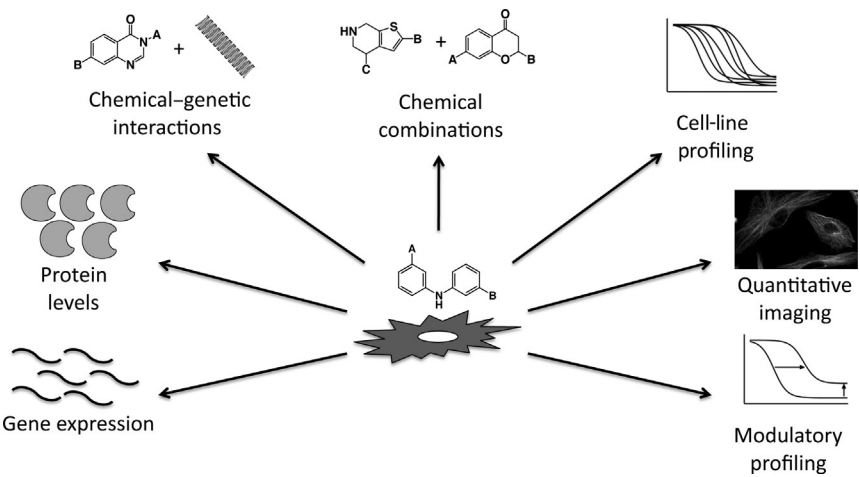


**Figure 11.2** Small-molecule profiling technologies. Overview of the different methodologies that have been used to create profiles for small molecules. Profiles are based on quantitative measurements of the effect of a small molecule on cells. These profiles can be used to define a small-molecule-induced process and to compare to other processes.

**Table 11.1** Small-molecule profiling modalities

| Measurement type | Throughput | Functional measurement | Application to mammalian cells | Application to diverse cell type | Single-cell resolution | Accessibility to scientific community | Reproducibility | References |
|---|---|---|---|---|---|---|---|---|
| Gene expression | High | No | Yes | Yes | No | Very high | Low | Hughes et al. (2000), Lamb et al. (2006) |
| Proteomic | Intermediate | No | Yes | Yes | Sometimes | Low | Low | Dix, Simon, and Cravatt (2008), Mahrus et al. (2008), Muroi et al. (2010), Sevecka and MacBeath (2006) |
| Gene–small-molecule interaction | High | Yes | Limited | No | No | Intermediate | Intermediate | Parsons et al. (2004, 2006) |
| Small-molecule combinations | Low | Yes | Yes | Yes | No | Low | High | Farha and Brown (2010), Lehar et al. (2007), Yeh, Tschumi, and Kishony (2006) |

*Continued*

**Table 11.1** Small-molecule profiling modalities—cont'd

| Measurement type | Throughput | Functional measurement | Application to mammalian cells | Application to diverse cell type | Single-cell resolution | Accessibility to scientific community | Reproducibility | References |
|---|---|---|---|---|---|---|---|---|
| Cell line profiling | Low | Yes | Yes | No | No | High | Low | Barretina et al. (2012), Basu et al. (2013), Garnett et al. (2012), Paull et al. (1989), Weinstein et al. (1997) |
| Quantitative imaging | Low | No | Yes | Yes | Yes | Low | Low | Perlman et al. (2004), Young et al. (2008) |
| Modulatory profiling | Intermediate | Yes | Yes | Yes | No | Low | High | Wolpaw et al. (2011) |

## 2. GENE EXPRESSION PROFILING

Gene expression profiling is a powerful tool to explore cellular states, development, and disease. Investigation of small-molecule mechanisms of action was among the first applications of gene expression profiling (Schena et al., 1996; Stockwell, Hardwick, Tong, & Schreiber, 1999). Given the informational richness of gene expression profiles and their widespread availability and relative affordability, this method has developed into the most widely utilized system for profiling and comparing small-molecule bioactivities.

### 2.1. Comparing small-molecule profiles

The initial study demonstrating the utility of comparing small-molecule-induced gene expression profiles was performed in yeast by Steve Friend and colleagues (Hughes et al., 2000). This landmark study was largely focused on gene expression changes induced by genetic deletion, but it also measured genome-wide profiles for 13 well-characterized small molecules. They clustered the compounds and the gene knockouts based on their expression profiles and found that knockouts of genes with similar cellular function clustered together and that the small molecules clustered with knockouts of their characterized targets. Additionally, they found that the changes induced by the small-molecule dyclonine clustered with the knockout of *erg2*. The target of dyclonine was unknown at this time, and the authors provided evidence that dyclonine in fact inhibited Erg2p.

Inspired by the success of this approach, Todd Golub and colleagues developed the "Connectivity Map," a compendium of gene expression profiles generated after treatment with 164 different small molecules, largely in two different human cell lines (Lamb et al., 2006). They applied a nonparametric, rank-based pattern matching approach to analyze the data. A unitless "query signature" was generated based on genes up- and downregulated in a biological process of interest. This signature was compared to the database of gene expression profiles; the compounds in the database were ranked based on how well they correlated to the query signature (see Fig. 11.3).

The authors first demonstrated that their system had platform independence by showing that signatures for compounds derived from gene expression experiments on other platforms had high "connectivity scores" to the compounds in the database with the same mechanism of action. They then demonstrated the utility in investigating the mechanism of action of an uncharacterized compound. They queried the database with a signature based on the gene expression

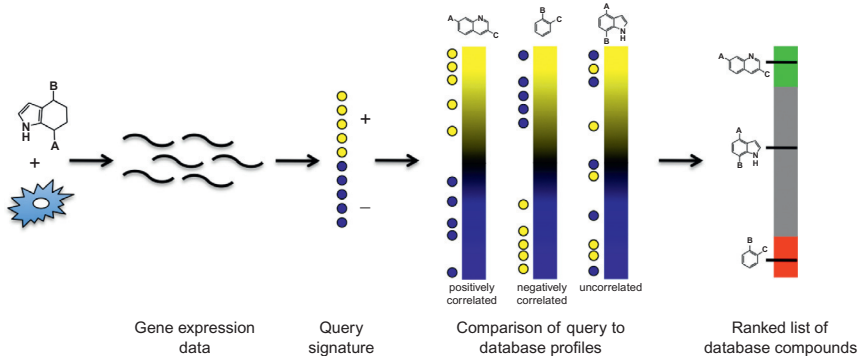


**Figure 11.3** Connectivity Map schematic. An outline of the process of querying the Connectivity Map is shown. Gene expression information is generated from an independent source, shown here as small-molecule treatment of cells. Those data are used to generate a unitless query signature which is then compared to the database of gene expression profiles. Small molecules in the database are ranked based on how well their profiles are correlated to the query signature.

changes induced by treatment with gedunin, a small-molecule natural product without a characterized mechanism of action, and found that it was highly correlated to HSP90 pathway inhibitors. They subsequently demonstrated that gedunin did in fact inhibit this pathway (Hieronymus et al., 2006). Lastly, the authors demonstrated that signatures generated based on disease states, such as obesity and Alzheimer's disease, could be used to identify compounds capable of inducing or reversing these states.

## 2.2. Protocol for the use of the Connectivity Map database

All of the data generated by the Connectivity Map project were made publicly available on their Web site (www.broadinstitute.org/cmap) with software tools allowing for the uploading of a user-generated query profile and interrogation of the database. Since the initial publication, the database has been updated to include profiles generated from treatment with 1309 compounds and a much larger expansion is planned, likely to be released prior to publication of this article. A stepwise protocol for querying the database is described below. Further instruction and guidance are available at the project Web site.

