Chapter 2

Quantitative High Content Imaging of Cellular Adaptive Stress Response Pathways in Toxicity for Chemical Safety Assessment.

This chapter has been published as:

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1. Abstract

Over the past decade major leaps forward have been made on the mechanistic understanding and identification of adaptive stress response landscapes underlying toxic insult using transcriptomics approaches. However for predictive purposes of adverse outcome several major limitations in these approaches exist. Firstly the limited number of samples that can be analyzed reduces the in depth analysis of concentration-time course relationships for toxic stress responses. Secondly these transcriptomics analysis have been based on the whole cell population, thereby inevitably preventing single cell analysis. And thirdly, transcriptomics is based on the transcript level - totally ignoring (post)translational regulation. We believe these limitations are circumvented with the application of high content analysis of relevant toxicant-induced adaptive stress signalling pathways using bacterial artificial chromosome (BAC) green fluorescent protein (GFP) reporter cell-based assays. The goal is to establish a platform that incorporates all adaptive stress pathways that are relevant for toxicity, with a focus on drug-induced liver injury. In addition, cellular stress responses typically follow cell perturbations at the subcellular organelle level. Therefore we complement our reporter line panel with reporters for specific organelle morphometry and function. Here we review the approaches of high content imaging of cellular adaptive stress responses to chemicals and the application in the mechanistic understanding and prediction of chemical toxicity at a systems toxicology level.

2. Introduction

The mode of action of a chemical entity encompasses its on-target but also off-target effects and both of these effects can lead to adverse outcomes such as drug induced liver injury,[51]′[52] renal failure,[53] skin allergies,[54] adverse respiratory arrhythmia,[55] neurotoxicity[56]′[57] or in the case of constitutively activated mitogenic signalling or mutagenic or inflammatory properties of the chemical entity ultimately leading to cancer.[58]′[59] Chemicals react or interact with cellular components leading to a perturbation of signal transduction networks as the cell tries to reestablish homeostasis. In case these perturbations are detrimental to cells adaptive stress responses are activated. However, if cellular stress is too severe these adaptive stress responses are unable to reestablish homeostasis; a threshold will be reached where the cell activates cell death responses to avoid cell community-level detrimental effects. Understanding these cellular adaptive stress responses to chemicals in detail is key for better drug development and safety assessment.[60]′[61] However, these adaptive stress responses are composed of a multitude of biochemical reactions and molecular events and are always in a dynamic flux to maintain cellular homeostasis in an ever changing environment. In addition, these responses exist at the intra- and inter-cellular level and must be fine-tuned and coordinated for cells to be able to perform their role in reestablishing tissue homeostasis. Such responses have been also named ‘toxicity pathways [62] or fully cover or are part of ‘adverse outcome pathways (AOPs)’.[63] We prefer the term ‘adaptive stress response pathways’, since this relates to the evolutionary defined genetic programs that are meant to adapt to new harmful environments. Ideally one would want to capture the central network hubs underlying these stress responses. We propose that high content imaging of key events that are pivotal in the development of specific toxicities will be essential. In this review we will address the different key adaptive stress response pathways in the context of chemically-induced liver injury and how components of these stress response pathways

can be used in high content imaging approaches. Moreover we will discuss how such tools can then be incorporated in further studies to assess the molecular mechanisms of toxicity as well as in more advanced high content analysis and modeling studies.

3. Drug-induced liver injury: concepts of adaptation and adversity of pathways of toxicity.

A research focus in our laboratory is on drug-induced liver injury (DILI). Liver toxicity is an important reason for drug attrition and a major cause of hospital admissions due to adverse drug reactions. Improved preclinical prediction of drug toxicities is essential for effective development of new and safer drugs. Classically, histopathology data and data on alanine aminotransferase (ALT) and total bilirubin increase obtained from animal models is the golden standard for identification of DILI, but will tell little about the molecular mechanisms involved in the pathogenesis after chemical exposure. Using these animal models for pre-clinical toxicity testing has led to poor predictions: hepatotoxicity is most often cited as the cause of withdrawal of a drug from the market.[8] Several reasons for the low predictivity of animal models for hepatic toxicities are thought to exist including the low occurrence of human toxicity (idiosyncratic DILI), the involvement of the immune system, differences in the metabolic capacities between animals and humans, genetic sensitivities and disease mediation.[64] At the cellular level indications for the type of injury can be resolved, *i.e.* phospholipidosis, steatosis, apoptosis or necrosis, but this does not lead to better mechanistic understanding of the initial cause of adverse outcomes. Biochemical analysis has allowed insight into major metabolic programs including cellular redox status, citric acid cycle metabolism and energy generation. Major developments in metabolomics now allow the detailed analysis of chemical-induced perturbations of the metabolome in close detail.[65] Such changes are likely rather reflections of earlier cellular perturbations that will then define and/or characterize the cellular status, *e.g.* steatosis or mitochondrial dysfunction than that they constitute the prime initiating event (Fig. 1). The initial cell state changes that are closest to the molecular initiating events are probably best described by the assessment of either key (cell-specific) biochemical or cell biological programs. This may include the measurement of enzyme activities in key programs or the evaluation of activation of cellular stress response pathways that will allow the cells to adjust to a new stressful situation. When these cell state changes cannot be met with a new rheostat, key breaking points in the cellular response programs may trigger the onset of adverse outcome and determine the fate of individual cells and eventually overall liver function. Only when exceeding certain concentrations adaptation cannot be met with cellular adaptive stress response programs and cell death or senescence will be initiated. In such a conceptual thinking, the monitoring of the activated adaptive stress response pathways after toxicant exposure would be an improved strategy to assess the underlying molecular mechanisms that link the initial molecular initiation events to toxic outcome. Preferably one would then follow individual markers of the activation status of such key adaptive programs including key-nodes of signalling events that are initiated once the threshold of the adaptive stress response has been met.

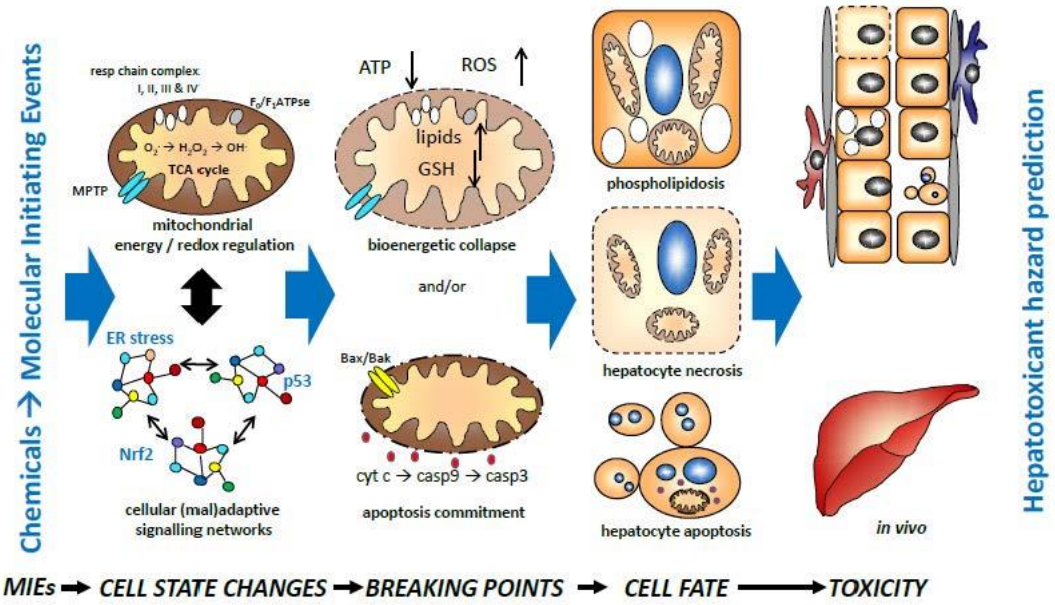


Figure 1: **A model for integrated understanding of chemical-induced liver injury.** Cellular adaptive stress response pathways sense and respond to environmental changes induced by the molecular initiating events of chemical induced toxicity. If the adaptive stress response programs cannot maintain a healthy homeostasis (breakpoint threshold is exceeded) cells will commit to *e.g.* apoptosis resulting in adverse outcome phenotypes such as phospholipidosis, massive hepatocellular necrosis and/or apoptosis leading to organ damage.

4. **From toxicogenomics to predictive classifiers of toxicity.**

Toxicogenomics forms an excellent tool to identify key stress response pathways.[45] Over the past decade an overwhelming number of toxicogenomics studies have been performed both in industrial settings as well as within the academic environment in the U.S., Europe and Japan (reviewed in).[66] While initially the promises and expectations of toxicogenomics were high and suggested to establish predictive tools for diverse types of organ toxicities, so far only limited success stories have been reported.[66-69] Regardless of the overall application of toxicogenomics in the industry and academia, in its current state it has been difficult to tie the gap between transcriptomics to the actual biological understanding. The reason for this has in particular been the cost factor and thereby the limitations for in depth concentration time course experiments for a wide range of target organ toxicities to establish direct cause-effect relationships between target gene expression and toxic outcome. An exception has been the detailed TG-GATES and DrugMatrix data for establishing in particular DILI toxicogenomics datasets.[70] These datasets have so far allowed the establishment of classifiers for specific types of DILI,[71-73] however the relationship between these classifiers and the molecular mechanisms of the phenotypes is still largely unclear. Moreover, there remains a wealth of information in these datasets that allows for hypothesis generation.[66] This should eventually lead to the identification of (additional) predictive classifiers that have more direct biological relevance to the mode of action of toxicity. We anticipate that some of such candidate genes could be excellent for establishing reporter cell systems, for which we propose to use GFP-based technology (see below). In particular the TG-GATES data is suited for this, as this dataset will allow the extraction of gene sets and/or biological (toxicity) pathways that are associated with DILI outcome. Genes that are part of these pathways

and expressed at low levels under control situation, but clearly upregulated after chemical treatment could serve as excellent markers to establish reporter models. Alternatively already available or future novel small molecular fluorescent probes could be applied in simple high content imaging approaches. Yet for further identification of transcriptomics-based reporters more detailed concentration-time resolved toxicogenomic studies are required, in particular where possible targeting specific cell types in relevant organs of toxicity, using human donors or if necessary alternatively in conjunction or replaced by improved *in vitro* models that recapitulate the human target organs. Clearly this should involve the latest next generation sequencing technology and should preferably be tightly integrated with direct assessment of additional critical markers of cellular function at the biochemical or phenotype level as well as toxicological outcome, including assessment of intracellular parent compound and metabolite concentration analysis to allow for future *in vitro* to *in vivo* extrapolations. Moreover, it will be essential to determine whether the eventual candidate markers of toxicity are an integral part in the regulation of adaptive stress response pathways using RNA interference-based functional genomics approaches.[74]

In the past years also alternative omics approaches have been used to better understand the initial mode of action of chemicals. In particular advanced SILAC-based proteomics (stable isotope labeling by amino acids in cell culture) has now uncovered a plethora of early post-translational signalling events through protein kinase-mediated phosphorylation which contributed to an improved molecular understanding of *e.g.* the DNA damage response.[75, 76] While such proteomics approaches are tedious and costly, they may lead to the identification of novel phosphorylation events in signalling networks that are key in the target organ specific toxicities. This would allow the generation of phospho-state specific antibodies as an extra type of event that could be integrated in high content imaging approaches and may facilitate bridging of the gap between the *in vitro* and *in vivo* (human) situation.

