Evaluating biological activity of compounds by transcription factor activity profiling

Alexander Medvedev1, Matt Moeser1*, Liubov Medvedeva1, Elena Martsen1, Alexander Granick1, Lydia Raines1, Ming Zeng1, Sergei Makarov Jr.1, Keith A. Houck2, Sergei S. Makarov1†

Assessing the biological activity of compounds is an essential objective of biomedical research. We show that one can infer the bioactivity of compounds by assessing the activity of transcription factors (TFs) that regulate gene expression. Using a multiplex reporter system, the FACTORIAL, we characterized cell response to a compound by a quantitative signature, the TF activity profile (TFAP). We found that perturbations of biological pathways elicited distinct TFAP signatures in human cells. Unexpectedly, perturbagens of the same pathway all produced identical TFAPs, regardless of where or how they interfered. We found invariant TFAPs for mitochondrial, histone deacetylase, and ubiquitin/proteasome pathway inhibitors; cytoskeleton disruptors; and DNA-damaging agents. Using these invariant signatures permitted straightforward identification of compounds with specified bioactivities among uncharacterized chemicals. Furthermore, this approach allowed us to assess the multiple bioactivities of polypharmacological drugs. Thus, TF activity profiling affords straightforward assessment of the bioactivity of compounds through the identification of perturbed biological pathways.

RESULTS

Obtaining TFAP signatures by the FACTORIAL assay

Using the FACTORIAL assay, we obtained TFAP signatures for perturbagens of various cell systems and biological pathways in human cells. As described in our previous publication (4), the FACTORIAL reporter system comprises a set of TF-responsive reporter constructs called reporter transcription units (RTUs). Each RTU has a TF-specific promoter linked to a reporter sequence. When they are transfected into assay cells, the RTUs produce reporter RNAs proportionate to the activity of their promoters. Thus, we can assess the activity of TFs by profiling the reporter transcripts (Fig. 1A). Significantly, owing to a rapid turnover of reporter RNAs, this approach enables the detection of not only activated but also inhibited TFs.

To ensure equal detection efficacy for the TFs, we used a “homogeneous” detection approach. In this approach, all RTUs have identical...
reporter sequences tagged by a unique endonuclease site (Hpa I) at a distinct position. The detection process entails reverse transcription polymerase chain reaction (RT-PCR) amplification of the reporter transcripts, followed by fluorescent labeling and Hpa I restriction. The labeled DNA fragments of predefined length are resolved by capillary electrophoresis (CE), producing a fluorescence profile that provides information about the activity of RTUs (Fig. 1A). The homogeneous detection approach enables robust and reproducible TF assessments (4).

The FACTORIAL assay used here was composed of 47 RTUs for TFs that mediate responses to a variety of stress stimuli and xenobiotics.
including nuclear factor κB (NF-κB), p53, AP-1, hypoxia-inducible factor–1a (HIF-1α), heat shock factor–1 (HSF-1), and many others (Fig. 1B). The RTU promoters contained one or multiple copies of TF binding sites for specified TF families. The reporter system also contained RTUs with minimal TATA and TAL promoters and cytomegalovirus (CMV) promoter.

Because chemicals have different cellular pharmacokinetics, we obtained the TFAP signatures after a prolonged (24-hour) exposure. We present these TFAP signatures in a radial graph format with 47 axes showing the stimulus-induced TF activity changes. By the definition, the baseline TFAP in unstimulated cells is a perfect circle with \( R = 1.0 \) (Fig. 1A). To assess the pairwise similarity of TFAPs, we used the Pearson correlation coefficient \((R)\). The probability that two random 47-endpoint signatures have a similarity of \( R > 0.7 \) is less than \( 10^{-7} \); thus, we presumed that two compounds elicited identical responses if their signatures’ similarity was greater than 0.7.