**I.** *Generate a query signature*

A "query signature" is a unitless list of up- and downregulated genes representing a biological process of interest, recommended to involve anywhere between 10 and 500 genes depending on the knowledge of the process. There are a number of ways to generate a signature. It can be derived from independent gene expression profiling experiments, it

can be manually generated based on prior biological knowledge or experiments, or it can be generated from the profiles contained within the Connectivity Map database itself. The Web site software allows for the use of up to three profiles for generation of a signature; however, the raw or partially processed data files can be downloaded and used independently to generate signatures.

**II.** *Upload signature*

In order to upload a custom-generated signature, up- and downregulated genes must be converted to the corresponding probeset name used in the Affymetrix HG-U133A array. This can be done using tools available at http://www.affymetrix.com/analysis/netaffx/index.affx. The list of probesets is then converted to a .grp file (this can be done using Microsoft Excel).

**III.** *Query the Connectivity Map database*

Separate .grp files for up- and downregulated probesets are uploaded and selected for use in the query.

**IV.** *Analysis of the results*

The results of a query can be viewed as a ranked list of either the separate instances (specific compound, cell line, concentration, time point) or as a ranked list of all instances of a specific compound, all instances of a compound/cell line combination, or all instances of compounds classified under the same ATC code. The "connectivity score" is calculated based on the enrichment of the upregulated query genes among the most overexpressed genes in the database instances (or groups of instances) and the enrichment of the downregulated query genes among the most underexpressed genes in the database instances (or groups of instances). The instances or groups of instances are ranked from those with the highest "connectivity score" (correlated) to those with the lowest score (anticorrelated) (see Fig. 11.3).

**V.** *Independent validation*

The authors make clear that the Connectivity Map is best used as a hypothesis generator. Finding based on a query must be independently validated in separate assays.

## 2.3. Applications in cell death

In the 7 years since the initial publication of the Connectivity Map database, it has been widely utilized, cited by nearly 800 scientific articles. These include a number of interesting new insights into cell death, a selection of which are summarized below.

One of the initial studies that utilized the Connectivity Map found that modulation of the antiapoptotic protein MCL1 was important for restoring glucocorticoid-induced apoptosis in acute lymphoblastic leukemia (ALL) cells (Wei et al., 2006). The authors first generated a query signature of genes whose expression distinguished between glucocorticoid-sensitive and glucocorticoid-resistant ALL samples. They used this signature to query the Connectivity Map database and found that multiple instances of the compound rapamycin were among the most highly ranked instances, suggesting that rapamycin may induce a glucocorticoid-sensitive state. They then showed in multiple cell lines derived from lymphoid malignancies that rapamycin sensitized to glucocorticoid-induced apoptosis, but not to other cell-death-inducing agents. They went on to show that rapamycin down-regulated MCL1, that overexpression of MCL1 conferred glucocorticoid resistance, and that MCL1 suppression conferred sensitivity. This study was thus able to use an insight gleaned from the Connectivity Map to demonstrate the specific dependence of glucocorticoid-induced apoptosis on MCL1 and not on other BCL2-family member proteins. A subset of the authors of this study used the Connectivity Map further in a subsequent study investigating MCL1 (Wei et al., 2012). They used the Connectivity Map to identify compounds that induce profiles similar to triptolide, a compound that they had shown represses MCL1 expression, but not the expression of proapoptotic proteins. They found a number of related compounds that also repress MCL1 and that all of these compounds were acting as transcriptional inhibitors. MCL1 repression was essential for these compounds' activity, thus suggesting a biochemical basis for the mechanism of cell death induced by such compounds.

One of the essential challenges in cancer therapeutic development is the identification of compounds that can induce cancer-cell-selective cell death. Hassane and colleagues used the Connectivity Map to identify compounds that selectively kill acute myeloid leukemia (AML) cells, and particularly AML stem cells (Hassane et al., 2008). The authors identified a gene expression signature from the treatment of cells with parthenolide, which had previously been shown to selectively induce death in AML cells (Guzman et al., 2005). They used this signature as a query to search multiple gene expression databases, including the Connectivity Map, and identified other compounds with similar profiles. They subsequently showed that these compounds were also able to kill AML cells, including AML stem cells, and that this death was dependent on inhibition of NF-κB and on the generation of oxidative stress. In a different study, Stumpel and colleagues used

the Connectivity Map to find compounds to target the particularly aggressive form of ALL harboring MLL rearrangements (Stumpel et al., 2012). They derived a query signature of genes selectively overexpressed in MLL-rearranged cell lines and found that a number of HDAC inhibitors produced profiles anticorrelated with the query. They went on to show that these compounds were selectively lethal in MLL-rearranged cells compared to MLL wild-type cells and that the compounds decreased expression of many of the signature genes and increased methylation at their promoters.

Endoplasmic reticulum (ER) stress plays a role in activating cell death (Rasheva & Domingos, 2009). Two recent studies have used the Connectivity Map to further investigate the role of ER stress in small-molecule-induced cell death. The first study investigated the difference in the mechanisms of action of two structurally related procaspase-activating compounds and found that at high concentrations one compound (PAC-1) activated ER-stress-mediated apoptosis, while the other (SPAC-1) did not (West et al., 2012). They created a signature query out of the 50 most highly up- and downregulated genes after treatment with a high concentration of PAC-1 and found that thapsigargin, a known inducer of ER stress, was the compound with the highest connectivity score. They then showed that high concentration of PAC-1 caused similar ultrastructural changes as thapsigargin and caused a similar increase in cytosolic calcium and a decrease in ER calcium, elucidating a side effect of this compound when used at high concentrations. A different study identified a novel, reversible ER stress response that is regulated in part by MCL1. This process was initially observed in response to treatment with apogossypol, a putative BCL-2 inhibitor. The authors created a query signature based on expression changes induced by apogossypol and used the Connectivity Map to identify 20 diverse compounds capable of initiating the same process, demonstrating its widespread occurrence.

## 2.4. Advantages and limitations in the study of cell death

Advantages of the use of gene expression profiles include their widespread availability and accessibility and the high dimensionality and information richness of the data. Gene expression profiling is already a widely available technology. With the recent rapid decrease in cost and increase in speed of sequencing technology, RNA-seq has replaced microarrays as the preferred method for gene expression measurement (McGettigan, 2013). This transition is likely to continue to decrease costs and increase the quality of gene

expression measurements. The ubiquity of these measurements has driven the development of a host of readily available tools for the processing and analysis of gene expression data. The Connectivity Map project made an explicit goal to allow for platform independence, validated this aspect in their initial publication, made all of their data publicly available, and developed Web-based tools for querying their data. Multiple successful independent studies, including some of those described above, have further confirmed the utility of applying independently generated expression data. This accessibility is particularly valuable to the broader scientific community and unique among the technologies described within this review. The high dimensionality of a genome-wide expression profile allows the opportunity to accurately characterize such a highly complex process such as cell death. In addition, the content of the individual profiles are information-rich and can be mined to identify specific genes pathways involved in a death process.