Besides toxicogenomics several additional efforts have been made on monitoring the underlying signalling pathways based on for example qRT PCR.[77] The primers for these high throughput assays are designed for profiling the expression of sets of genes that are proven or expected classifiers for the mode of action of chemicals, typically derived from toxicogenomics studies. Another approach to detect specific signalling events is the use of phosphorylation state-specific antibodies *e.g.* with phospho-specific flow cytometry.[78] The number of alternative non-animal testing methodologies have increased substantially, many improving on a number of substantial issues regarding *in vitro* toxicity screening like metabolic capacity and tissue-context structural attributes This review focuses on cellular stress response signalling based readouts for chemical safety evaluation; for an in depth review on recent developments on alternative testing methods see for example reviews by Hengstler *et al*.[16] and LeCluyse *et al*.[79]

5. High content imaging of adaptive stress response pathways.

Today's state of the art high content imaging systems combine (semi) high throughput (High Throughput Screening – HTS) with a high level of detail (High Content Screening/Imaging – HCS/HCI) which allows capturing the phenotypic cellular responses to many environmental conditions at the subcellular (organelle) and protein signalling level. This technology should overcome the past classical biochemical cell toxicity readouts that so far have captured different

types of cell dysfunction but usually give little information on causality, *i.e.* the mode of action of the chemical entity and the underlying chemical-pathway interactions and resultant cellular signalling events. The most well-known classical biochemical cell toxicity readouts include reduced activity of mitochondrial respiration (MTT and MTS assays),[80],[81] cellular ATP content depletion (e.g. ATPlite assay),[82] leakage of the enzyme lactate dehydrogenase (LDH) upon cell death[83] and quantitation of the fraction of surviving cells with or without intercalating DNA-staining dyes such as DAPI or Hoechst 33342 for living cells and propidium iodide [PI] for necrotic cells.

Now for the first time investigators are able to visualize the central signalling hubs controlling the adaptive cellular stress responses in a systematic manner, which allows its integration in toxicity screening strategies.[84-86] The true power of HCS using automated imagers lies in its ability to capture when and where specific molecular signalling events are taking place, enabling characterization of cellular responses to many different changes in the environment in a high time and spatial resolution, relatively high throughput (depending on exact setup) and on a single cell basis enabling detection of heterogeneity within populations.

Automated imaging systems can be roughly divided into four groups of increasing complexity: wide-field imagers for fixed cell samples, (spinning disk) confocal imagers to scale up the detail of the fixed images, imagers equipped with temperature and CO_2 control and confocal systems adapted for HCS and equipped with an environment chamber, reviewed in.[87] The latter two systems can be combined with a robotic plate exchange system to enable imaging of multiple multi-well plates containing living cells in parallel thus vastly increasing the throughput of high content live single-cell based imaging.

When current efforts of high content imaging in the context of the adverse outcome pathway concept[63] is viewed in relation to establish assays for adaptive stress response pathways, it becomes apparent that most assays are based on the final outcome of an adverse effect within a cell: the observed phenotype is the end result or downstream observable effect of the mode of action and adverse outcome molecular initiating event in the chemical- biological space. For example in a high content imaging assay utilizing cell viability stains like propidium iodide and annexin V-FITC[88] the readout is in effect based on dead cells only discriminating between the type of cell death, necrotic or apoptotic. Already a more detailed view of the type of cellular toxicity leading to cell death is obtained using for example mitochondrial membrane potential assays (*e.g.* TMRM, Rhodamine123 or JC-1), mitochondria superoxide detection (MitoSOX), reagents allowing mitochondrial permeability transition detection (calcein-acetoxymethyl ester (AM)) or detection of intracellular calcium levels (calcium binding probes), excess lipid droplet formation (nile red and BODIPY 493/503)[89] or an accumulation of phospholipids in the lysosomes during drug-induced phospholipidosis (*i.e.* LipidTOX Red or NBD-PE).[89, 90] One example of an implementation of such a HCI effort has been performed using several dyes and markers (calcium (Fluo-4 AM), mitochondrial membrane potential (TMRM), DNA content (Hoechst 33342) and plasma membrane permeability (TOTO-3)) on HepG2 cells to predict DILI.[91] This study mentions a sensitivity of 93% and specificity of 98% using 243 drugs, in comparison with 7 more conventional in vitro toxicity assays with a sensitivity of 25% and specificity of 90% for 611 compounds. However the underlying signalling events culminating in these adverse phenotypes are still not part of HCI. A step forward is the use of reporter cell lines. These can be on the genetic level (luciferase reporters) or on the protein level (fluorophore

coupled proteins) and even on interactome,[92] phosphor-proteomic[93] and metabolic level,[94] using fluorescence resonance emission transfer indicators (FRET). In toxicology luciferase reporters and fluorophore coupled protein reporters have been used and several are commercially available. A good example comes from the efforts within the Tox21 and ToxCast consortia where a set of 2870 compounds was screened using a β-lactamase reporter gene assay with reporter cell lines from GeneBLAzer®. These cell lines constitutively co-express a fusion protein comprised of the ligand-binding domains (LBD) of related human nuclear receptors coupled to the DNA-binding domain of the yeast transcription factor GAL4.[95] In addition more simple luciferase and GFP reporters are used in these large collaborative efforts. A drawback of these different reporter-based systems is that several critical endogenous regulation mechanisms are lacking including the entire promoter region as well as introns. This can often lead to less specific and or less sensitive readouts. In addition for many genes the promoter sequence is not entirely known, therefore fusion constructs are often based on CMV driven promoters leading to several factors of overexpression of the protein of interest which can lead to perturbed homeostasis in the reporter cell line. With the introduction of bacterial artificial chromosome (BAC) transgene-based cell lines these short comings are circumvented. By application of BAC transgenomics a large panel of BAC-reporter cell lines can be generated with relatively little effort.[48] The basic principle is based on the use of BACs that contain a genomic copy of a particular human gene including all exons and introns and at least 10 kB flanking DNA on each end of the gene which most likely encompasses the entire promoter region and other regulatory elements ensuring its normal physiological regulation of expression. A fluorescent or luciferase reporter construct can be introduced into the BAC by homologous recombination making use of homology arms on each end of the reporter construct. So far we showed that a small panel of BAC transgenic engineered mouse embryonic stem cells in combination with flow cytometry analysis could distinguish oxidative stress inducing chemicals from DNA damage inducing chemicals using the *Srxn1* and *Bscl2* genes.[13] This demonstrates how key nodes of stress response signalling networks can reveal mechanistic information on the mode of action of chemicals.

The next step is to utilize state of the art high content automated imaging coupled to automated image analysis with panels of reporter cell lines, enabling high throughput identification of specific key signal transduction nodes being perturbed by chemicals. We envision that high content automated imaging combined with a panel of endogenously regulated reporter cell lines will prove to be a powerful tool in early chemical safety assessment and will improve mechanistic understanding of chemicals in an early stage.

6. Key adaptive stress response pathways in chemical toxicity as BAC reporter systems.

Stress responses and the cellular signalling network in general cannot be regarded as an independent set of linear signal transduction routes. Therefore a panel of reporter cell lines to monitor multiple key nodes of adaptive stress response pathways is key. The major stress signalling routes activated in response to adverse chemical reactions that can be discerned from toxicogenomics studies include the antioxidant response element activation, heat shock response, unfolded protein response, metal stress response, the DNA damage response and CYP and other phase I, II and II enzyme/transporter induction by nuclear receptors. Time lapse analysis of these responses programs, where possible at a single cell level, would give valuable information on the

mode of action of novel chemical entities. Typically these programs would involve the activation of sensors that recognize the cell injury or stress followed by activation of single or networks of specific downstream transcription factors that modulate the expression of specific gene sets thereby affecting the outcome of the cellular stress response at the cell biological level (see Fig. 2 for examples). Below we will address the selection steps for reporters of the most relevant stress response pathways by describing in more detail their molecular activation as well as their involvement in liver injury. This then allows the identification of 'sensors', 'effectors' and downstream 'targets' that may be appropriate for DILI.

