Food for Thought...

Introduction

Resilience is the ability of a system (here, a cell) to cope with negative change. The concept has been used in many areas from ecology to material sciences, engineering and disaster research. Resilience can be seen as the opposite of vulnerability, though views differ dependent on the area (Linkov et al., 2014). In toxicology (especially *in vitro* toxicology), however, the term and the concept are not well developed. Cells, organs and organisms and their vulnerability are dependent on their capacity to cope with (disastrous) changes, i.e., exposure to a toxicant. Disaster research has been moving away from preparing for each and every possible hit toward a concept of resilience, especially involving critical infrastructures[1] (di Mauro et al., 2010). For example, one of the critical infrastructures of *in vitro* toxicology are mitochondria, an Achilles' heel of cells, where oxidative stress occurs in response to many hazards, triggering apoptosis by cytochrome C release. Given the endosymbiotic theory on the bacterial origin of mitochondria (Wallin, 1923), this could be interpreted as the late manifestation of a chronic infection of the cell.

It is tempting to develop testing strategies for hazardous substances based not on the apical manifestations but on the critical infrastructures that trigger the problem. This might be PRUHHIÀFLHQWWKDQGHQWLFLQJWKHPDQ\SRVVLEOHQWHU of substances (now called molecular initiating events (MIE) in the context of Adverse Outcome Pathways (AOP)) or characterizing the entire Pathway of Toxicity (PoT, Kleensang et al., 2014). We can interpret these critical infrastructures as the nodes of the PoT networks, which would lend themselves as biomarkers of toxicity (Blaauboer et al., 2012).

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Box 1: Definitions of specific terms used

Apoptosis
A highly regulated, genetically determined form of programmed cell death involving cell self-destruction and DNA fragmentation

Anastasis
Cellular reversal to life from late-stage apoptosis (Tang et al., 2015)

Cellular resilience
The ability of a system (here cells) to cope with perturbation and recover. In this case, it is the cell's capacity to withhold and recover from toxicant exposure with the possibility of developing tolerance to the next hit. Synonyms used on organism level are: recovery, tolerance, and adaptation to a new environment. The concept of 'resilience' should not be confused with adaptive (as opposed to 'adverse') responses of cells. Such adaptive responses may, e.g., be triggered at low, non-harmful toxicant concentrations, without leading to a change of cells or to resilience. They may also be triggered in parallel to adverse responses (PoT), but be insufficient to trigger resilience. The terms adaptive/adverse are mainly used for omics studies that allow the measurement of multiple cellular changes, but where it is difficult to determine which ones are adverse (i.e., are constituents of a PoT). Resilience is rather a physiological concept.

Epigenetic scar
Long-term changes in epigenetics induced by stressors, which affect regulation of gene expression. Synonym: Epigenetic memory

Homeostasis
The tendency toward a relatively stable equilibrium between interdependent elements, especially as maintained by physiological processes

Hormesis
A term used by toxicologists to refer to a biphasic dose response to an environmental agent characterized by a low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect (Mattson, 2008)

Pathway of Toxicity
A molecular definition of the cellular processes shown to mediate adverse outcomes of toxicants (Kleensang et al., 2014)

Pathway of Defense
A molecular definition of the cellular processes shown to mediate defense against adverse outcomes of toxicants, analogous to a Pathway of Toxicity

Vulnerability
The state of being open to injury

Disaster research aims to map and monitor critical infrastructures to identify services deemed vital for the functioning of society. The etymological root of "critical" is linked to the term "crisis," which refers to a change in the state of a system implying a time of great difficulty or dan[illegible] logical counterpart to identifying the critical infrastructure is to characterize the vulnerability that directly corresponds to resilience, i.e., the ability to cope with a possible hit. The probability of a hit determines the risk a[illegible]sess both for societies and, in our case, toxicology. We can only say how often something has been hit in the past, i.e., the prevalence of certain modes of action of substances. But, there can always be surprises, such as the so-called "black swans" Taleb. Black swan events are defined by the "*triplet: rarity, extreme impact, and retrospective (though not prospective) predictability*." Thalidomide, for example, was a toxicological black swan.