Notable disadvantages of the use of expression data in studying cell death include difficulty detecting time-dependent, cell type-dependent, and concentration-dependent phenomena; the high barrier to reproducing a similar system focused on cell death; and the difficulty distinguishing on-target from off-target transcriptional effects of small molecules. As noted by the authors of the Connectivity Map study, for reasons of feasibility they were forced to limit the time points of treatment, the number of different cell types, and the concentrations used. The profiles are therefore snapshots of a cellular state that may or may not successfully represent prior and future states. While the relatively early time that they chose (6 h) is appropriate for many cell death processes, others can occur more rapidly (Newman, Crown, Leppla, & Moayeri, 2010) or much more slowly (Turmaine et al., 2000). In addition, rapid enzymatic cascades, such as activation of preformed zymogens, may occur too quickly to be accurately represented by transcriptional changes. While the authors suggest that insights can be gained across cell types and species, cell death processes can be active only in specific cellular or genetic contexts and therefore not likely accessible with the use of only two cancer cell lines. Given the cost of producing the Connectivity Map database, it is not feasible to reproduce the system in a relevant cellular context to study a phenomena poorly accessed in the chosen cell lines. The Connectivity Map largely incorporated a single dose of compounds. This limitation may mask effects only activated at higher or lower concentrations. It also can exacerbate the problem of distinguishing off-target effects. Even reportedly specific small molecules can have pleiotropic actions on cells (Campillos, Kuhn, Gavin, Jensen, & Bork, 2008; Keiser et al., 2009).

It is not obvious or trivial to distinguish the transcriptional effects of a compound that are related to the process of interest from those that are related to an off-target effect. Thus, despite the proven value of Connectivity Map, additional tools are needed to probe cell death mechanisms with small molecules, using measurements with high dimensionality.

## 3. PROTEIN QUANTIFICATION

One potential improvement over gene expression measurements involves the direct detection of changes in protein abundance and protein modifications. While mRNA levels are often used as a surrogate for protein level, changes in mRNA can correlate poorly to changes in protein level (Haider & Pal, 2013). A number of methods have been developed for the widespread measurement of protein levels and modifications to those proteins, basally and in response to small-molecule treatments. Changes in protein levels and posttranslational modifications can be monitored with two-dimensional difference gel electrophoresis (DIGE; Cecconi et al., 2007; Unlu, Morgan, & Minden, 1997), sandwiched antibody microarrays (Schweitzer et al., 2002), antibody microarrays analysis of dual color-labeled proteomes (Haab, Dunham, & Brown, 2001; MacBeath, 2002), lysate microarrays (Nishizuka et al., 2003), fluorescence-based flow cytometry (Krutzik & Nolan, 2006), and more recently multiplexed mass cytometry (Bodenmiller et al., 2012). The activity and small-molecule binding to individual enzyme classes can be monitored in some cases with activity-based protein profiling (Cravatt, Wright, & Kozarich, 2008; Leung, Hardouin, Boger, & Cravatt, 2003). Cell-wide proteolytic events can be tracked using labeling and mass spectrometry-based techniques (Dix et al., 2008; Mahrus et al., 2008). All of these methods assess the effects of small molecule at the protein level, instead of the mRNA level, which is likely more relevant to the final phenotypic effects of small molecules.

### 3.1. Comparing small-molecule profiles

A limited number of studies have compared compounds based on signatures created from protein profiles. Using lysate microarrays, Sevecka and MacBeath compared 84 kinase and phosphatase inhibitors based on their ability to change the phosphorylation state of 12 proteins in the EGF receptor pathway (Sevecka & MacBeath, 2006). Osada and colleagues created profiles for 19 compounds based on changes in protein levels detected by DIGE after compound treatment of HeLa cells (Muroi et al., 2010).

Clustering of these profiles accurately grouped compounds based on their known mechanisms of action, similar to some of the results obtained using gene expression data. No direct comparison to gene expression data was made. Such a comparison would be highly valuable, and to our knowledge, no such study has been conducted.

## 3.2. Application in cell death

One protein-profiling technique, proteolytic profiling, deserves further attention due to its particular focus on cell death. Two studies introducing this technique were published simultaneously in 2008. James Wells' group used an engineered enzyme to biotinylate and subsequently enrich and identify proteins with free N-termini (Mahrus et al., 2008). They applied this technique to analyze 333 cleavage sites in 292 proteins identified in apoptosis induced by etoposide in Jurkat cells. They found that many of these cleavage sites were poorly predicted by *in vitro* caspase cleavage site selectivity measurements and that cleavage sites were enriched within interacting proteins. The Wells lab subsequently applied this technique to more comprehensively identify cleavage events in apoptotic cells in culture (Crawford et al., 2013) and to characterize the substrates of inflammatory caspases (Agard, Maltby, & Wells, 2010). They also compared the profiles generated in three different cell lines treated with three different small molecules to attempt to identify unique fingerprints for compound mechanisms of action (Shimbo et al., 2012).

In the second study, Cravatt and colleagues combined SDS-PAGE with LC–MS–MS in a technique they named Protein Topography and Migration Analysis Platform (PROTOMAP) (Dix et al., 2008). They used this technique to analyze proteolytic events induced by staurosporine treatment of Jurkat cells, identifying 91 characterized and 170 previously uncharacterized cleavage events. Analysis of these fragments demonstrated that many were persistent and preserved intact protein domains, raising the possibility of cleavage of proteins results in the generation of active fragments. More recently, the Cravatt group updated their PROTOMAP technology to also identify phosphorylation events (Dix et al., 2012). By identifying more than 700 cleaved proteins and 5000 sites of phosphorylation during apoptosis in Jurkat cells, they were able to demonstrate that these two modifications are intricately linked with phosphorylation-driving cleavage and vice versa.

### 3.3. Advantages and limitations in the study of cell death

Advantages of protein-profiling methods include high dimensionality, the direct measurement of the effector proteins rather precursor changes, the ability to distinguish and quantify posttranslational modifications of proteins, and the ability of some techniques to make single-cell measurements. While the dimensionality of protein profiles is typically lower than that of gene expression profiles, techniques are available for global monitoring of large numbers of proteins. These profiles have the advantage that they are direct measurements of the level of what is typically the functional entity (the protein) rather than the precursor mRNA, which is not always well correlated to the protein level (Nishizuka et al., 2003). They also can detect modifications of proteins, which increase the dimensionality of the data and allow for dissection of these critical events. This is particularly notable in cell death where changes in signaling cascades and cleavage events are essential (Kurokawa & Kornbluth, 2009). While flow and mass cytometry are limited to fewer simultaneous measurements (mass cytometry can detect up to 34) (Bendall et al., 2011), they have the ability to make single-cell measurements. Such measurements can be highly valuable in cell death, where there can be significant cell-to-cell variability in time-to-response and response to lethal stimuli among cells in a clonal population (Spencer, Gaudet, Albeck, Burke, & Sorger, 2009).

Protein profiling shares a number of the limitations of gene expression profiling, including the difficulty in identifying changes over time and concentration ranges and the difficulty separating primary from off-target effects of small molecules. Additional disadvantages include relatively high cost, low throughput, difficulty in detecting low-abundance proteins, and reliance on antibodies. While improvements in technology, particularly mass spectrometry and labeling techniques, have improved proteome coverage, costs are still high relative to gene expression profiling and detection of low-abundance remains problematic (Carragher, Brunton, & Frame, 2012). A number of techniques for the simultaneous measurement of proteins, including lysate arrays and flow and mass cytometry, require the use of antibodies and are therefore clearly limited by the availability and quality of the antibodies. Many of the protein-profiling technologies have had limited applications to date in cell death. An exception is the proteolytic profiling techniques employed by the Wells and Cravatt groups. These techniques are promising and it will be interesting to see the result of their application

to larger numbers of small molecules, particularly of inducers of non-apoptotic cell death pathways.