The invariant TFAP of mitochondria inhibitors

In one set of experiments, we assessed TFAPs for inhibitors of the mitochondrial electron transport chain (mETC) in human hepatocytic HepG2 cells. The mETC comprises functionally linked protein complexes within the internal mitochondrial membrane that transfer electrons from electron donors to oxygen. This process creates a proton gradient on the membrane that is converted into adenosine 5′-triphosphate (ATP) production. We evaluated a panel of specific inhibitors of different mETC complexes, including rotenone, pyridaben, and fenpyroximate (complex I inhibitors); antimycin A (a complex III inhibitor); and anionophore valinomycin. We assessed these chemicals at multiple concentrations. To compare multiple TFAPs, we developed a clustering algorithm that calculated an average (consensus) signature for the multiple TFAPs (see Materials and Methods). The clustering of TFAPs of mETC inhibitors revealed a single cluster. The consensus signature had high similarity \((R \geq 0.8)\) with the TFAPs of individual mETC inhibitors in a broad range of concentrations (Fig. 2A; for an alternative presentation of the TFAPs, see the heatmap in fig. S6). The consensus mETC TFAP comprised multiple TF responses, including activation of oxidative stress–responsive nuclear factor, erythroid 2-like 2 (NRF2)/antioxidant response element (ARE), which was consistent with published data (8).

HepG2 cells produce sufficient amounts of ATP via glycolysis to survive fully anaerobic conditions (9), and none of the mETC inhibitors caused cell death (fig. S1). Therefore, mETC perturbagens produced an invariant TFAP signature irrespective of their structural dissimilarity and their targets within the pathway.

The invariant TFAP of inhibitors of the ubiquitin/proteasome protein degradation pathway

The ubiquitin (Ub)/proteasome pathway (UPP) carries out the regulated degradation of cellular proteins (10). This process involves a consecutive action of a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a substrate-specific Ub ligase (E3) that attaches Ub residues to substrate proteins, targeting them for degradation by the 26S proteasome (PS) (10). Using the FACTORIAL assay in HepG2 cells, we obtained TFAPs for a panel of chemicals specifically inhibiting different nodes of the UPP. Those included PYR-41 (an E1 inhibitor with no activity at E2), NSC 697923 (a selective inhibitor of the E2 Ub-conjugating enzyme UBE2N), deubiquitinase inhibitors b-AP15 and WP1130, and five PS inhibitors, including lactacystin (an organic compound naturally synthesized by Streptomyces bacteria), peptide aldehydes MG132 and PSI, a boronic chalcone derivative (AM114), and a peptide boronate (bortezomib).

Clustering these signatures revealed a single cluster with a consensus TFAP signature that had high similarity \((R \geq 0.7)\) with the TFAPs of individual UPP inhibitors in a broad range of concentrations (Fig. 2B). The consensus TFAP comprised multiple TF responses, including activation of HSF-1 and NRF2/ARE RTUs and inhibition of the NF-κB RTU, which were in agreement with published data (11, 12). Representative signatures of the UPP perturbagens are also shown as a heatmap in fig. S6.

As UPP inhibitors can cause cell death, we monitored cell viability. At the end of 24 hours of incubation and the concentrations used, none of the UPP inhibitors caused cell death. Some UPP inhibitors induced cell death after a prolonged (48-hour) incubation (fig. S2A). Thus, the invariant TFAP of UPP inhibitors cannot be explained by cell death.

The FACTORIAL assay in other cell types [mammary epithelial MCF-7 and kidney epithelial human embryonic kidney (HEK) 293 cells] showed that different UPP inhibitors also produced common, yet cell type–specific, TFAP signatures (fig. S2B). The common TF responses that were observed in all cell types were the activation of HSF-1 and NRF2/ARE RTUs, while other TF responses varied among cell lines. Therefore, different UPP perturbagens produced invariant TFAP signatures in different cell types, irrespective of the perturbagens’ structure, their targets within the pathway, and their effects on cell viability.

The invariant TFAP of HDAC inhibitors

The enzyme families of histone acetyltransferases (HATs) and histone deacetylases (HDACs) catalyze the acetylation/deacetylation of histones and cellular proteins, which alters gene expression (13). Using the FACTORIAL assay in HepG2 cells, we evaluated a panel of structurally diverse HDAC inhibitors, including suberoylanilide hydroxamic acid, suberohydroxamic acid, CAY10398, M344, oxamflatin, pyroxamide, the cyclic peptide apicidin, and the mercaptoketone-based KD5170 (14). The clustering revealed a single TFAP cluster with a consensus TFAP that had high similarity \((R \geq 0.8)\) with the TFAPs of individual HDAC inhibitors at multiple concentrations (Fig. 3A). Representative TFAPs for HDAC inhibitors are also shown in the fig. S6 heatmap.