This article explores the resilience component of toxic action at the cellular level (Fig. 1). On an organism level, this is typically measured as recovery and reversibility and plays an important role in classification and labelling of substances. The scope of most studies does not include resilience at the cellular level, likely because of the emphasis on studying short-term effects, which puts the emphasis on cytotoxic actions of substances. This is, however, of limited relevance for most hazard manifestations, except for acute, high-dose intoxications.

The second part of this article goes one step further, suggesting that resilience is not just about the cell going back to "normal," but how the insult changes the cell and imprints on its future functionality and responses. The wounds leave a systemic memory effect, figuratively speaking a scar, which can be maintained among others by epigenetic mechanisms or mutations. A resilient cell is not necessarily a healthy cell; for example, it could be cancerous and very resilient towards chemotherapy. Some of the best examples for resilience are found in the field of chemotherapy. Some tumor cells develop a [illegible] resilience and become resistant to drugs despite being exposed to the same concentrations as their neighboring cells.

Such changes can be long-term, or even permanent; cel[illegible] can be beneficial and we will discuss [illegible] lar hormesis in this context. On the one hand, the concept of beneficial effects is more developed in biomedical r[illegible] particularly with respect to ischemia-reperfusion as a stressor to organs, and so called "pre-conditioning" (i.e., making cells more resilient to subsequent stress) is used experimentally and clinically (Wang et al., 2015; Clapp et al., 2012; Wu et al., 2012; Yellon and Hausenloy, 2005; Dunn et al., 2012; O'Neill et al., 2012). Tolerance is a similar concept, where small doses of a toxicant (e.g., the famous arsenic eaters of Styria, Heisch, 1860) or toxin (e.g., endotoxin, Lehner and Hartung, 2002) protects against subsequent stronger hits. These concepts from in vivo can be, to some extent, traced back to cellular changes (Hartung and Wendel, 1992). On the other hand, long-term effects can also be detrimental and lead to adverse outcomes. This will be critical for understanding late manifestations, changed susceptibilities and mixture toxicities, especially when exposure is of limited duration. The resulting "late consequences of early life stress," also termed the "Barker hypothesis" (Hales and Barker, 1992), have become a major theme in epidemiological research, public health and mechanistic research (McGowan et al., 2009; Suderman et al., 2012; Yehuda et al., 2015; Sebert et al., 2011; Lindblom et al., 2015; Aiastalo et al., 2013; Lau and Rogers, 2004).