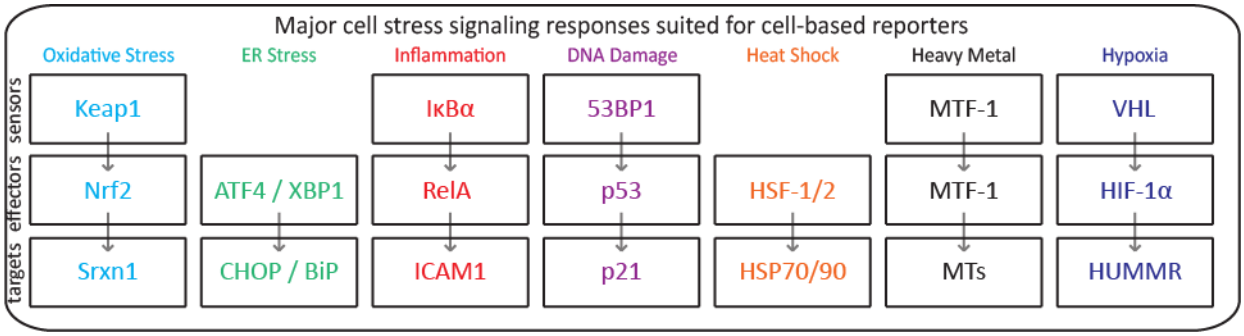


Figure 2: **Key stress reporter pathways for chemical safety assessment**. Key players in major adaptive stress response pathways that could be chosen for BAC reporter cloning – the early signalling 'sensors', 'effectors' transcription factors and downstream 'targets'.

6.1. KEAP1/Nrf2 signalling pathway.

Cellular redox homeostasis can be disrupted by internal metabolism, xenobiotic exposure, environmental factors and host immune cell defense mechanisms.[96-98] Although drug-metabolizing enzymes metabolize and detoxify electrophiles and oxidants,[99] metabolism of xenobiotics by *e.g.* P450 CYP enzymes can also lead to bio activation resulting in the formation of electrophiles. The most well-known example being acetaminophen overdose toxicity, which depletes cellular anti-oxidant glutathione levels and increases the levels of reactive oxygen species (ROS) in cells.[100] ROS are controlled by various constitutively expressed detoxifying enzymes such as glutathione-S-transferases, NADP(H):quinone oxidoreductase, glutathione peroxidases, catalase, superoxide dismutases, epoxide hydrolases, heme-oxygenase, UDP-glucuronosyl transferases, gamma-glutamylcysteine synthase and sulfiredoxin-1.[101-104] These ROS detoxifying genes are controlled by anti-oxidant response elements (ARE) in their promoter regions which are activated by so called xenobiotic-activated receptors (XARs) or by the nuclear factor erythroid 2-related factor 2 (NFE2L2 or Nrf2), which in turn are activated in response to specific chemicals or other environmental perturbations involving redox biology. Nrf2 is the key transcription factor required for ARE dependent drug metabolizing enzymes.[105] Many chemicals and substances induce ARE-dependent genes. Nrf2 mRNA is readily detectable in a wide range of cells, implying that transcription of Nrf2 is not a major mechanism by which Nrf2 is regulated.[106] The main mechanism of activation of the anti-oxidant response occurs by modification of specific cysteine thiol groups on Kelch-like erythroid cell-derived protein with CNC homology-associated protein (Keap1)[107] and Nrf2 – the cysteine groups function as electrophile and oxidant sensors.[108],[109] Under non oxidizing conditions Nrf2 is bound by homo-dimerized Keap1 acting as an adaptor protein for the Cul3-dependent ubiquitin ligase (E3) complex.[110] Cul3 is a scaffold protein for the binding with RING box protein 1 (Rbx1) which in turn recruits ubiquitin-conjugating

enzyme (E2) for polyubiquitination and degradation by 26S-proteasomes. Upon alkylation or oxidation of specific cysteine thiol groups on Keap1[111] Nrf2 translocates to the nucleus where it binds to promoter enhancer regions containing the ARE consensus sequences.[112] The Nrf2 signalling as adaptive stress response pathway has emerged as a vital signalling node. Nrf2-null mice are more sensitive to a wide range of chemicals, including butylated hydroxytoluene (BHT). Nrf2 protects against liver injury produced by numerous hepatotoxicants including acetaminophen *in vivo* and *in vitro*.*[113-115]* While constitutive Nrf2 activation can be detrimental in particular in cancer progression and drug resistance.[116],[117] We anticipate monitoring the anti-oxidant response using BAC transgene reporters combined with high content imaging will reveal important early clues to unexpected off-target effects and possible toxicity by *e.g.* reactive metabolites for early toxicity screening of chemicals and drugs. The magnitude and time dynamics of the Nrf2 pathway in relation to other adaptive stress response pathways is likely a relevant marker for early toxicity evaluation in pre-clinical compound screens. We have generated a set of oxidative stress BAC-GFP reporters (Fig. 3A) in a strategic manner to be able to monitor the oxidative stress 'sensor' Keap1, the transcription factor Nrf2 acting as the 'effector' and downstream 'target' anti-oxidant enzyme sulfiredoxin-1 (Srxn1). The latter is specifically controlled by Nrf2 and highly responsive to a wide range of DILI compounds in primary human hepatocyte transcriptomic analysis (unpublished results). After exposure to iodoacetamide the Keap1 accumulation in foci identified as autophagosomes is followed by the translocation of Nrf2 to the nucleus. Several hours later this is followed by a strong increase in the levels of Srxn1 (see Fig. 3A and supporting information 1).

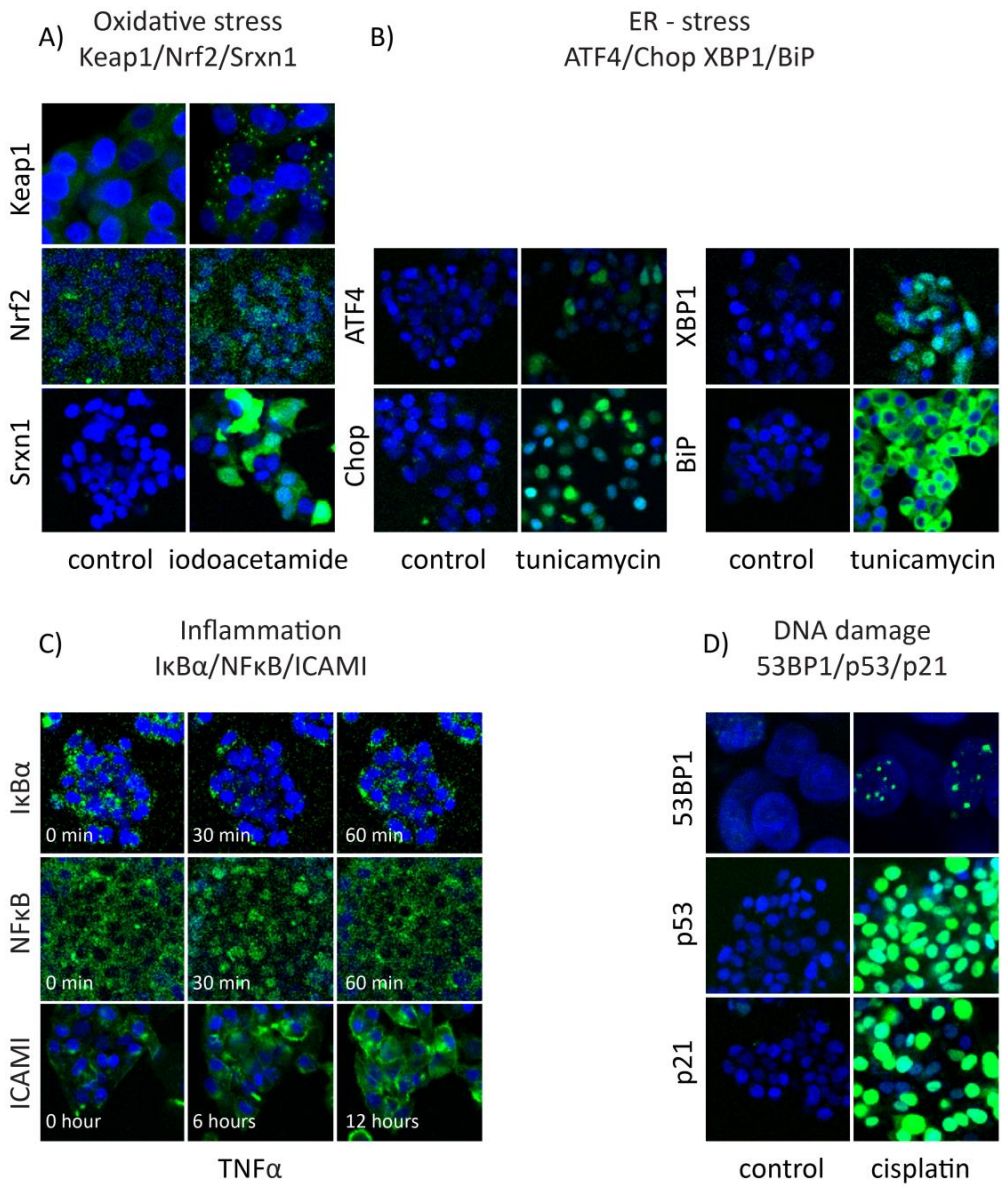


Figure 3: **Examples of BAC reporter cell lines of toxicity pathways in HCI. A)** Oxidative stress signalling: Keap1 as sensor; treatment with 10 µM iodoacetamide (an electrophile for covalent modification of nucleophilic residues on proteins, *e.g.* cysteines) leads to formation of Keap1 foci – these foci correspond to autophagosomes where the Keap1 proteins are degraded. Nrf2 as transcription factor: Endogenous Nrf2 levels are extremely low but the nuclear translocation after treatment is still quantifiable. Srxn1 as downstream target: Following Keap1 degradation and Nrf2 nuclear translocation downstream target Srxn1 protein levels are increased. **B)** ER-stress signalling: The apoptosis related ER-stress signalling arm of transcription factor ATF4 and downstream target CHOP v.s. the acute protective arm consisting of transcription factor XBP1and downstream target BiP. Both signalling arms are activated after 8 hours of 10 µM tunicamycin treatment. **C)** Inflammation signalling: The maximum and minimum of the first peak of the oscillatory NF-κB response after a 10 ng/ml TNFα treatment is shown. At 30 minutes the level of NF-κB inhibitor IκBα is decreased – followed by NF-κB translocation to the nucleus. The first peak has disappeared after 60 minutes. As a consequence downstream target ICAM1 levels steadily increase. **D)** DNA damage response: cisplatin-induced bulky lesions lead to formation of DNA-repair protein foci (*e.g.* 53BP1) which act as sensors for further DNA-damage repair signalling. This is followed by the nuclear translocation of transcription factor p53 leading to an increase in the level of non-apoptotic senescence downstream target p21.