## 4. GENE–SMALL-MOLECULE INTERACTIONS

Improvements in the generation and monitoring of gene knockouts in model organisms and the availability of RNAi technology in mammalian cells have led to their application in the investigation of small-molecule mechanisms of action (Brummelkamp et al., 2006; Hoon et al., 2008; Lum et al., 2004; Luo et al., 2008). These techniques have also been applied to create and compare profiles for small molecules.

### 4.1. Chemical–genetic profiling in yeast

Charlie Boone's lab, Guri Giaever's lab, and Cori Nislow's lab have developed and utilized a system called "chemical–genetic profiling" that measures the hypersensitivity to small molecules conferred by the full collection of viable haploid deletion mutants in yeast. The Boone lab initially compared 12 small-molecule profiles (Parsons et al., 2004) and then extended their analysis to 82 compounds (Parsons et al., 2006). Using hierarchical clustering and sparse matrix factorization, they showed that compounds with similar mechanisms of action had similar profiles and were able to identify that the estrogen modulator tamoxifen can cause increases in intracellular calcium concentrations. In their initial as well as a subsequent study (Costanzo et al., 2010), they showed that by integrating chemical–genetic profiles with genetic interaction profiles, they could identify the mechanism of action of previously uncharacterized compounds based on the similarity of the profiles of a compound and the profile from the deletion of its target.

### 4.2. Applications of yeast profiling in cell death

Subsequent studies have used this technology in the investigation of cytotoxic compounds. The first examined the mechanism of action of two previously uncharacterized and structurally similar antifungal compounds (Yu et al., 2008). By comparing their chemical–genetic profiles to those from the studies published above, they found that one compound clustered with mitochondrial inhibitors while the other clustered with DNA-damaging agents. They subsequently validated these mechanistic predictions. Spitzer and colleagues compared chemical–genetic profiles for compounds found to potentiate the activity of the antifungal fluconazole (Spitzer et al., 2011). Comparing

these profiles led them to identify two principle mechanisms of synergy and allowed them to identify additional synergistic compounds. The Nislow and Giaever group took an analogous approach to investigating the mechanisms of action of DNA-damaging agents (Lee et al., 2005). They created chemical–genetic profiles for 12 compounds against ~4700 homozygous deletion strains. As a part of a broader analysis, they clustered the compounds and found that compounds with similar mechanisms of DNA damage clustered together.

## 4.3. Chemical–genetic profiling in mammalian cells

Citing the difficulty in applying the yeast system to study cancer chemotherapeutics due to the lack of conservation of certain drug targets, Hemann and colleagues developed a profiling system using RNAi in mammalian cells (Jiang, Pritchard, Williams, Lauffenburger, & Hemann, 2011). They measured the ability of 29 shRNAs (targeting either the Bcl2 family or p53 and its activating kinases) to alter the lethality in a murine lymphoma cell line of 15 chemotherapeutic compounds, each used at a single dose. Clustering of these profiles accurately grouped compounds according to their known mechanism of action. They went on to show that a subset of eight shRNAs was sufficient to accurately classify the compounds and that profiles generated with those eight shRNAs for additional compounds were able to successfully classify both compounds with mechanisms already represented within their database as well as compounds with unique mechanisms.

## 4.4. Advantages and limitations in the study of cell death

Chemical–genetic profiling systems can generate large amounts of high-quality, functional information about the mechanism of action of a compound. Each profile is information-rich and can be individually mined for mechanistic data as well as used as a fingerprint of compound action that can be compared to other profiles. The use of a functional assay is a key advantage over gene expression and protein-profiling methods. Although efflux pumps and multidrug resistance genes can be complicating (Parsons et al., 2004), the use of a functional assay removes the difficulty in distinguishing relevant from off-target effects. Functional assays are able to give information about what took place over the time course of the experiment, rather than taking a snapshot of one point in the process. The yeast system is well established and robust and the pooled barcoding systems allow for genome-wide coverage with good throughput. There are large available data sets including extensive gene–gene interaction data to which

chemical–genetic profiles can be compared (Costanzo et al., 2010; Hillenmeyer et al., 2008). The RNAi system is less widely used but is promising for its applicability in mammalian cells. Hemann and colleagues used only 29 genes, but pooled RNAi approaches may make it feasible to create large numbers of genome-wide profiles (Luo et al., 2008). Their approach is also appealing for the ability to readily generate mini-profiles focused on a process of interest. For example, Hemann and colleagues used a set of established apoptosis-related genes, but other choices could be made to create a systems concentrated on a different cell death process.

The limitation of the yeast system is primarily that the technology can only be applied in yeast. This is a particular drawback in studying non-apoptotic cell death, which is poorly conserved even between mammals and other metazoans (Tait & Green, 2008). While the shRNA system is applicable in mammalian cells, it may be difficult to apply to specialized cell types that are not easily transfected. There are also off-target concerns with RNAi, exemplified in the Hemann study by their inability to fully reproduce the clusters when each clone in their eight-gene set was replaced with a different clone targeting the same gene. Compounds in both systems are generally used at a single dose, masking concentration-dependent changes and raising the likelihood of using too high a concentration and increasing the likelihood of pleiotropic effects.

## 5. SMALL-MOLECULE COMBINATION INTERACTIONS

Combinations of drugs are the foundation of treatment for a number of diseases including HIV, tuberculosis, and multiple types of cancer. There is a long history of the analysis and interpretation of the interactions between small molecules (Keith, Borisy, & Stockwell, 2005). More recently, these effects have been quantified and compared to help understand compound mechanisms of action.

### 5.1. Profiles based on small-molecule interactions

Small-molecule interactions have been used as fingerprints both in model organisms and in mammalian cells. Kishony and colleagues scored pairwise interactions between 21 antibiotics as antagonistic, additive, or synergistic, based on their combined effect on the growth of *E. coli* (Yeh et al., 2006). These data allowed them to accurately group the compounds according to their known mechanisms of action and suggest a novel mechanism for one

poorly characterized compound. Farha and Brown took a similar approach, screening ~200 compounds capable of inhibiting *Escherichia coli* growth for synergy with 14 well-characterized antibiotics (Farha & Brown, 2010). They compared these profiles and were able to make mechanistic inferences about novel compounds. Lehar and colleagues created dose matrices for combinations of 10 sterol inhibitors in yeast (Lehar et al., 2007). Analysis of the shape of these interaction maps allowed distinction between compounds acting in the same pathway and compounds acting in different pathways. They performed a similar analysis on data from a screen of all combinations of 90 characterized compounds in a human colon cancer cell line and found that compounds with similar mechanism of action were more likely to have similar interactions with the rest of the set.

## 5.2. Advantages and limitations in the study of cell death

Analysis of small-molecule combinations is valuable for its versatility and widespread applicability. Unlike genetic changes or RNAi, it can be applied across species and in specialized cell types. It has the potential to generate high-dimensionality data, it typically uses a functional assay as the output, and the profiles can be information-rich and mined for further insights. While some studies used single doses, larger dose matrices like those used by Lehar et al. can be used to capture dose-dependent effects. Additionally, as demonstrated by Lehar and colleagues in their yeast experiments with sterol inhibitors, focused, information-dense mini-profiles can be readily generated for specific processes of interest.