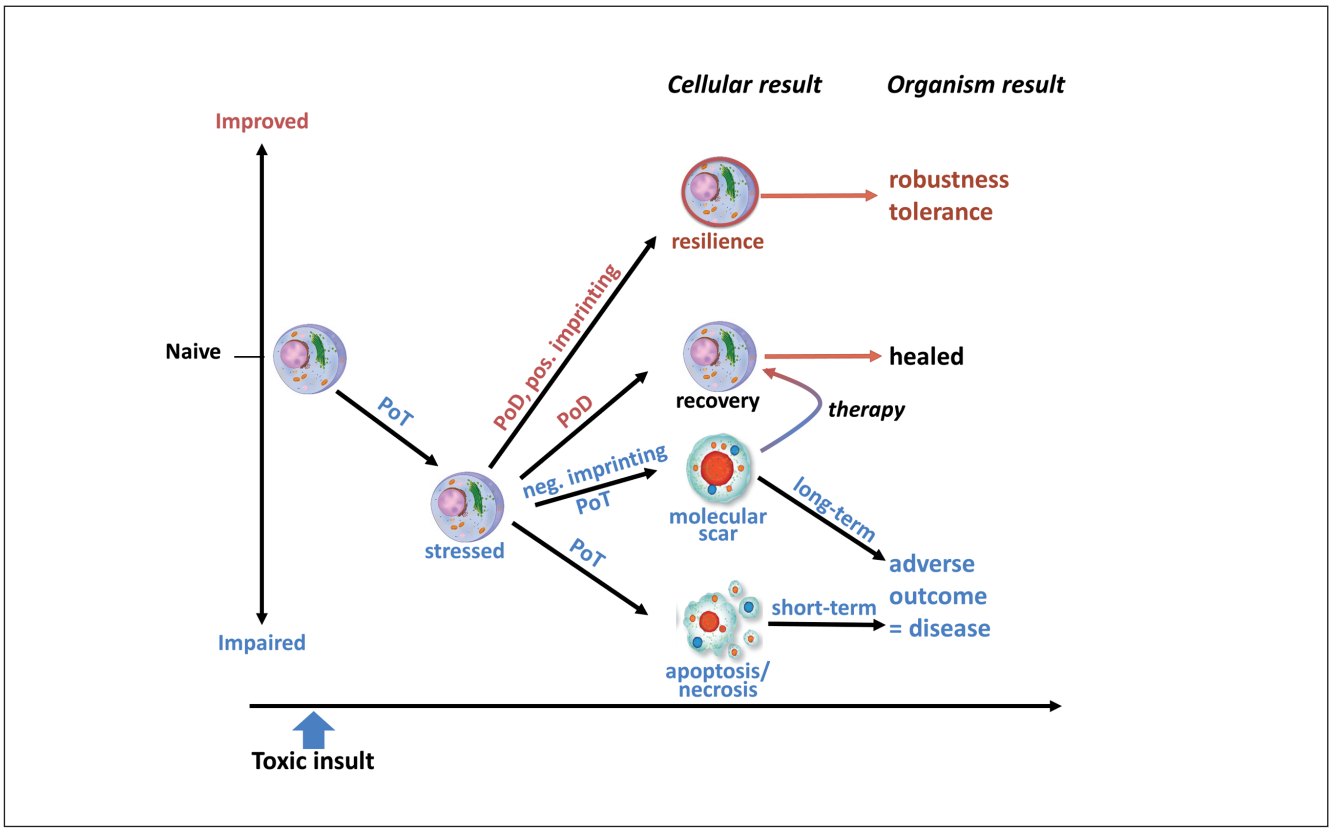

Fig. 1: The Cellular Resilience Concept

Survivable toxic insults create cellular stress; Pathways of Defense (PoD) might allow cells to return to a normal state; imprinting programs, however, often leave cells in an altered state, e.g., with an epigenetic scar, which may contribute to long-term manifestations of hazard (but could also be a target for therapeutic strategies) or improved resistance against future hits. So-called "adaptive responses" circumscribe all changes of cellular parameters that are not directly linked to short-term adverse outcomes. They can involve PoD as well as different imprinting reactions. The concept of resilience is a clearly distinct concept, describing a small spectrum of cellular responses that will normally result in improved stress management.

Consideration 1: It is not important whether you fall, but whether you get up again

This is not only true for the boxer, but for each and every hit

The *in vitro* toxicological literature is thin with respect to such questions at a cellular level. Some aspects were addressed in recent EU projects such as SEURAT-1, ESNATS, Predictomics, etc., but their focus was still largely on the initial damage

sponse, as cancer cells have evolved a number of strategies to

therapy. These involve upregulation of anti-apoptotic proteins

Hansson et al., 2003; Hanahan and Weinberg, 2011). The design of toxicological studies at the organism level, however, addresses such questions very well. Morphological changes in the target organ, as well as behavioral abnormalities, often are addressed immediately after exposure as well as after a recovery period. Similar design of toxicological tests at molecular and cellular levels provides a major advantage in understanding molecular mechanisms of organ/organism recovery and

normal, and this requires sensing and counter-regulations. A number of cellular stress responses have been described (rearrangements in energy metabolism, oxidative stress response, activation of anti-apoptotic pathways and DNA repair mecha-

their actual contributions to reestablishing homeos-

clear. These stress response pathways (SRP) include hypoxia signaling via HIF-1, the heat shock response

antioxidant response via NRF-2, stress kinase signaling via JNK and AP-1, DNA damage responses via p21 or BSCL2, and the unfolded protein response/amino acid starvation response via ATF-4/ATF-6 (Limonciel et al., 2015; Jennings, 2013; Wink et al., 2014; Hendriks et al., 2012). Earlier in this series we discussed homeostasis under stress (Hartung

et al., 2012), which is what we often measure when characterizing toxic signatures by omics technologies. The restoration process that occurs when removing the stressor, however, is addressed less frequently.

We hypothesize that these are actually the processes that determine long-term manifestations of hazard or recovery. Most toxicants are encountered at doses far below cytotoxicity but at levels high enough to affect biology. This understanding of perturbation and restoration should drive our analysis of pathogenesis and reversibility.