6.2. Unfolded protein response (UPR) and endoplasmic reticulum (ER) damage.

The endoplasmic reticulum (ER) is the major cellular organelle involved in protein synthesis, modification, folding and sorting.[118] Cells have evolved an adaptive protective mechanism to cope with perturbations in the protein processing capacity of the ER called the unfolded protein response (UPR). The UPR is separated in three branches: the inositol requiring enzyme 1α (IRE1α) branch, the activating transcription factor 6 (ATF6) branch and the protein kinase RNA-like ER kinase (PERK) branch.[119] They all have one sensing molecule in common: the ER-resident chaperone BiP. Under normal conditions, BiP binds to the transmembrane transducers IRE1α, ATF6 and PERK on the ER luminal membrane. BiP has a relatively strong binding affinity with unfolded proteins, when unfolded proteins start to accumulate in the ER lumen the transducers are thought to go through a conformational change because of the resultant free BiP binding sites. Dissociation of BiP triggers PERK to homodimerize and autophosphorylate.[33, 120] Activated PERK phosphorylates eukaryotic translation initiator factor 2α (eIF2α). This leads to an attenuation of general translation, however also leads to increased translation of a specific mRNA species that encodes the transcription factor ATF4. ATF4 in turn activates genes involved in amino acid metabolism, redox balance, protein folding and autophagy.[121, 122] IRE1α is also activated via homodimerization and autophosphorylation triggered by BiP dissociation. The activated ribonuclease domain of IRE1α catalysis the excision of a 26 nucleotide intron from ubiquitously expressed XBP-1 mRNA which causes a frame shift in the XBP-1 coding sequence resulting in its translation. X-box binding protein 1 (XBP1) then translocates to the nucleus and induces transcription of ER-associated degradation (ERAD), phospholipidosis to promote ER-membrane expansion, and protein folding by expression of chaparones like p58, ERdj4 and BiP.[123] In addition activated IRE1α activates programs including regulated IRE1 dependent decay (RIDD; selective degradation of mRNA of proteins located in the ER), macroautophagy and inhibition of translocation of proteins into the ER-lumen.[124-126] Following BiP dissociation ATF6 translocates to the golgi apparatus where it is cleaved into the transcriptionally active form (ATF6f).[127] ATF6f subsequently activates genes involved in ERAD (endoplasmic reticulum associated degradation) and protein folding.[128] Thus, all three UPR axes (PERK, IRE1α and ATF6) initially contribute to the adaptation of the cell to overcome the overload of unfolded proteins. However, when the amount of unfolded proteins keeps accumulating during sustained stress conditions, the UPR switches to pro-apoptotic mechanisms. A key transcription factor in this switch is C/EBP-homologous protein (CHOP, also known as GADD153). CHOP is mainly activated via the PERK-ATF4 axis,[129] however, there is also evidence for a non-specific activation via (one of) the other two branches.[130, 131] CHOP regulates transcription of a variety of pro-apoptotic genes including Death Receptor TRAIL receptor 2 (DR5)[132] and Bcl-2 family member Bim,[133] thereby sensitizing cells to apoptosis. In addition CHOP also de-attenuates the general translation program by inducing expression of GADD34 which dephosphorylates eIF2α. This can result in an accumulation of premature proteins in the ER, which is shown to induce accumulation of reactive oxygen species (ROS) and subsequent mitochondrial damage and apoptosis.[134] Recent publications demonstrate a crucial role for ER-stress in hepatosteatosis, cholestasis and hepatotoxicity. Elevated levels of ATF4 and spliced XBP1 were observed in fatty liver samples compared to normal and steatotic liver samples.[135] Also *in vivo* evidence for a role of UPR in cholestasis was recently observed where CHOP-null mutants developed much less liver fibrosis

compared to wild type livers.[135] In addition, CHOP knock-out mice are less susceptible to acetaminophen-induced liver injury.[136] Altogether there is clear evidence for different UPR/ER stress programs in liver injury responses. Concurrently it is critical to establish reporters for the different anti-apoptosis and pro-apoptosis UPR/ER stress signalling pathways. We have established BAC-GFP reporters for ATF4 and CHOP on the one hand, and XBP1 and BiP/HSPA5 on the other hand (Fig. 2 and Fig. 3B) which are highly responsive for prototypical UPR inducers, such as tunicamycin. These individual reporters would be valid to be incorporated in advanced high throughput microscopy approaches to assess chemically-induced UPR onset.

6.3. Inflammatory signalling through the cytokine-NF-kB pathway.

The liver contains around 20-40% non-parenchymal cells including resident immune cells from both the adaptive and innate immune system (Kupffer cells (KC), Natural Killer (NK) cells and dendritic cells (DCs)) and as such has a unique immunological environment. Paradigmatically, this ensures both the tolerogenic nature of the liver and defense against bacterial or viral infections.[137-139] Pro-inflammatory cytokines like TNFα and IL1 are produced mainly by immune cells and facilitate intercellular communication within the liver to mediate (immune) cell activation, migration and recruitment. TNFα is produced by Kupffer cells upon pathogen challenge and danger-associated molecular pattern (DAMP) exposure and has been convincingly shown *in vivo* to be a key-component in the development of DILI, for instance in trovafloxacin and sulindac liver injury.[140, 141] Stimulation with TNFα activates the TNF receptor and induces the formation of a receptor complex activating kinase TAK1 and the IKK kinase complex. Phosphorylation of IκBα followed by its ubiquitination and subsequent proteasomal degradation[142] leads to NF-κB nuclear translocation. Nuclear NF-κB can activate gene transcription of early, middle and late target genes, including IkBα and A20 establishing a strong negative feedback loop.[143] Similarly, IL1β stimulation induces NF-κB translocation by activating TAK1 and the IKK complex. However, signalling upstream of TAK1 differs and ultimately NF-κB translocation by IL1β signalling leads to transcription of a different set of target genes. In both cases NF-κB nuclear translocation is a dynamic process which involves an oscillatory response where the duration of the overall nuclear localization time is one of the factors determining the transcriptional activity and downstream effects. Either drug exposure itself or drug exposure combined with pathogen challenge can lead to liver inflammation and pro-inflammatory cytokine production.[144-146] Current research suggests that drug-mediated perturbations in cytokine or DAMP signalling pathways cause synergistic drug/cytokine-induced cell death.[38, 147] Therefore, unraveling cytokine signalling in DILI will form one of the corner stones in the understanding of DILI. We have approached this by measuring drug-induced effects on NF-κB translocation using high content live cell microscopy using BAC-GFP reporter cell lines of a signal 'sensor' IkBα, an 'effector' protein RelA and several 'target' genes including ICAM1. Such experiments provide information in a time-resolved, quantitative and single cell fashion on NF-κB activation.

6.4. The DNA damage response.

Cells in our body are exposed to exogenous and endogenous sources of DNA damage inducing agents, *e.g.* UV light, genotoxic substances and metabolic processes causing single or double strand breaks, base modification or intra or inter-strand crosslinks.[148] A set of highly conserved

cell cycle check point and DNA damage repair systems has evolved that allows cells first to repair inflicted DNA damage before replication commences with the risk of mutation induction.[149] The signalling involved in sensing the types of DNA damage, halting cell division at the cell-cycle check point and repairing the damaged DNA is fitted to the various types of lesions. DNA damage is detected by specific damage sensing mechanisms and by enzymes involved in DNA replication and transcription.[150] Crucial early regulators in the DNA damage response are the PI3-K-related protein kinases ataxia-telangiectasia mutated (ATM), ATM and RAD3 related (ATR) and DNA-dependent protein kinase (DNA-PK).[151] From these proteins the DNA damage signal is thought to be transmitted via CHK and CHK2 (check point kinase 1 and 2, respectively), aided by scaffold proteins such as MDC1 (mediator of DNA-damage checkpoint 1), 53BP1 (p53-binding protein 1) and BRCA1 (breast cancer 1 early-onset).[152-154] Among others ATM and ATR can activate p53 by phosphorylation of p53 or its inhibitor - the E3 ubiquitin ligase Mdm2.[155, 156]

p53 is mainly known as a tumor suppressor, but numerous additional roles have been reported. At least 129 direct transcriptional targets of p53 exist.[157] Under conditions of severe stress, p53 tumor suppression activity leads to irreversible apoptosis programs by activating extrinsic and intrinsic apoptosis targets including *BAX*, *FAS*, *NOXA* and *PUMA.*[158] The best-studied pro-apoptotic protein required for apoptosis induction by p53 is PUMA, a p53 target gene that is required to release cytoplasmic p53 from the antiapoptotic protein Bcl-X_L, followed by mitochondria outer membrane permeabilization.[159] Alternatively, under conditions of low-level stress, p53 mediates its tumor suppression function via cellular growth arrest by activating the expression of cyclin-dependent kinase inhibitor p21, giving individual cells the possibility to repair DNA damage.[160] The most well described downstream targets of p53 have been reviewed by *e.g.*[161, 162] It is important to note that p53 also mediates numerous roles under non-stressed conditions which involves diverse cellular process including cellular migration, metabolism, cellular redox state, autophagy, angiogenesis inhibition, innate immunity and differentiation.[160] This is likely related to sub-lethal stress conditions that can also activate p53. Thus stress severity and type leads to different functional roles of p53. Functionality of p53 is modulated by its concentration, conformation and translocation into the nucleus. p53 contains nuclear localization- (NLS) and nuclear export signals (NES) that are located adjacent to and within the oligomerization domain of p53, respectively, leading to the possibility that p53 oligomerization is an important mediator of nucleo-cytoplasmic transport.[163] Nucleo-cytoplasmic shuttling is controlled by Mdm2, which interacts with p53 in the nucleus targeting it for nuclear export and degradation.[163]' [164] Moreover, phosphorylation of p53 by kinases such as ATM, ATR, DNA-PK, and casein 1-like kinase (CK1) regulates p53 nuclear import or export.[155, 165-168] To monitor the DD response in individual cells using fluorescent reporter imaging several candidate genes can be proposed. In the case of double strand breaks or stalled replication forks the ATR, ATM and DNA-PK kinases are activated followed by recruitment of a large variety of DNA repair proteins that localize to the damaged sites forming distinct DD loci.[169] Well known markers of these foci are phosphorylated histone variant H2AX and p53 binding protein 1 (53BP1).[152, 170] 53BP1 undergoes nuclear relocalization to focal structures of unknown architecture at double strand breaks or large adduct loci, presumably to facilitate the checkpoint and repair functions.[152] 53BP1 based foci formation are a useful high content imaging readout to identify candidate genes that modulate the DD response and allowed the identification of NUP153 as a novel factor

specifically required for 53BP1 nuclear import.[171] Therefore 53BP1 can perform a role as early DD response 'sensor'. Next, the nucleo-cytoplasmic translocation and concentration of p53 as one of the most relevant 'effectors' in the DD response. Finally, well known downstream 'targets' of p53 could be monitored, including Mdm2 which functions as a feedback loop inhibitor of p53 activity[172], [173] or p21 which is known to be very sensitive to small increases in p53 levels.[160]