Using combinations of small molecules has several drawbacks, primarily centered on throughput and coverage. Larger systems in which all pairwise interactions are tested are limited by the exponential increase in the number of required experiments. The same problem prevents the use of multiple doses and higher-order combinations of small molecules. While with genetic techniques it is possible to barcode and pool experiments, this is not theoretically possible with small-molecule combinations. Additionally, genetic approaches can achieve genome-wide coverage while chemical interaction approaches are limited by the availability of relevant small molecules. It can require the testing of large numbers of combinations to identify statistically significant interactions. For example, Farha and Brown detected an interaction with only 45 of the 186 compounds tested against their 14-compound reference set.

## 6. CELL LINE VIABILITY PROFILING

George Gey and colleagues first successfully cultured human tumor cells (HeLa cells) in 1952 (Gey, Coffman, & Kubicek, 1952). Since that time, cultured tumor cells have been a central pillar of biological investigation in general and cell death in particular. While attempting to identify potential chemotherapeutic agents, researchers at the NCI noted that differential cell line toxicity was a useful marker of compound mechanism of action (Shoemaker, 2006). Cell line viability profiling has continued today as one of the most frequently utilized profiling methodologies.

### 6.1. NCI60 screen

In the late 1980s, the National Cancer Institute developed a screen of 60 human tumor cell lines from 9 different tumor types (NCI60) with the intention of identifying disease-specific lethal compounds. The NCI60 screen was instituted as a primary drug screening platform in the 1990s and then transitioned to a research tool in 2000. By 2005, over 350,000 compounds had been screened and compounds continue to be screened at a reduced rate of approximately 3000 per year (Shoemaker, 2006). Early on in the screening program, it was noted that the patterns of relative potency across the cell lines could be used as a marker of compound mechanism of action. They subsequently developed their "COMPARE" algorithm to further analyze these patterns and allow for querying of their large database of compound sensitivities (Paull et al., 1989). In this initial work, they demonstrated that the algorithm was able to accurately link alkylating agents, topoisomerase inhibitors, and antimetabolites to other compounds from the same mechanistic class. See Fig. 11.4 for an example of the display format and use of the COMPARE algorithm from a recent study (Yang et al., 2012).

#### *6.1.1 Notable applications of the NCI60 screen*

One of the first successful applications of the COMPARE algorithm was in the investigation of halichondrin B, a cytotoxic natural product without a known mechanism of action. The NCI team found that halichondrin B had a sensitivity profile similar to known microtubule destabilizers and subsequently demonstrated that halichondrin B was also a microtubule destabilizer (Bai et al., 1991). Later the database was used to investigate the mechanism of action of recombinant anthrax lethal factor. The

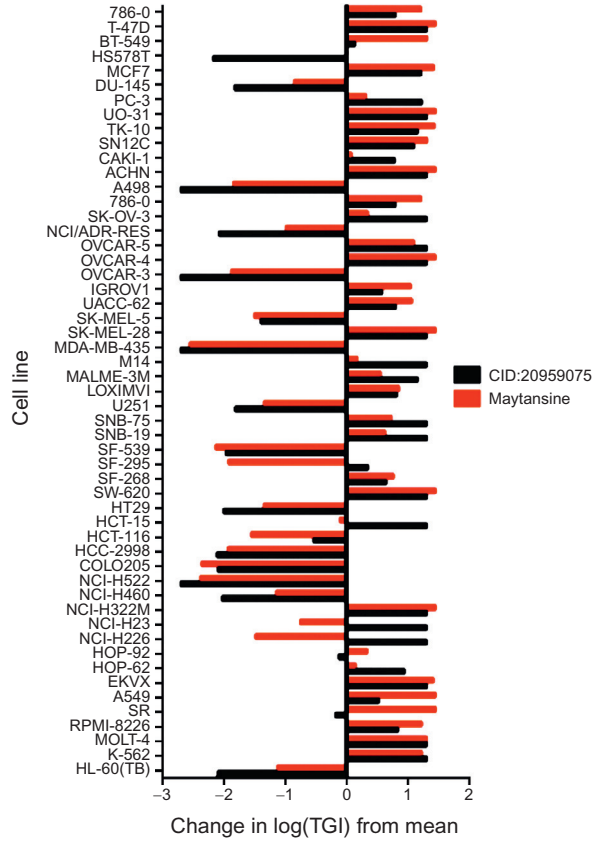


**Figure 11.4** NCI60 data display and COMPARE analysis. Yang and colleagues (Yang et al., 2012) identified the highly potent cytotoxic compound 20959075. Its profile across the NCI60 cell lines is shown in the black bars. The bars represent the distance of the total growth inhibition (TGI) from the mean of all cells lines tested. COMPARE analysis identified maytansine, a known microtubule destabilizer, as the most highly correlated compound in the database with a correlation of 0.808. Its profile is shown in the red bars. The authors went on to show that 20959075 is in fact a microtubule destabilizer.

sensitivity profile was similar to the profile of PD98059, an MEK inhibitor. Anthrax lethal factor was then shown to act as a protease that cleaves MEK1 and MEK2 (Duesbery et al., 1998).

The NCI60 screen was also valuable in identifying compounds with unique profiles in the database, therefore suggesting that such compounds acted through a novel mechanism of action. This was notable the case for the proteasome inhibitor bortezomib (Holbeck & Sausville, 2004).

Bortezomib and several analogues were tested in the NCI60 screen. Initial COMPARE analysis demonstrated that the compounds were similar to each other but had profiles distinct from the database compounds, supporting the novel mechanism of action of bortezomib and its analogues. In addition, the potency of the analogues in the cells lines correlated well to the *in vitro* potency of the compounds against the proteasome, supporting proteasome inhibition as the lethal mechanism of action of the compounds.

## 6.2. Use of molecularly characterized cell lines

The use of multiple cell lines can be conceptualized as a chemical–genetic profile, akin to testing an unknown combination of an unknown number of genetic (and epigenetic) mutations. Researchers at the NCI noted the value in defining those mutations in order to then be able to link molecular characteristics of the cell lines to compound activity. The advent of genome-wide profiling techniques has enhanced this ability as it has become possible to molecularly characterize larger numbers of cell lines. Programs have since been initiated to greatly expand on the approach taken by the NCI60 and profile compounds in hundreds or thousands of well-characterized cell lines.

### *6.2.1 Molecular characterization of NCI60 cell lines*

In the mid-1990s, the NCI60 group began annotating their cell lines with "molecular targets"—genetic mutations, mRNA levels, protein levels, and enzymatic activity (Weinstein, 2006). In a pioneering study, Jonathan Weinstein and colleagues developed visualization and computational tools to analyze the relationship between cell sensitivity profiles and molecular characteristics (Weinstein et al., 1997). In this study, they showed that they could identify compounds that were likely multidrug resistance transporter substrates. They also identified compounds whose activity was either dependent on or independent of wild-type p53. Further studies have integrated genome-wide expression data (Ross et al., 2000; Scherf et al., 2000), proteomic profiles (Nishizuka et al., 2003; Park et al., 2010; Shankavaram et al., 2007), and mutation data (Ikediobi et al., 2006). The NCI has made all of their data and analysis tools available on their web site (http://dtp.nci.nih.gov/mtargets/mt_index.html).