Consideration 2: Anastasis – awaken from the dead

Quite surprisingly, cellular suicide attempts can be stopped. The term "anastasis" (Greek for "rising to life") has recently been coined (Tang et al., 2012, 2015). The group observed:

> "... *Unexpected reversal of late-stage apoptosis in primary liver and heart cells, macrophages, NIH 3T3 fibroblasts, cervical cancer HeLa cells, and brain cells. After exposure to an inducer of apoptosis, cells exhibited multiple morphological and biochemical hallmarks of late-stage apoptosis, including mitochondrial fragmentation, caspase-3 activation, and DNA damage. Surprisingly, the vast majority of dying cells arrested the apoptotic process and recovered when the inducer was washed away. Of importance, some cells acquired permanent genetic changes and underwent oncogenic transformation at a higher frequency than controls. Global gene expression analysis identified a molecular signature of the reversal process.*"

Transcriptional responses were found to be critical for this reversal, and inhibition of classical survival genes BCL-2, XIAP, 00RU+63 VLJQLÀDQWO\VXSSUHVVHG UHYHUVDO RI DSRSWR VLV7KRXJKWKLVPD\VHHPDQLVRODWHGÀQGLQJWKHUHDUHIUHTXHQW reports in the literature that cells can survive apparently lethal damage, such as rupture of the plasma membrane (Roostalu und 6WUlKOH -DLVZDO HW DO UHOHDVH RI F\WRFKURPH & to the cytoplasm (Potts et al., 2003; Deshmukh and Johnson, 1998), membrane blebbing (Foghsgaard et al., 2001) or cas-SDVHDFWLYDWLRQ/HLVWDQG-llWWHOl,WQHHGVWREHIXUWKHU FODULÀHG ZKHWKHU VXFK FHOO FXOWXUH REVHUYDWLRQV DUH HTXDOO\ in *vivo* and whether such cells would be removed by phagocytosis EHIRUH WKH\FDQ UHFRYHU /HLVW DQG -llWWHOl +LUW HW DO 2000; Hirt and Leist, 2003), but at least in *Drosophila*, transient caspase activation has been documented in cells that were not removed (Tang et al., 2015).

So, even after the most extreme impact, programmed cell death, when initiated, is reversible to a considerable extent. Reversibility, however, may not return the cell exactly to the ground state but to altered cellular states, for instance related to senescence (Jurk et al., 2012) or involving permanent DNA damage (Ono et al., 2003; Vijg et al., 1997; Tang et al., 2012).

Consideration 3: All cells are equal(ly vulnerable)

Astonishingly, cells are very similar in their susceptibility to toxicants at the level of cytotoxicity, as was demonstrated by several studies where different cell types have shown comparable responses to the toxicants regardless of the tissue of origin, and VLJQLÀDQWFRUUHODWLRQEHWZHHQ *in vitro* and IC$_{50}$ *in vivo*: *LOOLÀDOOHZDVOLNHOWKHÀUVWWRQRWLFHWKDWGLIIHUHQWFH* display cytotoxicity to a given chemical at very similar concentrations. He started the Halle register, a large manual collection of IC$_{50}$ FRQFHQWUDWLRQVIURPSXEOLVKHGFHOOH[SHULPHQWV reported in 1988 (Halle and Goeres, 1988) and later translated and published by ECVAM (Halle, 2003). The principal idea of this work was to use the geometric mean of the collected IC$_{50}$ values (in mmol/l medium) and the corresponding acute oral LD$_{50}$ for rats or mice (in mmol/kg) to calculate a simple linear regression model. There was clearly a positive correlation, though this was not good enough to predict LD$_{50}$ values in later validation attempts (NIH, 2006), or even the then-recommended prediction of start doses for LD$_{50}$ testing (Schrage et al., 2011). It is quite remarkable, still, that this approach works to some extent, especially for the prediction of substances that are not acutely toxic, for which it is now recommended by ECVAM[2] (Prieto et al., 2013). Halle concluded (2003):

> "*The results of linear regression analysis showed that the biostatistical parameters obtained with IC$_{50}$/LD$_{50}$ values for xenobiotics taken from various publications ... and from the US National Institute for Occupational Safety and Health's Registry of Toxic Effects of Chemicals (NIOSH RTECS) are comparable within a certain range, despite the fact that the various laboratories used different cell types, Standard Operating Procedures (SOPs), and cytotoxic endpoints.*"

Here, especially, the aspect that a mean of different cytotoxicity assays can serve as a value characterizing the toxicity of a substance is of interest.