6.5. Additional stress response pathways.

There are additional known stress response pathways in drug-induced liver injury that we will only briefly touch. These include hyperthermia (heat shock response), heavy metal insult, hypoxia and nuclear hormone receptors. The heat shock protein family (HSP) is currently recognized for their role in reaction to a broad variety of physical and chemical insults, including drug-induced liver injury inducers.[174]

Physical and chemical insults may cause an accumulation of unfolded and denatured cellular proteins. This triggers heat shock factors (HSF-1 or HSF-2) to trimerize and translocate to the nucleus.[175] HSFs induce expression of five different heat shock protein families: the HSP70 family, HSP90 family, HSP110 family, HSP40 family and the small HSP family.[176] The heat shock proteins function as chaperones and bind proteins to prevent denaturation and to refold denaturized proteins.[177]

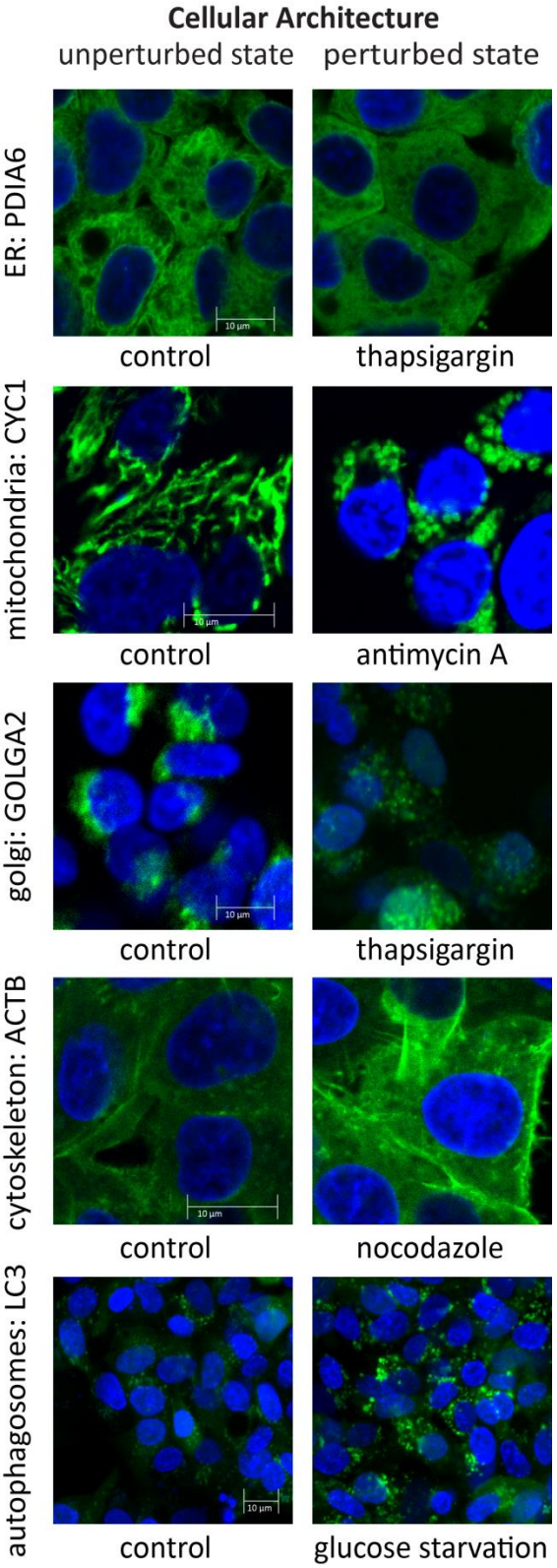
Cells are also able to adapt to heavy metal stress. Heavy metals, like zinc, copper and cadmium, are sensed by MTF-1. A conserved cysteine cluster is responsible for the homodimerization of MTF-1 after activation with heavy metals.[178] MTF-1 translocates to the nucleus where it induces transcription of metallothioneins. Metallothioneins are responsible for the detoxification of heavy metals.[179] MTF-1 is shown to be essential for adult liver detoxification in mice.[180] This indicates an important role for the heavy metal stress pathway in drug-induced liver injury.

Reduced oxygen tension (hypoxia) in cells can induce cell perturbations and cell death. Therefore, the intracellular oxygen tension is constantly monitored by prolyl hydroxylases, which catalyze the hydroxylation of proline residues of transcription factor hypoxia-inducible factor 1α (HIF1-α). The hydroxylation of HIF-1α enables the Von Hippel-Lindua (VHL) protein to bind to HIF-1α. This complex translocates to the autophagosomes where it is degraded under conditions of normal oxygen tension. Under hypoxic conditions, hydroxylation of HIF-1α proline residues ceases, enabling HIF-1α to translocate to the nucleus and activating downstream targets.[181] One such target is HIF-1α-inducible protein (HUMMR) which alters mitochondrial distribution and transport.[182] HIF-1α is been shown to play a role in DILI; HIF-1α deficient mice exposed to an acetaminophen overdose showed significantly less hepatotoxicity in the early stages after administration.[183]

Finally, another protein family which is essential in hepatotoxicity is the nuclear hormone receptor family.[184] The members of this family are highly expressed in the liver and include PXR, RXR, CAR, AHR and HXR. They can bind a broad spectrum of ligands, including various xenobiotics. When bound, the nuclear receptors are released from their co-repressors and bound by their co-activators. Together, these complexes activate gene transcription of cytochrome P450 enzymes, and other phase I, II and III enzymes responsible for bile salt and fat metabolism and which are very well known for their drug metabolizing capabilities.[16] We consider the components of the

above pathways, including HSFs, Hsp family members, MTF-1, metallothioneins, HIF1α and xenobiotic nuclear hormone receptors and some of their downstream target genes, also likely candidates for BAC-GFP reporter constructs. These can then contribute to monitor stress response pathways related to toxicity, in particular DILI.

6.6. Markers for critical cellular organelles and cell function.



Transcriptomics analysis has revealed the detailed cellular adaptive stress response landscapes and the diversities of organelles that are likely involved. Indeed, various organelles undergo cellular perturbations and/or remodelling upon injury such as the mitochondria (fission/fusion),[185] endoplasmic reticulum (ER)[186, 187] and the actin cytoskeleton[188], which are critical in the onset of cytotoxicity. We found that such phenotypic alterations occur well before the typical ultimate outcome of cell stress: cell death by apoptosis.[189] It seems essential to classify the cellular perturbations also on the basis of imaging-based analysis of disruption of cell organelle morphology and function. Similar as for the stress response reporters, specific target genes used for BAC tagging enable visualization of these organelles and their perturbations as response to diverse cellular stress conditions and can serve as markers for sub-cellular compartments and cell organelle function. Such reporters should preferably be selectively localized in these organelles and remain associated with the organelle membrane or lumen even under stress conditions. Several relevant markers are indicated in figure 4.

***Figure 4*: Examples of morphology markers and cell death markers.** Panel 1 to 5 (top to bottom): **1)** endoplasmic reticulum folds disperse after a 8 hour treatment with 1 µM thapsigargin, a endoplasmic reticulum Ca^{2+} ATPase inhibitor; **2)** Mitochondria swelling and network disintegration after 8 hour treatment with 5 µM Antimycin A, Cytochrome reductase inhibitor; **3)** golgi polarization is dispersed after a 8 hour treatment with 1 µM thapsigargin; **4)** formation of stress fibers after a 4 hour treatment with 5 µM nocodazole (microtubili polymerization inhibitor); and **5)** autophagosome increase after 8 hours of glucose starvation.

7. From microscope images to quantitative data.

How can one derive the relevant quantitative data that describes all the relevant toxicological features from high throughput microscopy experiments to assess chemical safety? The simultaneous imaging capabilities of several high content live cell imagers in a screening laboratory easily leads to 100 GB of images (20,000-30,000 images) overnight. Therefore automated high content image acquisition must be coupled to an integrated automated multiparameter-image analysis tool for fast and accurate quantification of the acquired images, for a review on popular tools see.[145] As an example of how to handle such large data streams and computational overhead we describe our own customized automated image analysis pipeline based on an integration of custom made ImageJ[190] plugins, CellProfiler,[191] HDF5[192] and quantitative data processing R-scripts (Fig. 5). Image loading, image metadata definitions, intensity- and most morphological feature measurements, and the initial tracking of single cells is performed by standard CellProfiler modules. The segmentations are performed by a custom made ImageJ plugin based on the Watershed Masked Clustering Algorithm,[193] in addition some morphological (*e.g.* skeleton measurements) are also performed by ImageJ plugins. These plugins have been integrated in the CellProfiler environment by making use of the python-javabridge utility provided by CellProfiler. The latest version of CellProfiler includes the option to store quantitative data output in a hierarchical data format: HDF5.[192] An often overlooked aspect is data format standardization for accessibility and inter laboratory data exchange, for a promising implementation for the high content imaging community see cellH5.[194] CellH5 is based on the HDF format – a hierarchical file based system to store (biological) data.[195] Together with a plate layout text file the quantitative data is analyzed and graphically displayed in an automated fashion using R-scripts, which will become available as a R-package in the near future. The automated analysis includes reorganization and modification of the tracked objects and linkage of cellular features/phenotypes to cell mobility on the single cell level. In addition a database for storage and accessibility of imaging data and preferably a pipeline to streamline the workflow is needed. Several tools exist for this purpose the most well-known open source variations are the database, analysis and management package OMERO[196] and for analysis pipeline and software tool integration KNIME.[197] However quicker and easier to implement database-management systems originate from commercial vendors.