The FACTORIAL assay in HEK293 and MCF-7 cells also revealed common, albeit cell type–specific, signatures of HDAC inhibitors in these cells (fig. S3A). After a 24-hour incubation, none of the HDAC inhibitors caused cell death (fig. S3B). Thus, structurally dissimilar HDAC inhibitors produced invariant TFAPs.

The invariant TFAP of cytoskeleton disruptors

Microtubules are tubular polymers of tubulin that, along with microfilaments and intermediate filaments, constitute the cytoskeleton of eukaryotic cells. Microtubules are dynamic structures whose length is regulated by the polymerization and depolymerization of tubulin (15). Using the FACTORIAL assay in HepG2 cells, we evaluated a panel of structurally dissimilar microtubule polymerization disruptors (MTDs), including colchicine, nucodazole, vinblastine, vincristine, and vinorelbine. Cluster analysis revealed a single cluster with a consensus TFAP signature that had high similarity to the TFAPs of individual MTDs at multiple concentrations \((R \geq 0.8)\) (Fig. 3B). The most prominent TF responses of the consensus TFAP were an up-regulation of AP-1 and CMV RTUs and down-regulation of T cell factor (TCF)/β-catenin and estrogen response element RTUs. These responses were
in agreement with published data (16–18). Representative TFAPs of MTDs are also shown in fig. S6 as a heatmap. At the end of a 24-hour incubation and the concentrations used, none of the MTDs inhibited cell viability (fig. S4). Therefore, structurally dissimilar MTDs produced an invariant TFAP.

**The invariant TFAPs of DNA-damaging agents**

To obtain TFAP signatures of DNA damage, we irradiated HepG2 cells by ultraviolet C/ultraviolet B (UVC/UVB) light or treated them with chemicals with different modes of action. Those included topoisomerase I inhibitor camptothecin; topoisomerase II poison auramine O; DNA cross-linking chemicals oxaliplatin, cisplatin, and mitomycin C; and antimetabolites 5-fluorouracil and 5-fluoro deoxyuridine (Fig. 4A).

Cluster analysis revealed two distinct TFAP clusters with a low similarity of consensus signatures \( r = 0.52 \); Fig. 4B). The characteristic features of the consensus TFAP were an activation of AP-1, CMV, and p53 RTUs. The other cluster contained the TFAPs for higher UV doses and high concentrations of chemicals ("strong DNA damage"). The consensus TFAP showed multiple responses [including activation of AhR, TCF/β-catenin, and interferon-stimulated response element (ISRE)] and down-regulation of liver X receptor (LXR) (Fig. 4C). Representative TFAPs of the DNA-damaging agents are also shown by the heatmap in fig. S6. Some of these TF responses to DNA damage (for example, p53, AP-1, and AhR) are well known (19–21). Therefore, the low and high doses of DNA-damaging agents produced two different TFAPs. 5-Fluorouracil and floxuridine elicited only the weak-damage TFAP, while auramine elicited only the strong-damage TFAP at all tested concentrations (Fig. 4A).

The DNA-damaging agents had disparate effects on cell viability; at the end of incubation (24 hours), only some of the treatments (the
highest doses of UV and cisplatin) had significantly reduced cell numbers, indicating that the cytotoxicity cannot account for the common TF responses (fig. S5). The fact that DNA-damaging agents produced two distinct TFAPs is consistent with the known biphasic phenotypic response to DNA damage, in which low-level, repairable damage causes a transient arrest of DNA replication, whereas more extensive damage induces permanent arrest of replication (22).

In summary, the assessment of specific perturbagens of various cell systems and biological pathways showed that each class of perturbagens had a distinct TFAP. Moreover, perturbagens of the same pathway produced an invariant TFAP, regardless of where and how they interfered.