The next similar attempt was the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) program (Clemedson and Ekkwall, 1999), which showed a good correlation (around 70%) between *in vitro* basal cytotoxicity data and human lethal blood concentrations. In MEIC, 50 reference chemicals were tested in 61 *in vitro* assays (Ekwall, 1999). A principal component analysis indicated:

> "... *High general similarity (around 80%) of all the results from the 61 methods. According to the new 'random probe' analysis, this similarity must depend on the high correlation of results from assays with different cell types (mean R^2 0.81) and/or different viability endpoints (mean R^2 0.85). Main factors contributing to the 20% dissimilarity of results were different exposure times and the use of phylogenetically distant test objects in the non-analogous ecotoxicological assays (Clemedson and Ekwall, 1999).*"

[2] https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/files-3t3/ReqNo_JRC79556_lbna25946enn.pdf

To study the relevance of *in vitro* results, IC_{50} values were compared with human lethal blood concentrations (LCs) by linear regression. An average IC_{50} for the ten 24-hour human cell line tests predicted peak LCs better (R^2 0.74) than other groups of tests (Ekwall, 1999). This claimed predictivity formed the basis for the A-cute-Tox project (Clemedson, 2008). In this FP6 EU project, the correlation of *in vitro* cytotoxicity with animal LD_{50} data and human lethal blood concentrations was further evaluated, and clearly lower correlations were found. Many different cell lines were compared and found to give a similar correlation in IC_{50} values independent of the cell type used (Kinsner-Ovaskainen et al., 2013).

Recently, Lin and Will (2011):

> "*... Investigated the utility of hepatic-, cardiac-, and kidney-derived cell lines to (1) accurately predict cytotoxicity and (2) to accurately predict specific organ toxicities. We tested 273 hepatotoxic, 191 cardiotoxic, and 85 nephrotoxic compounds in HepG2 (hepatocellular carcinoma), H9c2 (embryonic myocardium), and NRK-52E (kidney proximal tubule) cells for their cytotoxicity ... The majority of compounds, regardless of their designated organ toxicities, had similar effects in all three cell lines. Only approximately 5% of compounds showed differential toxicity responses in the cell lines with no obvious correlation to the known* in vivo *organ toxicity.*"

Another study showed that neuronal cells do not react differently to neurotoxicants than non-neuronal cells (Stiegler et al., 2011). Differences in sensitivity to toxicants, however, have been reported for mouse embryonic stem cells differentiated into other lineages (Visan et al., 2012; Seiler and Spielmann, 2011), suggesting, that the developing system (differentiating cells) could be an exception and possibly linked to the fact that they are more vulnerable to toxicants than mature or undifferentiated cells. Another exception could be higher sensitivity of cells in S-phase of mitosis to drugs and toxicants broadly used in cancer therapy.

One reason for non-selectivity on the level of cytotoxicity testing is that the majority of chemicals are promiscuous with respect to toxicity targets, as observed in ToxCast[3], the US EPA high-throughput screening project, which states: "*... The majority of chemicals represented in the ToxCast phase I library likely act via nonselective interactions with cellular macromolecules*" (Thomas et al., 2013). The project continues: "*976 structurally and categorically diverse chemicals in the ToxCast library across 331 biological assays: a quarter of the 976 compounds tested showed no demonstrable activity (AC_{50}) in any of the assays ... specific or promiscuous activities ... a chemical affected 10 assays on average, ranging from 0 (274 chemicals) to 90 (1 chemical)*" (Sipes et al., 2013).

Taken together, these studies make a very strong case that different cells of the same species are similar with regard to cytotoxicity and do not explain organ-selectivity of toxicants. Obvious exceptions are the few compounds that show differential effects in fresh primary hepatocytes due to metabolic activation or deactivation not taking place in other cells. The limited predictivity of *in vitro* assays for animal toxicity in 28 day or longer-term studies (Thomas et al., 2012) means that another component is necessary to explain why a given substance targets [illegible] functional endpoints and activation of stress-response pathways at sub-cytotoxic concentrations. Unfortunately, not many studies have compared functional cellular endpoints at subcytotoxic concentrations in a high throughput manner so far. An analysis of the ToxCast dataset seems to be most promising. ToxCast does include eight cytotoxicity tests. It should be noted that the effective concentrations of different assays for the same chemical were very close: the concentration at which a substance was [illegible] where it activated 10% of the assays it was positive in, differed only by a factor less than three (Thomas et al., 2013); this shows that chemicals typically trigger many pathways at more or less the same toxicant concentration.