### *6.2.2 Expanded cell line databases*

Three recent projects have tested small molecules in large numbers of molecularly characterized cell lines. Garnett and colleagues tested 130 compounds in an average of 368 cell lines that had been characterized by

sequencing of 64 commonly mutated cancer genes, evaluation of 7 commonly rearranged cancer genes, genome-wide copy number analysis, and genome-wide expression analysis (Garnett et al., 2012). The Cancer Cell Line Encyclopedia (CCLE) project tested 24 anticancer drugs against 479 cell lines that were characterized genetically by sequencing of >1600 genes and testing of 392 recurrent mutations in 33 known cancer genes, DNA copy number evaluation, and genome-wide expression analysis (Barretina et al., 2012). Using a subset of 242 of the cell lines in the CCLE, Basu and colleagues profiled 354 highly selective small molecules (Basu et al., 2013). The data from all three projects are publicly available. Basu and colleagues designed a Web portal for querying their database (http://www.broadinstitute.org/ctrp).

All three of these studies were primarily focused on identifying genomic markers that could be used to predict responsiveness to a chemotherapeutic drug. While characterizing compound mechanism was not the central goal, they do provide rich data sets to investigate compound activity. Garnett and colleagues and the CCLE project did cluster their compounds based on the cell line responses. The CCLE had a small number of compounds that shared a common target, and these groups did cluster together. Garnett and colleagues had a larger number of such compounds, many of which clustered together (MEK1/2 inhibitors, EGFR, IGFR1) although others did not (SRC inhibitors, microtubule stabilizers). Basu et al. clustered the compounds based on the most significant genomic features correlated with compound activity and highlighted several compounds with common targets that clustered together.

## 6.3. Advantages and limitations in the study of cell death

Cell line profiling of small molecules is the first established profiling technique and has a number of attractive features. The primary advantage is the collection of a large amount of publicly accessible, information-rich data. The lifespan of the NCI60 project has allowed for the profiling of an unparalleled number of compounds. The three more recent projects described above have profiled fewer compounds but in an impressive number of cell lines with extensive molecular characterization. Similar to chemical–genetic and chemical interaction profiling, the assays are functional and therefore more likely to detect relevant effects, even when used at a single time point. A recent study suggests that the concentration–response data produced by such studies is even more information-rich than previously appreciated

and that additional features can be mined for mechanistic information (Fallahi-Sichani, Honarnejad, Heiser, Gray, & Sorger, 2013). The data dimensionality depends on the number of cell lines tested, but even given similar dimensionality, the data are likely to be more information-dense than a chemical–genetic profile. Single genetic changes often produce no effect on a compound, but the complex combination of alterations represented by each cancer cell line is much more likely to alter compound response, resulting in fewer null values. Lastly, genomic characterization of cell lines allows for the mining of the profiles for mechanistic information.

Limitations of cell line profiling include dependence on available cancer cell lines and the requirement for the use of large numbers of cell lines to achieve representation of uncommon targets and pathways. One of the driving motivations for the assembly of the larger cell line profiling projects was the realization that 60 cell lines was insufficient to allow for the statistical detection of some selective agents, particularly those dependent on mutations found in only of fraction of cancers of a particular lineage (Sharma, Haber, & Settleman, 2010). Unlike in chemical–genetic profiling in which genome-wide coverage can be achieved, cancer cell line profiling is likely to only appreciably access changes relevant to cancer. The dependence on cancer cell lines is particularly a limitation in the study of cell death, since inactivation of cell death is one of the hallmarks of cancer (Hanahan & Weinberg, 2011). It also limits the ability to study highly specialized processes only accessible in specific cell types.

## 7. QUANTITATIVE IMAGING

As described earlier, morphology has long formed the foundation of cell death characterization. While qualitative descriptions of morphology are problematic, advances in microscopy and computational analysis have allowed for the extraction of quantitative parameters from images that can be used to create profiles to compare compounds.

### 7.1. High-content imaging in cell culture

The first large-scale imaging-based compound profiling system was developed by the Altschuler and Wu lab. They generated profiles for 100 compounds by testing a range of concentrations in HeLa cells and after 20 h staining with 11 fluorescent probes and imaging to capture up to 8000 cells per well (Perlman et al., 2004). They derived 93 descriptors from the images and used them to compare the 61 compounds for which they

detected a significant phenotype. They showed that replicates of compounds and compounds with similar known mechanisms of action clustered together (with some notable exceptions including protein synthesis inhibitors). They were also able to predict the mechanism of one poorly characterized compound and showed that their profiles were more informative than the analysis of only the intensity of the staining. A second study used 3 cellular stains and measured 36 cytological features from which they derived 6 factors by combining highly correlated features (Young et al., 2008). Using this system, they screened a library of over 6000 compounds at a single dose and time point and did further analysis of the 211 compounds that caused the largest perturbations of the 6 factors. Clustering of these compounds accurately classified compounds with known biological activity.

## 7.2. Advantages and limitations of image-based profiles in studying cell death

Imaging systems continue to decrease in cost and increase in throughput and quality. They are generally accessible across species and cell types that can be cultured. Such systems are valuable for studying cell death because links can be made between quantitative metrics and traditional cell death phenotypes. For example, one of the factors defined by Young and colleagues combined chromatin condensation and decrease in nuclear size and was thought to correspond to apoptosis. Another appealing feature of imaging systems is the possibility of obtaining single-cell data, which could make it feasible to identify and characterize a death process that occurs in only a subset of a cell population.

Image-based profiles are limited in that they collect observational and not functional data, which raises the possibility that the changes observed are not driven by the same process that leads to cell death. While factor analysis like that performed by Young and colleagues attempts to connect biological meaning to the extracted parameters, image-based data do not readily correlate to underlying biochemistry. In fact, in cell death, morphological changes do not necessarily represent unique biochemistry. While the additions of cellular stains increase the dimensionality and sensitivity of the data, many compounds may not cause a significant morphological change, particularly at a chosen time point. This was demonstrated in all of the studies described above where only a minority of compounds displayed an appreciable phenotype.

## 8. MODULATORY PROFILING

To address some of the deficiencies in the above-described methods and assist in the characterization of small-molecule-induced cell death, we recently developed a system called "modulatory profiling" that creates information-dense, functional profiles for small molecules based on quantification of the degree to which various agents are able to perturb the death induced by lethal compounds (Wolpaw et al., 2011).

### 8.1. Design and validation

To understand a process, one needs a way to perturb it. From this principle, it follows that a rigorous description of a process could be generated from cataloging various agents capable of inhibiting or enhancing it. To apply this concept to cell death, we assembled a collection of 32 chemical and genetic "modulators" of known cell death pathways and quantified their ability to change the extent of death induced by lethal compounds in two cell lines. We did this by testing a single, literature-derived concentration of the modulator against a 12-point dilution series of the lethal compounds and extracted two parameters, the change in potency and the change in efficacy (see Fig. 11.5). As a proof-of-principle, we tested 28 well-characterized lethal compounds and showed that clustering based on their "modulatory profiles" correctly grouped together compound replicates and compounds with the same mechanism of action. Clustering based on modulatory profiles was shown to more accurately group compounds according to their established mechanisms compared to clustering using a similar algorithm applied to gene expression profiles or chemical structure.

To further demonstrate the value of this approach, we generated profiles for 25 poorly characterized or uncharacterized lethal compounds and clustered them with the characterized compounds (see Fig. 11.6). Three compounds had profiles that clustered with known microtubule destabilizers, and we subsequently showed that these compounds do in fact destabilize microtubules in cultured cells. Other compounds grouped together and based on those groupings were determined to act through nonspecific methods, either compound reactivity or detergent-like membrane disruption. Lastly, poorly characterized and uncharacterized compounds in a cluster without any well-characterized compounds were shown to act through a nonapoptotic process that involved the mitochondria.