**The invariant TFAP signatures enable identification of compounds with specified bioactivity**

The finding of the invariant TFAPs suggested that these signatures could be used to identify chemicals with specific bioactivities among uncharacterized compounds. To test this assumption, we queried a data set of TFAP signatures for the environmental chemicals evaluated by Attagene under the U.S. Environmental Protection Agency (EPA) Toxicity Forecaster (ToxCast) project. These chemicals were screened at multiple concentrations using the FACTORIAL assay in HepG2 cells (5, 23). The ToxCast chemicals were also independently evaluated by other groups using different assays. A Tox21 group has screened this collection for mitochondria inhibitors using a mitochondria membrane potential (MMP) assay, also in HepG2 cells (24). The MMP assay data can be found in the EPA Aggregated Computational Toxicology Resource (ACToR) database (25). Using these data, we compared the assessments of mitochondria perturbagens using the invariant mETC TFAP and the functional assay.

The ACToR database has data on 2793 ToxCast chemicals that were evaluated by both the MMP and FACTORIAL assays. Of those, the MMP assay scored 518 compounds as mitochondria inhibitors (Fig. 5A). Querying signatures of these 2793 chemicals by the invariant mETC TFAP endpoints.

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**Fig. 3. The invariant TFAP signatures for HDAC inhibitors and cytoskeleton disruptors.** Assay cells (HepG2) were incubated with HDAC inhibitors or MTMs for 24 hours. Each TFAP represents the average data of three independent FACTORIAL assays. The consensus TFAPs were calculated by clustering those of individual perturbagens, as described in Materials and Methods. (A) Representative TFAP signatures of HDAC inhibitors. (B) Representative TFAP signatures of MTMs. Tables A and B show the similarity values r for the TFAPs of perturbagens at indicated concentrations versus the consensus TFAPs, calculated as a Pearson correlation coefficient r. Note that the values of TF changes are plotted in a log scale. The radial graphs show TFAPs of perturbagens overlaying the consensus TFAPs. (C) TFAP endpoints.
TFAP retrieved a large number of chemicals. For some chemicals, we retrieved multiple TFAPs at different concentrations. To avoid redundancy, we counted each chemical only once.

The concordance of the assays’ data was calculated as the percentage of MMP-positive chemicals among the retrieved chemicals. To avoid redundancy, we counted each chemical only once.

The concordance rate correlated with the TFAP similarity threshold. For example, ~69% of retrieved chemicals with a similarity of \( r \geq 0.900 \) were MMP-positive. The chemicals with similarity values within \( 0.800 \geq r \geq 0.700 \) had a concordance rate of ~42% (Fig. 5A, middle panel). Representative retrieved signatures are shown in Fig. 5B (top panel).

As we found a significant fraction of MMP negatives among the retrieved chemicals, we searched the literature for retrieved chemicals with high similarity values. With the similarity threshold at \( r \leq 0.800 \), we retrieved 199 chemicals (listed in table S1). Of those, 118 were
MMP-positive, and 81 were MMP-negative (concordance, 59%; Fig. 5A and table S1). The PubMed search showed that at least 25 of the 81 MMP-negative chemicals were known mitochondria disruptors, such as azoxystrobin (26) and kresoxim-methyl (27). Figure S7 shows the references and representative TFAP signatures for these MMP-negative chemicals. These data indicate that the actual accuracy of TFAP-based predictions was better than we estimated using the MMP assay as a reference. One plausible explanation for this discrepancy is the difference in the screening conditions: We assessed the TFAPs after a 24-hour incubation with concentrations up to 200 μM, whereas the MMP screening entailed a 1-hour incubation at concentrations below 60 μM.

To estimate the cumulative recall of the MMP-positive chemicals, we calculated how many of all 518 MMP-positive chemicals we retrieved by the TFAP query. The recall inversely correlated with the similarity threshold. With a threshold of 0.900, only 15 MMP-positive chemicals were retrieved (~2% recall). The recall increased to ~49% with the threshold set at 0.800 (Fig. 5A, middle panel). Thus, setting a high similarity threshold for the query improved prediction accuracy, whereas lower thresholds facilitated a broader coverage.