Consideration 4: Kinetics cannot explain all organ selectivities

Some toxicants, especially environmental chemicals, may have a promiscuous effect on many organs, but some are very target-[illegible] toxicokinetics, i.e., differences in absorption, distribution, metabolism and excretion (ADME) of chemicals across different body locations, create organ selectivity, as in:

- Topical (local) toxicities of skin, eye, lung, etc.
- [illegible] ics absorbed in the gut and in the liver
- Differences in metabolic activation, again especially known for the liver and kidney
- Biological barriers, such as the blood-brain barrier or the blood testes barrier or the placenta
- [illegible] paraquat (lungs), MPP+ (dopaminergic neurons)
- And others

If kinetic and ADME can be addressed *in vivo*, however, the combination of some rough pharmacokinetic modeling with *in vitro* cytotoxicity data is challenging and does not always improve *in vivo* hazard prediction from high-throughput *in vitro* toxicity assays. In fact, Wetmore et al. (2013) found that: "*Adjusting the* in vitro *assays for pharmacokinetics did not improve the ability to predict* in vivo *effects as either a discrete (yes or no) response or a low effect level (LEL) on a continuous dose scale*." This may again be due to the simple cytotoxicity assays being non-optimal starting points.

One example of organ selectivity not linked to pharmacokinetics is the selective toxicity of the neurotoxicant 1-methyl-4-phenylpyridinium (MPP+) to dopaminergic neurons of the

[3] http://www.epa.gov/comptox/toxcast/

nigrostriatal pathway (Efremova et al., 2015), in which the neighboring mesolimbic pathway is hardly affected. The different types of dopaminergic neurons seem to cope with this chemical insult in different ways.

Consideration 5: Are differences in cellular resilience responsible for organ selectivity of toxicants?

There are two common explanations why many chemicals show organ selectivity *in vivo* as discussed above: (1) the XQLTXHSUHVHQFHRIVSHFLÀWDUJHWVWUXFWXUHVORHDGLQJWRGLIIHU-ent susceptibilities and (2) differences in substance kinetics allowing concentrations of the substance or its toxic metabolite to reach higher levels in a certain part of the body. Differences in susceptibility of different cell types *in vitro*, however, as discussed, are often not very pronounced, but most cells used *in vitro* do not have the same phenotype as *in vivo*, especially ZLWKUHJDUGWRWKHVSHFLÀWDUJHWVRIWR[LFLWDQGPHWDEROLVP-metabolism (Coecke et al., 2006). Systemic levels of the toxicant can be the same and adjustment for tissue concentrations did not dramatically improve the *in vitro* to *in vivo* extrapolations. This does not belittle the role of kinetics in extrapolation from effective *in vitro* to corresponding *in vivo* dose (Basketter et al., 2012; Leist et al., 2014), but points out its incomplete explanation of the organ selectivity of substances. Therefore, we suggest a third alternative: perhaps it is less the susceptibility to a toxicant, but the ability to recover from its hit that makes the difference. The condensed hypothesis put forward is that all cells are equally vulnerable, but some are more resilient than others.

The concept of cellular resilience, the differing ability of cells to cope with damage, includes properties such as: the ability to mobilize alternative energy sources and other redirections of metabolic resources; the elasticity of the metabolic network; the synthesis of defensive molecules such as anti-oxidants and other stress response elements; as well as the induction of repair.