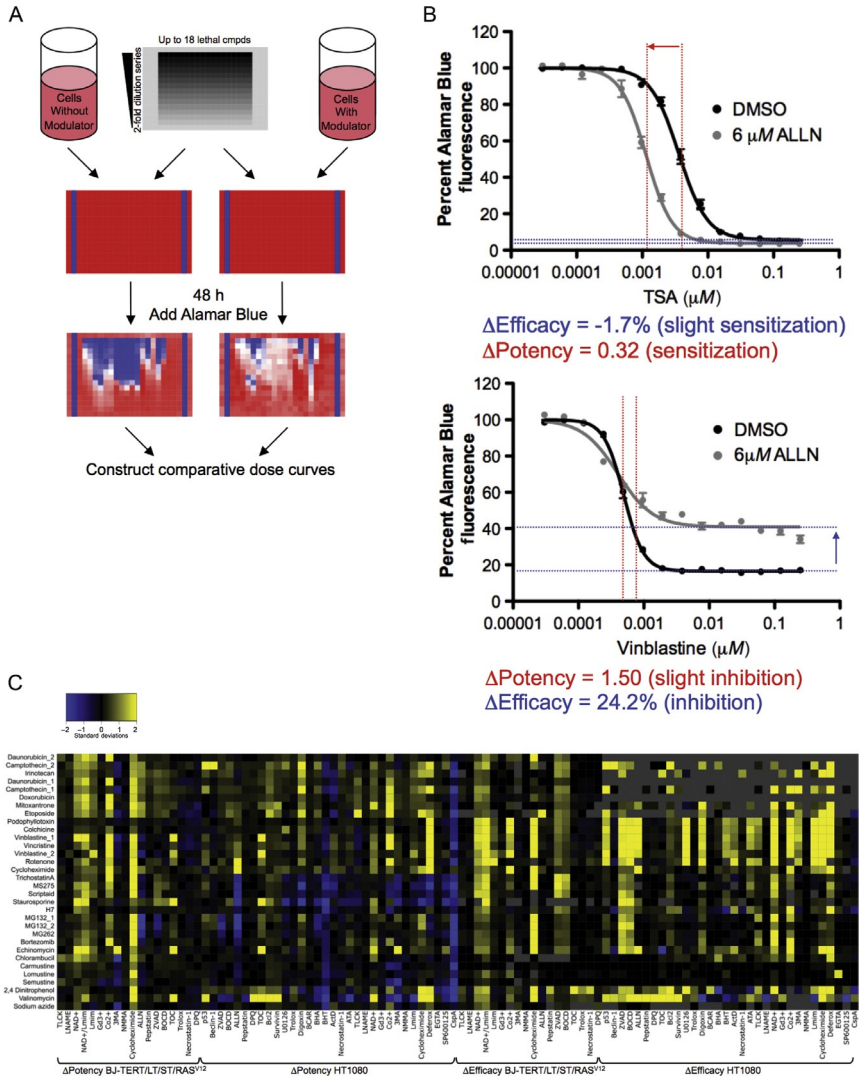


**Figure 11.5** Creating modulatory profiles. (A) Cells with or without modulator were seeded into 384-well plates and lethal compounds were added in a dilution series. Viability was measured after 48 h with Alamar Blue and comparative concentration–response curves were constructed from the data. (B) Two examples of comparative dose curves. These illustrate the two parameters extracted from each pair of curves, the change in potency and the change in efficacy. (C) Heat map illustrating the modulatory profiles for a number of characterized lethal compounds. Each row represents the modulatory profiles for a different compound. *Reproduced with permission from Wolpaw et al. (2011).*

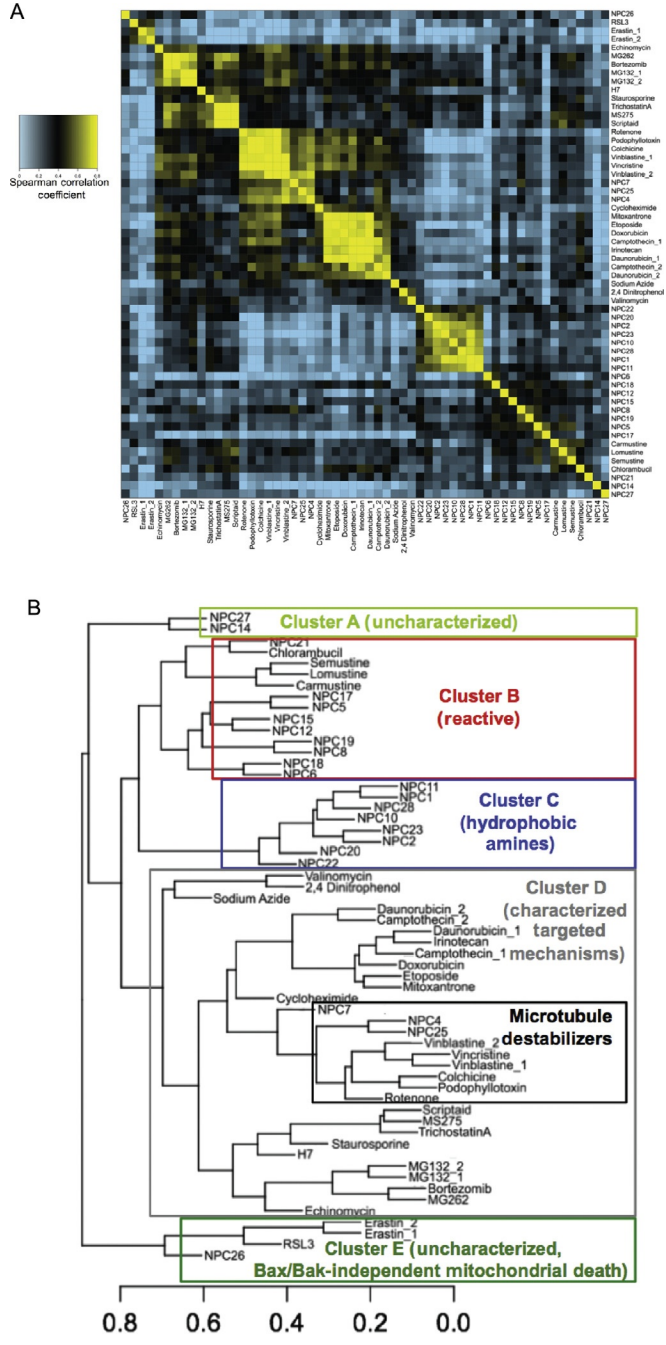


**Figure 11.6** Comparing and clustering modulatory profiles. (A) Heat map of the similarity matrix showing the Spearman correlation between modulatory profiles of both characterized and uncharacterized lethal compounds. (B) Dendrogram derived from

## 8.2. Modulatory profiling protocol

A detailed protocol for performing modulatory profiling was published previously (Wolpaw et al., 2011). Below we will describe a summary of our protocol and identify opportunities for repurposing the system to study specific cell death processes.

**I.** *Selection of modulators, reference lethal compounds, and cell lines*

We initially selected literature-reported modulators of established cell death pathways including reactive oxygen species scavengers, calcium channel blockers, protein synthesis inhibitors, and protease inhibitors, among others. A different set of modulators could in principle be chosen to focus more clearly on a specific process of interest. We used four genetic modifiers (two shRNAs and two cDNAs), which left significant opportunity for expansion. We used a diverse group of well-characterized lethal compounds, but alternative reference lethal compounds could be chosen. For quality control purposes, it is advantageous to select multiple compounds from the same mechanistic class. We chose two cell lines, the human fibrosarcoma HT-1080 line and an engineered tumorigenic line, derived from human foreskin fibroblasts (BJ-TERT/LT/ST/RAS$^{V12}$). Both cell lines grow rapidly in and uniformly in culture, HT-1080 cells are easily manipulated with genetic tools, and BJ-TERT/LT/ST/RAS$^{V12}$ have defined genetic changes and therefore do not have mutations in poorly characterized death pathways that may be found in a cancer cell line. Different cell lines could be chosen in order to focus on a cell death process specific to a certain cell type.