We also found that other invariant TFAP signatures enabled the identification of compounds with the specified biological activities. For example, querying the Attagene data set by the invariant signatures for UPP perturbagens yielded known UPP inhibitors, including an organotin triphenyltin (28), the antialcoholism drug disulfiram (29), and cadmium chloride (30). Akin to that, the query by the HDAC TFAP also retrieved known HDAC inhibitors, including short-chain fatty acids (isobutyric and isovaleric acid) (31) and trichostatin (14) (Fig. 5B, bottom panel). Therefore, the invariant TFAPs afforded the identification of compounds with the specified biological activities.
The TFAPs enable the assessment of multiple bioactivities of polypharmacological compounds

Assessments of specific perturbagens of the biological pathways showed that these chemicals produced unchanged TFAPs in a broad concentration range (Figs. 2 to 4). However, we found that many ToxCast chemicals elicited different TFAPs at different concentrations. One such example is glitazones, antidiabetic drugs that target the nuclear receptor peroxisome proliferator–activated receptor γ (PPARγ) and modulate gene expression related to lipid storage, cell differentiation, and inflammation (32). Glitazones also share another PPARγ-independent activity that affects the mitochondria function (33, 34). Glitazones bind to mitochondrial membranes and specifically inhibit the mitochondrial pyruvate carrier and respiratory function (35). The mitochondria malfunction caused by these drugs has been associated with drug-induced liver injury (DILI) (33, 36). Troglitazone produced the most frequent occurrence of hepatotoxic events and was eventually withdrawn from the market (33), while pioglitazone and rosiglitazone are still on the market.

Here, we assessed the TFAPs for troglitazone, which is a most-DILI-concern drug, and pioglitazone, a less-DILI-concern drug. The glitazones produced multiple TFAPs at different concentrations (Fig. 6). At low concentrations, both glitazones produced an identical TFAP, which was consistent with their on-target activity (PPAR activation; Fig. 6, A and B, left panel). With increased concentrations, the primary TFAPs of glitazones transformed into different secondary TFAPs, suggesting the influence of off-target drug activities (Fig. 6A). With a further increase in concentration (60 μM), the troglitazone TFAP again transformed into a different tertiary signature (Fig. 6A). The highest concentrations of troglitazone (180 μM) caused cell death (Fig. 6A).

The evident similarity of the secondary signatures of troglitazone and pioglitazone suggested that these drugs had the same off-target activity. Querying the Attagene TFAP database showed that the secondary TFAPs were identical to the invariant mETC TFAP (r > 0.8; Fig. 6B).

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Fig. 6. Assessing the on-target and off-target activities of polypharmacological drugs by TF activity profiling. The TFAPs for the glitazones in HepG2 cells (a 24-hour treatment). Each TFAP represents an average of three signatures by independent FACTORIAL assays. Representative data of the three experiments are shown. (A) The TFAP signature transition with increased concentration indicates an influence of off-target drug activities. (B) The TFAP signatures enable the identification of the on-target and off-target activities of glitazones. Left: The identical TFAPs for low-concentration glitazones reflect on-target activity (PPAR activation). Middle: Identical secondary TFAPs for pioglitazone and troglitazone at higher concentrations indicate mitochondria malfunction. Right: At the highest concentration (60 μM), the troglitazone TFAP is identical to that of hydrogen peroxide, indicating oxidative stress. (C) Assessing dose response of the PPAR RTU to glitazones to determine the AC50 values for the on-target activity. Each data point is an average of three independent measurements.
middle panel). Therefore, the secondary TFAPs reflected mitochondria malfunction. Querying the TFAP data set by the TFAP of troglitazone at 60 μM showed high similarity (r > 0.8) with the TFAP for hydrogen peroxide (Fig. 6B, right panel). Therefore, high concentrations of troglitazone produced oxidative stress, which is consistent with reports by others (34).

Our results show that the assessment of TFAP signatures of glitazones afforded the identification of the primary and off-target activities. The signatures at low concentrations reflected the primary drug activity at PPARγ. The reported AC₅₀ (concentration at 50% of maximum activity) values for the PPARγ activation by troglitazone and pioglitazone are of ~200 nM (37). These data are consistent with the dose responses of the PPAR RTU to these drugs (Fig. 6C). The secondary TFAP indicated mitochondria malfunction and was consistent with the inhibition of the mitochondrial pyruvate carrier by low micromolar concentrations of glitazones (35).