8. Examples of the application of cellular stress response fluorescent reporter systems.

The BAC GFP cellular stress response reporter cell lines can be applied in various settings. Firstly, this may involve large compound screens using end-point measurements to simply monitor overall activation a cellular stress response pathway after chemical exposure. Secondly, it can include the live cell imaging of dynamic signalling responses of for example transcription factor activation and the consequences of chemicals on such a response. Thirdly, for those signalling routes that are of high relevance in toxicology large scale RNA interference approaches may address the underlying signalling networks that control the respective cellular stress responses and thereby the cellular outcome, *e.g.* enhanced activation of adaptive programs with limited cytotoxicity, or suppression of adaption with increased susceptibility for cell killing. Below we will describe in more detail these applications.

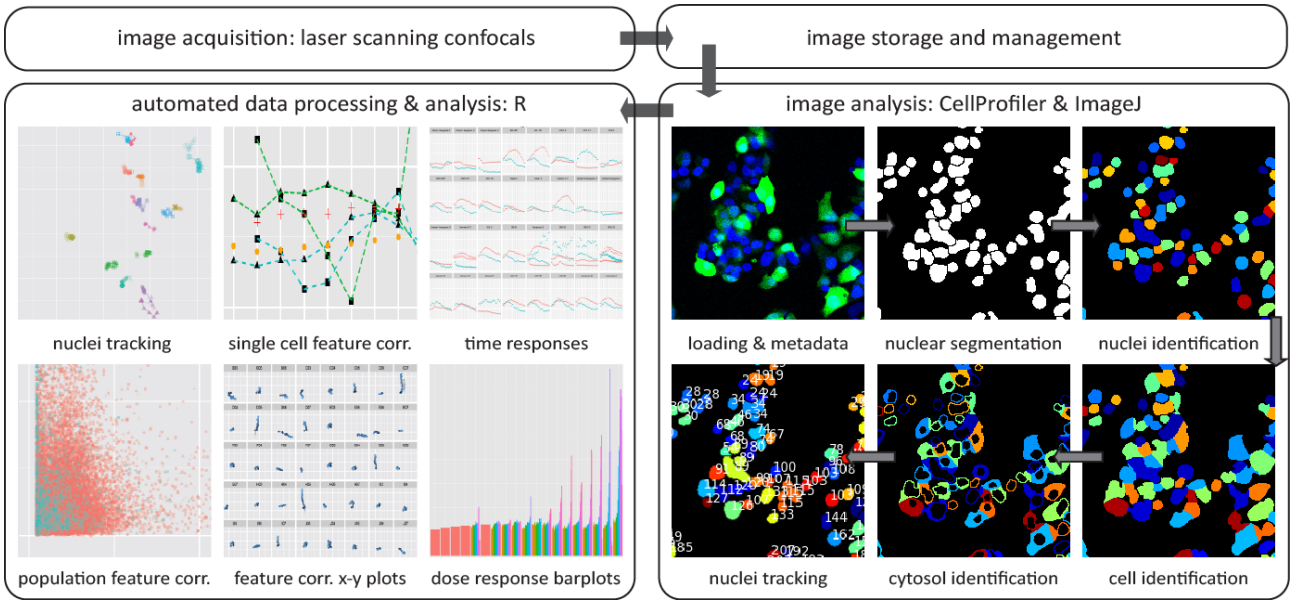


Figure 5: **Pipeline of high content imaging of BAC-GFP reporter cell lines.** Image acquisition by laser scanning confocal microscopes is followed by storage in a central data storage utility including a database management system. Image analysis is performed using CellProfiler and integrated ImageJ plugins. Raw images are loaded and metadata (*i.e.* well locations) is defined. After image processing (*e.g.* noise filtering) the nuclei are segmented using the nuclei-stained channel followed by single nuclei identification to enable analysis of population distributions. These identified nuclei are used as seeds to detect the cell-boundaries using the GFP channel. Further image objects can be defined (*e.g.* foci, cytoplasm, and organelles). Single-cell tracking is usually included to enable single-cell based analysis over time. Image analysis output (*i.e.* quantitative data) is stored in HDF5 files. R is used to interact with the data in HDF5 in an automated manner; several summary statistical output text files are generated and in addition a collection of plots to investigate the quantitative data are generated. Legend: Nuclei tracking: for quality control purposes of the tracking performance, the x & y-axis represent the x and y coordinates in the original images; single cell feature corr.: single cells are followed in time(x-axis) for two selected measurements(y-axis), the two selected measurements are for example cell speed and cytosolic intensity of the reporter cell lines; time responses: the selected percentile of all the single-cell measurements(y-axis) in each well in the multi-well plate are plotted over time(x-axis). Population feature corr.: two selected measurements(x & y-axis) can be compared on a single cell basis on the entire plate – for identifying dependencies (*e.g.* cell density and cytosolic intensity measurement); feature corr. x-y plots: linear regression analysis for two selected measurements for each condition in the multi-well plate to identify correlations; dose response barplots: area under the time-curve summary statistic of a selected measurement(y-axis) for each condition(x-axis) in the multi-well plate with increasing concentration(sub parts x-axis).

8.1. Compound screening to assess chemically-induced cellular stress response activation.

The application of the above described and proposed reporter systems can be positioned in mechanistic toxicology, early pre-clinical drug discovery for compound classification or compound ranking. Alternatively they can be applied in more large compound screening campaigns to build reference databases for future compound classification or as part of for example large consortia such as ToxCast[198] to complement the current set of HT assays. Such large compound screens could then be integrated with QSAR analysis. We have so far evaluated the feasibility of such an approach by testing the effect of the Spectrum Collection compound library including 2,350 FDA

approved drugs and active natural product compounds on the modulation of the Srxn1-GFP reporter in HepG2 cells. HepG2-Srxn1-GFP cells were exposed to individual drugs at 10 µM and then treated with a specific activator of the Keap1/Nrf2 route, CDDO-Me,[199] and then fixed after 7 hr or left untreated and then fixed after 24 hr. We quantified the effect of all compounds Srxn1-GFP activation and determined the Z-scores to rank all compounds compared to control conditions. We observed compounds that strongly enhance the Nrf2/Srxn1 response induced by CDDO-Me, which interestingly contained colchicine, vincristine and vinblastine that all effect microtubule polymerization as a common pharmacological effect (Fig. 6). In addition, we observed compounds that inhibit the CDDO-Me-mediated GFP-Srxn1 upregulation, which also included compounds from the same pharmacological class that inhibit Na+/K+-ATPase ion channels. Such reference compound data allow the identification of off-target effect related to modulation of adaptive stress response pathways. Also QSAR analysis of such a dataset may allow the identification of toxicophores that affect the Nrf2/Srxn1 response.

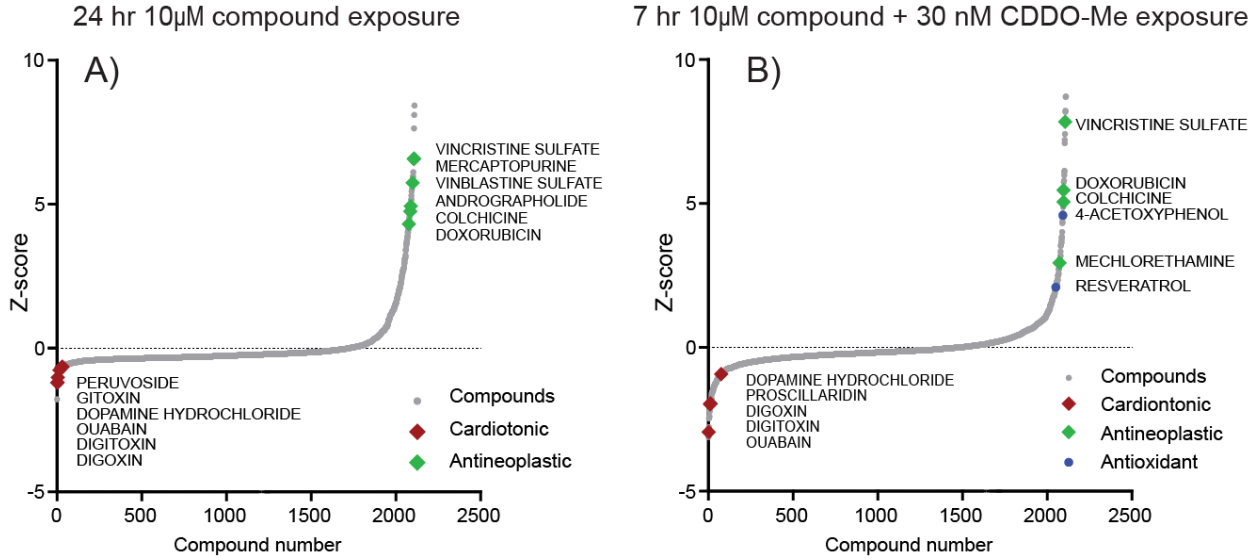


Figure 6: **Compound screen for Nrf2-mediated Srxn1 induction.** To monitor NRF2 activity upon drug exposure, BAC Srxn1-GFP HepG2 cells were used. Imaging was performed three days after cell seeding in 96-well µ-clear imaging plates, Spectrum library compounds were transferred by automated pipetting to a final concentration of 10 µM in 6 replicate plates. A) Three plates were incubated for 24hr, fixed and imaged by automated confocal microscopy. B) Three other plates were co-exposed to 30 nM CDDO-Me, a potent Nrf2 activator, and fixed after 7h incubation; this set of plates allowed us to identify compounds that inhibit or enhance the Nrf2 response. The average cellular Srxn1-GFP intensity was determined by ImageJ-based image analysis. The Z-score was calculated based on the population average. Per well, on average 5000 cells were quantified. If this number was less than 500, a compound was marked as severe toxic and excluded from the analysis. For a detailed description of the screen see supporting information 2.