It is often assumed that the robustness of many complex systems is rooted in their redundancy, which for networks represents the existence of many alternative paths that can pre-VHUYHFRPPXQLFDWLRQDPRQJQRGHVVXFKDVPHWDEROLF-and regulatory gene networks), even if some nodes are absent. 5HNDDQG%DUDEDVLUHYLHZWKHVWDWHRIWKHDUWLQ-RIIFRPSOH[FRPPXQLFDWLRQQHWZRUNVDQGKLJKOLJKWVWKHQHHGIRU-that previous research attempting to address this issue in quantitative terms failed to uncover the degree in which redundancy plays a role. It is quite surprising that many gene knock-outs actually have no or little phenotype without inactivation of another gene or additional environmental stress (Melton, 1994; Barbaric et al., 2007), illustrating the biological robustness of the system. The rate of knock-outs without phenotype is diffÀXOWWRHVWLPDWHLQPLFHEHFDXVHQHJDWLYHGDWDDUHRIWHQ-published; in yeast, for example, the rate is approximately 40-60%. Often stresses to the system, such as infection, hypoxia, temperature changes or toxicity is required to show that responses are impaired. But do some cells have fewer redundanFLHVWKDQRWKHUV7KLVLVQRWFOHDUÀUHGXQGDQF\DQG-ness refer more to the initial set-up than to the difference in coping with the hit, this does not really further the argument. The question seems to be whether cells reach a tipping point before collapse (Scheffer et al., 2012) and whether this point is different for different cell types depending on their resilience programs.

Components contributing to cellular resilience likely include: the stress responses of the cell, which include repair enzymes; cell membrane repair (Steinhardt, 2005); the mechanisms to remove damaged proteins and other cellular trash; heat-shock proteins (Velichko et al., 2013); anti-apoptotic mechanisms %ULQNHWDOUHOHDVHGÀPPDWRUPHGLDWRUV)LQFKHW-al., 2010) and growth factors; damage limiting (e.g., anti-oxidative) components; the mobilization of additional energy, etc. Which raises the question: what happens in the cells after the hit but before they enter into necrotic or apoptotic cell death ZZRVWXGLHVUHODWLQJ3DUNLQVRQ'LVHDVH,QWKH-(Krug et al., 2014), dopaminergic neurons were exposed to the Parkinson's toxicant MPP+, the metabolite of the illicit drug (meperidine) contaminant 1-methyl-4-phenyl-tetrahydropyridine (MPTP). MPTP is not toxic itself, but owing to its high lipophilicity, it is able to cross the blood brain barrier, where it is metabolized in astrocytes by monoamine oxidase B (MOA-B) to MPP+, which is then transported selectively by the dopamine transporter into neurons where it inhibits the mitochondrial electron transport chain, ultimately leading to oxidative stress and apoptosis.

In this project (Krug et al., 2014), human dopaminergic neuronal cells (LUHMES) where exposed to MPP+ and were then analyzed using combined metabolomics and transcriptomics approaches to identify the earliest cellular adaptations to stress. When mitochondrial parameters were at control levels, strong transcriptome and metabolome changes, such as depletion of phosphocreatine and oxidative stress (e.g., methionine VXOIRLGHIRUPDWLRQZHUHREVHUYHG6WUHVVOXFRVHÀ-showed a complex pathway of toxicity. This included the interference of energy metabolism, ROS formation, ER stress, gene expression and ultimately led to mitochondrial cytochrome-C release and apoptosis. A strong increase of S-adenosyl-methionine (SAM) and early activation of the transsulfuration pathway with increased glutathione levels. Bioinformatic analysis ZRUNVDQGKLJKOLJKWHGWKHWUDQVFULSWLRQIDFWRU6)D-stream regulator of early responses. Findings on this signaling pathway and on adaptive increases of glutathione production ZHUHFRQÀUPHGELRFKHPLFDOO\0HWDEROLFDQGWUDQVFUL-SURÀOLQJFRQWULEXWHGFRQVLGHUDEO\WRIRUPDWLRQRI-primary and secondary changes that contribute to the cellu-ODUUHVSRQVHWR033)KDVDOVREHHQGHVFULEHGDVDNH-transcriptional factor in MPTP toxicity by others (Ye et al., 2013). This investigates how the cells struggle to survive before apoptosis sets in, representing a likely PoD in the resilience of these cells.