**II.** *Cell plating and addition of reagents*

Cells were cultured as previously described (Yang & Stockwell, 2008). Cells were trypsinized, counted, and seeded in 384-well plates with or without a specific modulator. Lethal compounds were added from a separate 384-well plate containing 12-point, twofold dilutions of the compounds and DMSO-only control wells (see Fig. 11.5A).

clustering the similarity matrix shown in (A). Five broad clusters are highlighted and lettered. In addition, microtubule destabilizers are shown in black, a cluster that includes three previously uncharacterized compounds. Other features that are not highlighted include clustering of characterized compounds according to their known mechanisms of action—alkylating agents, mitochondrial poisons, topoisomerase inhibitors, histone deacetylase inhibitors, and proteasome inhibitors. *Reproduced with permission from Wolpaw et al. (2011) and slightly altered.*

After 48 h, the cell viability dye Alamar Blue (Nociari, Shalev, Benias, & Russo, 1998) was added and plates were read 16 h later on a Victor 3 plate reader (Perkin Elmer). All liquid transfers were performed using a Biomek FX AP384 module (Beckman Coulter). All assays were performed at least in triplicate.

**III.** *Calculation of changes in potency and efficacy*

Background fluorescence (no cells with modulator or no cells with lethal compound) was subtracted and values were normalized to vehicle or modulator-only controls. GraphPad Prism was used to calculate logistic best-fit curves based on four parameters—$EC_{50,}$ Top, Bottom, and Hill slope. Curves with and without modulator were compared. Changes in the Top (change in survival in very low concentrations of compound) were appropriately not observed and therefore the Top was set as equal to one (100% survival). We found that changes in the Hill slope were particularly error prone and uninformative (Kenichi Shimada and Brent Stockwell, unpublished). We therefore used a modification of the changes in the $EC_{50}$ (potency) and the Bottom (efficacy) of these curves as the parameters with which to create the profiles, giving each parameter equal weight (see Fig. 11.5B). While most studies have used potency alone or a combination of potency and efficacy (area under the curve), a recent study validated our approach, demonstrating the utility of efficacy as a separate marker of compound activity (Fallahi-Sichani et al., 2013). These data-processing steps were performed primarily using MatLab.

**IV.** *Comparing, visualizing, and clustering modulatory profiles*

Potency and efficacy changes were individually normalized so that each had a standard deviation of one (for all compounds, not for each compound). This removed the units and gave each parameter equal weight. Spearman correlations were then calculated for each pair of compounds to produce a similarity matrix like the one shown in Fig. 11.6A. This was done within the R programming environment. The R function hclust was then used to cluster the similarity matrix, using the group average method for defining new clusters (Kaufman & Rousseeuw, 1990). This produced a dendrogram such as the one shown in Fig. 11.6B.

**V.** *Further validation and exploration of findings*

Similar to other profiling methods, modulatory profiling suggests connections and mechanisms that then must be confirmed through independent experimental validation.

## 8.3. Application of modulatory profiling to the investigation of ferroptosis

Our lab recently described a nonapoptotic cell death process, "ferroptosis," induced by the small-molecule erastin and dependent on intracellular iron (Dixon et al., 2012). This study used two focused variations of modulatory profiling to demonstrate the uniqueness of ferroptosis. Six chemical modulators of ferroptosis were tested for their ability to alter the death induced by 16 diverse lethal compounds, including two small-molecule inducers of ferroptosis. This analysis showed that ferroptosis modulators were largely inactive against other lethal compounds, and clustering showed that the two ferroptosis inducers were similar to each other and distinct from other lethal compounds. A similar analysis was performed using seven shRNAs, targeting genes that were found in a screen to be required for ferroptosis. These hairpins were tested against seven lethal compounds (including one ferroptosis inducer, erastin) and were only active against erastin. Clustering also placed erastin in a unique cluster, demonstrating the uniqueness of the genetic network required for ferroptosis.

A further study from our lab investigated the role of glutathione peroxidase 4 (Gpx4) in ferroptosis (Yang et al., 2014). This study created a modulatory profile for Gpx4 siRNA and showed that it was similar to the profiles of the ferroptosis-inducing compound RSL3, providing evidence that Gpx4 is the relevant cellular target of RSL3. This demonstrates the ability of modulatory profiling to link small molecules to their targets based on the similarity of their modulatory profiles, similar to what has been done in yeast by combining genetic interaction and chemical–genetic interaction profiles.

## 8.4. Advantages and limitations in the study of cell death

Modulatory profiling was specifically designed for the study of small-molecule-induced cell death and has some distinct advantages. Perhaps its most important feature is its flexibility, as illustrated in its application to ferroptosis. Modulatory profiles can be readily applied to a specific cell death process of interest, including in specialized cell types. In addition, it uses a functional assay (changes in cell death) and therefore is more likely to identify the relevant mechanism of action of a compound. It uses a broad concentration range, allowing for the detection of dose-dependent effects. It utilizes human cell lines, allowing for the study of processes not found in lower organisms. Modulatory profiling uses a combination of genetic and chemical modulators. Combining both modalities improves resolution,

allows investigation in cell types that are difficult to access with genetic methods, and allows access to processes not well targeted currently by small molecules. The chemicals used in modulatory profiling are selected and optimized prior to the profiling experiment. This eliminates the need for a dose matrix like those used in small-molecule interaction profiling and reduces the potentially enormous number of experiments required to test all pairwise interactions among a large collection of small molecules.

Modulatory profiling is limited by the availability of appropriate modulators. If relevant modulators for a given process are unknown or unavailable, a broad-based system can demonstrate the uniqueness of that process, but it will not be able to offer any detailed mechanistic information or differentiate between inducers of that process. In addition, the use of chemical modulators requires individual wells, which makes it difficult to increase throughput. The ability to create profiles for genetic lethal agents, as described above for Gpx4, is promising but has only been demonstrated in specific circumstances. Its widespread applicability remains to be demonstrated.

## 9. CONCLUSIONS

Over the past 15 years, technological advances and miniaturization have decreased cost and allowed for the implementation and expansion of high-dimensionality profiling systems for the analysis of biological processes. As we have described, these systems take advantage of the ability to quantify transcript and protein levels, create and test large numbers of genetic mutants, perform large numbers of assays required for cancer cell line profiling or chemical combination experiments, and extract quantifiable features from high-content imaging data.

Cell death is an essential and diverse process with a highly complex and interconnected underlying cellular signaling network and significant species and cell-type specificity. High-dimensionality profiling systems are appealing in the study of cell death for their potential to create fingerprints for processes that can be used both for comparison to other pathways and definition of a specific pathway. While unfocused systems like genome-wide expression profiling are valuable for their universality, there is also an important role for smaller, process-focused profiling modalities. We have used modulatory profiling both broadly and specifically, to try to create a map of all of the death pathways available to the cell, but also to create focused systems to

analyze specific pathways or contexts. Such systems should continue to play an important role in future cell death research.

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