8.2. Effects of chemical exposure on the dynamics of cellular signalling.

A good example of a highly dynamic adaptive signalling response is the nuclear translocation the transcription of factor NF-κB after inflammatory cytokine signalling. This is highly relevant in DILI as already described above and therefore monitoring the effect of chemicals on this oscillatory response is highly relevant. We have previously demonstrated such an effect for the drug diclofenac, a widely used non-steroidal anti-inflammatory drug, which causes hospitalization upon

liver failure in 23/100000 users according to a prospective study among arthritis patients.[200] Diclofenac is hepatotoxic in animal models at very high doses,[201] yet a combination of very low

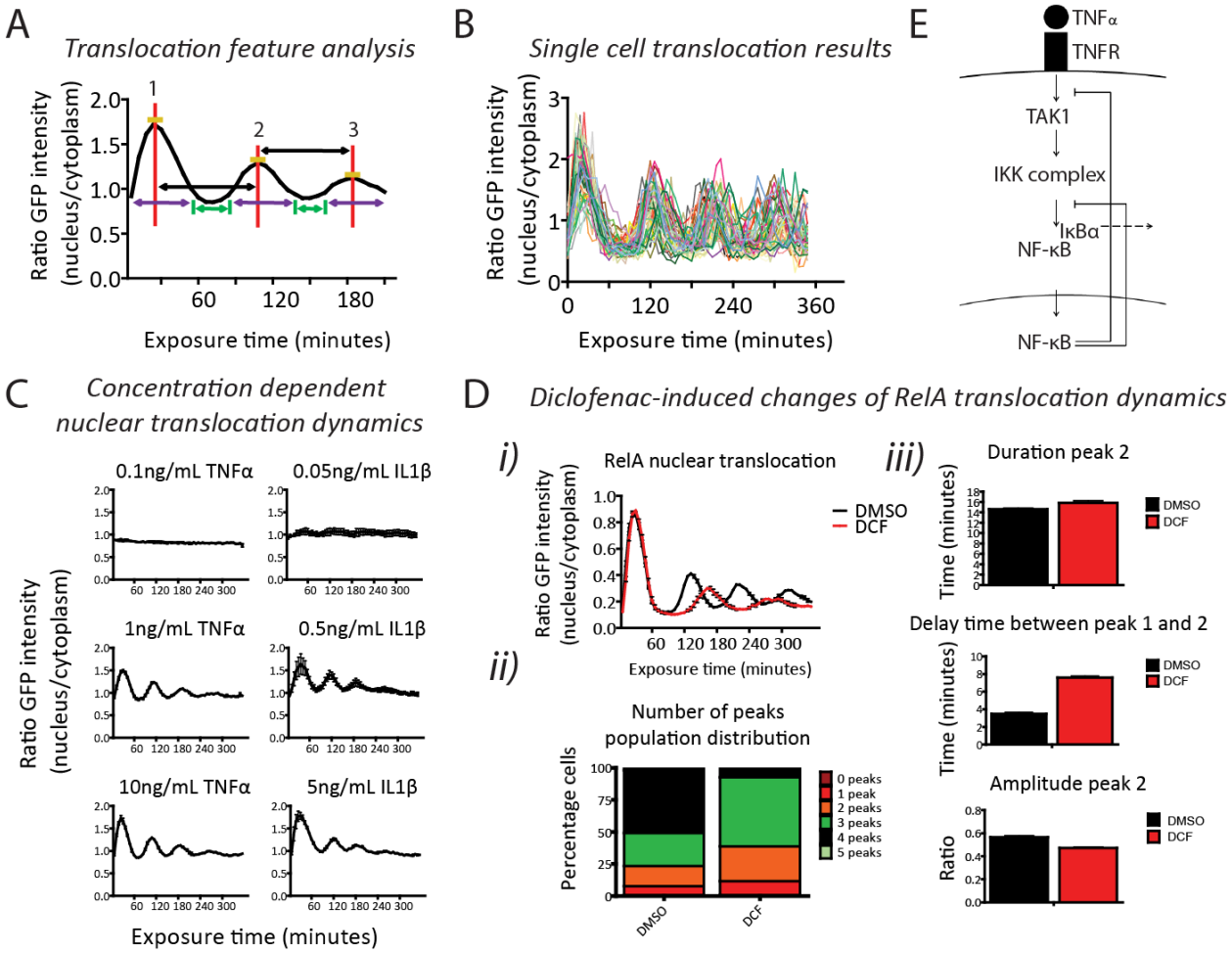


Figure 7: **Multiparametric analysis of BAC RelA-GFP HepG2 cells for nuclear translocation. A)** Characterization of nuclear oscillation features. Per single cell the time of the peaks, the duration of the peaks, the delay time between peaks, the time between peaks, the peak amplitude and the slopes of nuclear entry and exit kinetics. **B)** Single cell nuclear oscillation plots of cells with 4 nuclear translocation events**. C)** Nuclear translocation dynamics of RelA-GFP under a concentration range of TNFα and IL1β. **D i)** Normalized RelA-GFP nuclear translocation upon 10ng/mL TNFα stimulation. Cells were pre-incubated under 2% DMSO control or 500 µM diclofenac (DCF) conditions. **ii)** Population distribution of the number of peaks under 2% DMSO control or 500 µM diclofenac (DCF) conditions. **iii)** Nuclear oscillation features of cells under 2% DMSO control or 500 µM diclofenac (DCF) conditions. Duration of peak 2, the delay time between peak 1 and 2 and the amplitude of peak 2 are displayed**. E)** Schematic overview of NF-κB signalling pathway including feedback loops. For a detailed description of the experiment see supporting information 3.

doses of diclofenac and LPS exposure in rats leads to the exposure of DAMPs and pro-inflammatory cytokines IL1β, TNFα, and CINC1.[147] Furthermore, in vitro assays showed that in HepG2 cells TNFα stimulation and in primary human hepatocytes a mixture of TNFα, IL1α, IL6 and LPS leads to synergistically induced apoptosis or cell death, respectively.[38, 146] To assess this interaction further we established a high content analysis assay in HepG2 cells to measure the activation status of NF-κB induced by TNFα. For this we generated BAC-GFP-RelA HepG2 cells. TNFα exposure results in a concentration dependent nuclear translocation of the NF-κB complex

containing the GFP-RelA subunit (Fig. 7). NF-κB transcriptional activity leads to IkBα expression and subsequent shuttling of NF-κB back into the cytoplasm. Continuous TNFα stimulation however stimulates NF-κB to a second nuclear translocation, characterized by the typical nuclear oscillation pattern. Importantly, diclofenac inhibits the oscillatory response. Multiparametric analysis of NF-κB oscillation at single cell level allows the identification of different parameters, accurately describing the cell- population distributions of the nuclear translocation responses and the effect of diclofenac (Fig. 7). Our current data fit the mathematical models of feedback mechanisms that control NF-κB activity.[202] High throughput microscopy will now allow us to integrate this quantitative single cell NF-κB oscillation data in mathematical models to predict the mechanism by which chemicals interfere with the NF-κB signalling pathways and thereby suppress overall survival signalling. Indeed, depletion of NF-κB in HepG2 cells sensitizes cells towards diclofenac-induced cell death [38].

8.3. RNA interference to unravel signalling networks that control cellular stress responses.

Above we described the regulatory mechanisms by which various physiological adaptive stress response pathways are controlled. This is based on the current scientific knowledge and may not per se establish how chemicals affect these pathways. Chemical cell injury is associated with extensive activation of various protein kinases and ubiquitinases that mediate post-translational modification of various proteins and thereby affect their activity. This may impinge as well on the modulation of these adaptation programs. Hence understanding the entire complexity of the signalling networks that drive adverse drug reactions requires functional genomics RNA interference-based approaches as well. The integration of RNA interference with HCI of the GFP-reporters is a powerful approach. We first addressed this concept to assess the role of alternative mechanisms by which the Keap1/Nrf2 pathway is controlled. Indeed alternative mechanisms of Nrf2 regulation have been reported: *e.g.* binding of selective substrate for autophagy p62 to the KIR motif on Keap1 leads to activation of Nrf2 [203] or a Keap1 independent degradation of Nrf2 possibly by phosphorylation of specific Nrf2 serine sites, *e.g.* by glycogen synthase kinase 3 (GSK-3) and protein kinase C (PKC).[204]'[205] We sought to identify novel candidate signalling molecules that control the activation of Srxn1-GFP expression by a specific activator of Keap1, CDDO-Me. As a first step we evaluated the role of epigenetic modifiers (~150 target genes) and performed knockdown of individual genes by Dharmacon siRNA smartpool mixes followed by CDDO-Me treatment for 7 hr. Nrf2 knock down as a positive control blocked CDDO-Me-induced Srxn1 expression as expected, while Keap1 knockdown enhanced the response. Interestingly, SMARCA2 was found to almost fully block the adaptive stress response program, while LRCH4 enhanced the response (Fig. 8). These results exemplify that RNAi screens can further contribute our understanding which signalling networks control pathways of toxicity. Genetic polymorphism in candidate regulators may determine the amplitude of cellular adaptive stress response programs and thereby the susceptibility to particular adverse drug reactions. Much work will be required in this area.