Furthermore, our data may explain the unusually high DILI frequency by troglitazone. The maximum plasma concentrations of troglitazone and pioglitazone in humans are 6.4 and 2.9 μM, respectively. The drugs’ TFAPs indicate that these concentrations are below the thresholds for off-target effects (20 and 180 μM for troglitazone and pioglitazone, respectively; Fig. 6A). However, because of variable genetic and environmental factors, it is possible that drug concentration may exceed the mitochondria malfunction threshold in some individuals. As compared to pioglitazone, therapeutic concentrations of troglitazone are much closer to the off-target threshold, increasing the probability of hepatotoxicity. These results demonstrate the utility of TFAP-based assessments for the evaluation of compounds’ polypharmacology.

**DISCUSSION**

Elucidating the biological activity of compounds as relevant to their efficacy and toxicity is an essential objective of biomedical research. In the presented approach, we assessed the bioactivity of compounds by evaluating the responses of signal transduction pathways that regulate gene expression. As a readout, we used the activity of TFs that connect the signaling pathways with the regulated genes. Using the FACTORIAL reporter assay, we characterized cell response by a quantitative signature, the TFAP. A major advantage of the TF activity profiling approach is that it generates simple quantitative signatures that provide clear quantitative metrics to compare the bioactivity of compounds.

The main findings can be summarized as follows: (i) Perturbations of biological pathways/cell systems produce distinct characteristic TFAP signatures; (ii) perturbagens of the same biological pathway elicit an invariant TFAP regardless of where or how they interfere; (iii) the invariant TFAPs enable the identification of compounds with the specified bioactivities among uncharacterized chemicals; and (iv) the assessment of TFAPs of polypharmacological compounds enables the identification of their multiple biological activities.

This approach has clear ramifications for the drug development process. By assessing the TFAP signatures of a chemical, one can identify its potential therapeutic uses and forecast its toxicity. By detecting the transformations of TFAP signatures with varying concentrations, one can compare the off-target activities of drug candidates and the concentration windows wherein the primary activity dominates. This enables a streamlined solution for hit-to-lead selection. Moreover, the TFAP signatures provide insights into drug polypharmacology. While unintended polypharmacology can compromise safety, drugs that modulate multiple disease-relevant targets can be unprecedentedly efficacious. In this regard, the TFAP signatures allow researchers to distinguish between unwanted and desirable polypharmacology. Furthermore, the TFAP assessment enables a straightforward approach to the identification of new indications for approved drugs.

The finding of the invariant TFAPs indicates that disruptions of biological pathways and cell systems cause coordinated changes in the activity of multiple TFs. That implies the existence of specific response programs. As cellular systems operate under permanent pressure from environmental and internal stresses, there must be mechanisms to cope with the possible malfunctions. Some of these mechanisms have been described, such as the retrograde mitochondrial signaling that alters nuclear gene expression to adapt to mitochondria malfunctions (38). Another example is the "survival response" that coordinates changes in gene expression to accommodate the global epigenomic disruption by HDAC inhibitors (39). In this regard, the invariant TFAP signatures for mETC and HDAC perturbagens (Figs. 2A and 3A) epitomize the signals that regulate these programs.

Here, we have identified the invariant TFAPs for mETC, UPP and HDAC inhibitors, cytoskeleton disruptors, and DNA-damaging agents. This diversity suggests that these invariant signatures should exist for perturbagens of other biological pathways and systems. Our methodology provides the framework for their discovery. Identifying the full complement of these invariant signatures may lead to a new ontology for the bioactivities of compounds.

**MATERIALS AND METHODS**

**Experimental design**

**Cells and reagents**

For the FACTORIAL assay in HepG2 cells [American Type Culture Collection (ATCC) #HB-8065], we used the HGI9 subclone (Attagene) that was selected for an elevated xenobiotic metabolic activity (5). MCF-7 (ATCC #HTB-22) and HEK293 cells (ATCC #CRL-1573) were from the ATCC. Chemical inhibitors were purchased from Cayman Chemical Company (www.caymanchem.com) and Selleck Chemicals (www.selleckchem.com). The ToxCast chemicals were provided by the ToxCast project (EPA). All chemicals were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in cell growth medium did not exceed 0.2%.

**UV irradiation**

Cells were irradiated using a calibrated Spectroline EF-180 UV lamp (Fisher Scientific).