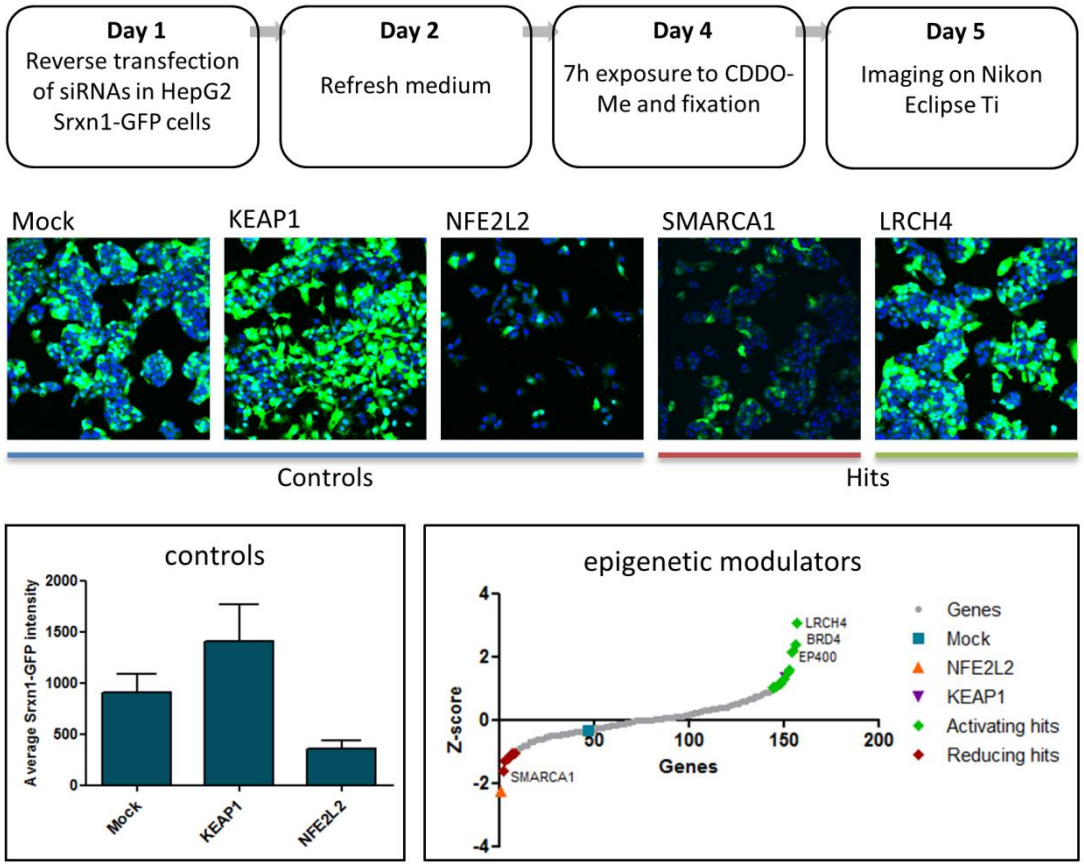


Figure 8: **siRNA screen for Nrf2-mediated Srxn1 induction.** To monitor the epigenetic modifiers that modulate Nrf2 activity we performed a siRNA screen of in total ~150 candidate genes in BAC Srxn1-GFP HepG2 cells. Cells were transfected with siRNA for 72 hr followed by exposure to CDDO-Me for 7 hr. Then cells were fixed and imaged on a Nikon confocal microscope and image analysis of Srxn1-GFP expression in individual cells using Image-J. Z-score was determined for the effect of individual genes. Note that SMARCA2 knock down inhibits Srxn1 expression while LRCH4 is enhanced Srxn1 expression. NFE2L2 (Nrf2) and Keap1 were used as controls. For a detailed description of the experiment see supporting information 4.

9. Future perspectives.

In this review we described the approaches and advantages to apply high content imaging to monitor the dynamics of adaptive stress response pathways that are critical in toxicity in relation to compound screening, mechanistic RNAi studies and dynamic modeling of such responses. So far these studies are based on the analysis of 2D cell culture of HepG2 cells. While such systems are likely fit for purpose for various applications, these cells contain limited reminiscence with human hepatocytes in the *in vivo* situation and have for example limited xenobiotic metabolism capacity.[206, 207] Nevertheless, there are improved methods to culture HepG2 cells in 3D cultures which will improve their differentiation and increase their metabolic capacity.[208] When such 3D cultures, in combination with other relevant liver cells, are applied to the HepG2 reporter cell lines, we may well get better prediction of liver toxicity. Automated live cell imaging of 3D cultures requires fast confocal imaging approaches and is rather in its infancy.

Alternatively, for improved modeling of the stress responses in relation to toxic outcome, the above reporter assays could be combined with alternative fluorescent probes that detect various biochemical parameters such as mitochondrial membrane potential, ROS or cell death.

This will then allow to more closely dissect the relationship between levels of stress activation and ultimate onset of cytotoxic events and help to assess the safety window of stress response activation.

An ultimate goal for an efficient monitoring of stress response signalling would be to integrate different reporters in one cell system, by labeling the different reporters with different fluorescent proteins. This would then allow the evaluation of the relationship between activation of different stress responses at the cellular level, and again to determine the maximal levels of stress responses in relation to toxic outcome. Alternatively, different cell reporters that contain different colors could be mixed into a 'rainbow' cell stress reporter platform that would capture the different stress response in one well, yet not in the same cells.

Certainly HepG2 may on the long run not be the optimal cell model and stem cell technology seems to be the future. While current differentiation protocols are at the most feasible to generate hepatocyte-like cells from either human pluripotent stem cells or induced-pluripotent stem cells,[209] it will be of high relevance to generate iPS cells with critical stress response markers. These can then be differentiated in hepatocyte-like cells and used in high content imaging approaches.

How can we eventually integrate these models in compound screening? As mentioned, these HepG2 reporter systems could be a highly valuable tool in large screening efforts including the ToxCast and Tox21[210] efforts. In particular the single cell analysis of stress responses as well as the evaluation of dynamic responses using live cell imaging will be a major asset to better dissect the principal mode of action of chemicals. Since these reporter systems allow fixation, large compound screens are feasible. On a more limited scale these reporter systems can be used in a pre-clinical drug development program to classify smaller compound library for lead prioritization: *i.e.* compounds that in a concentration escalation response provide minimal activation of various stress responses are likely to have also the least interference with the normal cellular physiological homeostasis and hence a reduced liability for adverse drug reactions.

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Supporting information 1

[Movies of several BAC-reporter cell lines have been uploaded. The DNA-damage response BAC-reporters 53BP1-GFP, p53-GFP & p21-GFP were exposed to 25µM Etoposide. The UPR BAC-reporters ATF4-GFP, XBP1-GFP, BiP-GFP and Chop-GFP were exposed to 10 µM tunicamycin. The oxidative stress BAC-reporters Nrf2-GFP, Keap1-GFP and Srxn1-GFP were exposed to 10µM iodoacetamide. The inflammatory BAC-reporters RELA-GFP, ICAM1-GFP and IKBalpha-GFP were exposed to 10 ng/ml TNFα. All movies are over a time course of 24 hours with equidistant time intervals between consecutives frames]. This material is available free of charge via the Internet at http://pubs.acs.org.

Supporting information 2

[Figure 6: Human hepatoma HepG2 cells were obtained from American Type Culture Collection (clone HB-8065, ATCC, Wesel, Germany). HepG2 cells stably expressing Srxn1-GFP were created by 500ug/mL G418 selection upon transfection of GFP tagged Srxn1 cloned into bacterial artificial chromosome (BAC) construct, using LipofectamineTM 2000 (Invitrogen, Breda, Netherlands). For all experiments the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 25 U/mL penicillin and 25 µg/mL streptomycin between passages 5 and 20. The screening was performed on µClear Greiner 96 well plates on a Nikon TI eclipse A1 MP confocal microscope. Spectrum library compounds were transferred by automated pipetting to a final concentration of 10 µM in 6 replicate plates**.** Three of these plates were incubated for 24hr, fixed and imaged by automated confocal microscopy. The three remaining plates were co-exposed to 30 nM CDDO-Me, a potent Nrf2 activator, and fixed after 7h incubation. The spectrum library 2320 was obtained from Microsource Discovery Systems, Gaylordsville. This collection consists of 2320 compounds of which 60% drug components, 25% natural products and 15% other bioactive components. The compounds are dissolved in DMSO at 10 mM and diluted to 10 µM final concentration (0.1% DMSO)]. This material is available free of charge via the Internet at http://pubs.acs.org.

Supporting information 3

[Figure 7: Human hepatoma HepG2 cells were obtained from American Type Culture Collection (clone HB-8065, ATCC, Wesel, Germany). HepG2 cells stably expressing RelA-GFP were created by 500ug/mL G418 selection upon transfection of GFP tagged RelA cloned into bacterial artificial chromosome (BAC) construct, using LipofectamineTM 2000 (Invitrogen, Breda, Netherlands). For all experiments the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 25 U/mL penicillin and 25 µg/mL streptomycin between passages 5 and 20.

Prior to imaging, nuclei were stained with 100ng/mL Hoechst 33342 in complete DMEM for 45 minutes. Next, cells were exposed to 500µM Diclofenac (Sigma-Aldrich, Zwijndrecht, The Netherlands) or 0.2% DMSO for 8 hours. Then, the cells were stimulated with the indicated amount of human TNFα (R&D Systems, Abingdon, UK). Nuclear translocation was followed for 6 hours by automated confocal imaging every 6 minutes (Nikon TiE2000, Nikon, Amstelveen, Netherlands). Quantification of the nuclear/cytoplasmic ratio of GFP-RelA intensity in individual cells was performed using an algorithm for imageJ]. This material is available free of charge via the Internet at http://pubs.acs.org.

Supporting information 4

[Figure 8: Human hepatoma HepG2 cells were obtained from American Type Culture Collection (clone HB-8065, ATCC, Wesel, Germany). HepG2 cells stably expressing Srxn1-GFP were created by 500ug/mL G418 selection upon transfection of GFP tagged Srxn1 cloned into bacterial artificial chromosome (BAC) construct, using LipofectamineTM 2000 (Invitrogen, Breda, Netherlands). For all experiments the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 25 U/mL penicillin and 25 µg/mL streptomycin between passages 5 and 20. Knock down of a certain gene was done by performing reverse transfection. For the transfection mix, pooled siRNAs (50nM) where mixed with INTERFERin (0.3% end dilution, Polyplus, Leusden, Netherlands) and serum free medium. The used siRNAs were siNFE2L2, siKEAP1 and the libraries kinases, ubiquitinases, deubiquitinases, epigenetic modulators, transcription factors and toll-like receptors which were all derived from Dharmacon (Lafayette Colorado, USA). As control, Mock was used in which no siRNA was added to the transfection mix. Medium was refreshed 24 hours after transfection or 100 µL medium was additionally added 7 hours after reverse transfection]. This material is available free of charge via the Internet at http://pubs.acs.org.