**Cell viability**

Cell viability was evaluated by the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] assay (ATCC) in HGI9/ HepG2 cells. As a baseline, we used cells that were treated with corresponding dilutions of the vehicle (DMSO). The viability data are an average of two replicates. The viability threshold was set at 80% viability.

**The FACTORIAL assay**

The FACTORIAL assay was reproduced as described (4, 5). The mix of 47 RTU plasmids was transiently transfected in a suspension of assay cells using TransIT-LT1 reagent (Mirus). The transfected cells were plated into 12-well plates. Twenty-four hours later, cells were rinsed and incubated for another 24 hours with the evaluated compounds in a Dulbecco’s modified Eagle’s medium (DMEM) growth medium supplemented with 1% charcoal-stripped fetal bovine serum and antibiotics. Total RNA was isolated, and the RTU activity profiles were
assessed by consecutive steps of RT-PCR amplification, Hpa I digest, and CE, as described (5, 6).

**TFAP signatures**

The profile of changes of the transcriptional activity of TFs (the TFAP signature) was calculated by dividing the RTU activity values in compound-treated cells by those in vehicle-treated cells. TFAP signatures were plotted as radial graphs comprising 47 axes showing the fold changes of corresponding RTUs on a logarithmic scale. A value of 1.0 indicated no effects on the TF activity.

**Statistical analysis**

**Assessing the similarity of TFAP signatures**

A TFAP signature can be viewed as a vector in a 47-mer space with coordinates $x_i$ that are log-transformed fold-induction TF values ($\log(\text{RTU})$). The length of the vector is calculated as $|x| = \sqrt{\sum_{i=1}^{47} (\log(\text{RTU}))^2}$. The pairwise similarity of TFAP signatures is calculated as Pearson correlation coefficient $r$ (5) that can vary in a range from −1.0 to 1.0.

**Calculating the consensus TFAPs for multiple perturbagens**

We have modified the average linkage method (40) to develop an algorithm for a recurrent agglomerative hierarchical clustering. We start with $N$ clusters $\{C_i\}$, $i = 1$ to $N$, each containing a single TFAP, to find the clusters $\{C_d\}$ and $\{C_m\}$ with the highest similarity $r$. These clusters are merged into a $\{C_{km}\}$ cluster. The coordinates of the $\{C_{km}\}$ cluster are calculated as an average of $x_k$ and $x_m$ vectors, taken with the weights equal to the size of clusters $N_k$ and $N_m$, normalized to their lengths $|x_k|$ and $|x_m|$, and multiplied by the average length of these signatures, as follows

$$x_{km} = \frac{|x_k| \cdot N_k + |x_m| \cdot N_m}{(N_k + N_m)^2}, \left(\frac{x_k}{|x_k|} \cdot N_k + \frac{x_m}{|x_m|} \cdot N_m\right)$$

The resulting TFAP signature was considered as the consensus (average) TFAP for the chemicals within the cluster. The iterative clustering continued until the distance between clusters exceeded a certain similarity threshold $r^*$. Here, this threshold was set at $r^* = 0.70$.

**Comparing the TFAP-based predictions of mitochondria perturbagens with the functional MMP assay data**

We queried the data set of TFAPs for ToxCast chemicals using the consensus TFAP for mETC inhibitors (Fig. 2A) and counted the number of retrieved chemicals with similarity values $r$ within certain intervals ([$r^*$, $r$ $\geq r^*$]). The concordance with the MMP assay was calculated as the percentage of MMP-positive chemicals among the retrieved chemicals. To calculate the cumulative recall of the MMP-positive chemicals, we calculated the percentage of the 518 MMP-positive chemicals among the retrieved chemicals at different similarity thresholds.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/9/eaar4666/DC1

Fig. S5. The effect of microtubule DNA-damaging agents on the viability of assay cells. Fig. S6. An alternative presentation of TF responses to perturbagens as a heatmap. Fig. S7. The list of known mitochondria disruptors with a high ($r$ ≥ 0.800) similarity to the invariant mETC TFAP. Table S1. ToxCast chemicals with a high ($r$ ≥ 0.800) similarity to the invariant mETC TFAP. References (41–65)

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