In the second project (Maertens et al., 2015), we analyzed microarray data derived from brains from MPTP treated mice (Miller et al., 2004) and carried out weighted gene correlation network analysis (WGCNA), supported by text mining, and other systems-level technologies to construct a genetic a regulatory network for MPTP toxicity. The paper was discussed in two guest editorials (Rahnenführer and Leist, 2015; Andersen et al., 2015). Several modules of connected genes, which overrepresented annotations for neurodegenerative diseases, ZHUH LGHQWLÀHG 7UDQVFULSWLRQ IDFWRU DQDOVLV LGHQWLÀHG 63 which is known to regulate the dopamine transporter (Wang and Bannon, 2005) and is involved in several neurodegenerative diseases, as key regulator (Qiu et al., 2006; Santpere et al., 2006). Interestingly, SP-1 was not detected as an important player using conventional statistical methods of gene expression analysis. In addition to SP-1, the network hubs consist of candidates well known for their role in Parkinson's disease 67$-8165(%) DOVR LGHQWLÀHG LQ WKLV VWXG KDV SUH-YLRXVO EHHQ LGHQWLÀHG DV D ULVN ORFXV IRU VSRUDGLF 3DUNLQVRQ disease and, in a recent RNAi screening study, was implicated in the control of the PTEN-induced kinase 1 (PINK1)/Parkin pathways that control the autophagic destruction of mitochondria (Ivatt and Whitworth, 2014). One hub, HDAC1, has been implicated in cell survival in neurotoxicity to dopaminergic neurons *in vitro* and ischemia *in vivo* (Kim et al., 2008), and is thus a candidate PoD. The protein LANCL1, also suggested by the WGCNA network, was connected to both HDAC1 and STAT3 and binds glutathione. It also is believed to play a role in neuronal survival following oxidative insult (Zhong et al., 1RWDEO $) LGHQWLÀHG LQ WKH FHOO FXOWXUH H SHUL-ments above, was also present as a hub in the WGCNA. This study shows that WGCNA – though here *in vivo* – can help identify not only the components of the toxic insult, but also the initiation of PoD as elements of cellular resilience.

Thus, combined omics analysis is a new, unbiased approach for unraveling the earliest metabolic changes, the balance of which decides the cell's fate. Similarly, we now hope to unravel the pathway of defense and resilience when the stressor is withdrawn. A prerequisite for this was the development of a 3D organoid culture of LUHMES cells (Smirnova et al., revised), which allows culturing of cells for longer durations and transfer of the organoid into uncontaminated culture dishes for toxicant withdrawal and recovery studies.

Consideration 6: How to challenge the concept?

7KH ÀUVW SURSRVHG VWHS ... WKH FKDUDFWHUL]DWLRQ

its return to normal/new homeostasis, preferably by a combination of omics technologies that include non-coding RNAs and epigenomics to generate high-content data sets. Such largely untargeted characterization comes with many challenges, as detailed by the Human Toxome Project (Bouhifd et al., 2015a). Central issues are the signal-to-noise problem and the "small n" IDOODF LW LV YHU GLIÀFXOW WR LGHQWLI D IHZ PHDQLQJIXO out of the almost 30,000 when there is a lot of biological and technical variability and a limited number of possible measurements (Krug et al., 2013). Other omics technologies, such as metabolomics, are even less standardized (Bouhifd et al., 2013; Ramirez et al., 2013; Bouhifd et al., 2015b this issue of *ALTEX*). One way forward is by tracing the signatures of toxicity back to their mechanisms (Hartung and McBride, 2011), but incomplete mapping of pathways in different databases is a major challenge .OHHQVDQJ HW DO :RUNÁZV OLNH WKRVH VXJJHVWHG HDU-lier (Maertens et al., 2015), however, can help derive candidate pathways from such untargeted characterizations, and from our experience, WGCNA analysis represents a key tool for overcoming the aforementioned shortcomings. Targeted follow-up PHDVXUHPHQWV WUDQVFULSWLRQ IDFWRU DQDOVLV DQG TXD results by linguistic search engines and systematic literature reviews, also help.

The next step will be the systematic intervention in these pathways with gene-silencing technologies or pharmacological inhibitors, i.e., "mechanistic validation" (Hartung et al., 2013). With resilience pathways, the expectation would be that these delay or hinder the restoration of homeostasis or functional capacity to levels before the hit, limit the protective effect against a second hit (see below), and might possibly result in a shift of the concentration-response curve of cytotoxicity as a proxy of organ selectivity.

The ultimate step will be dynamic modeling of the perturbed cell and its resilience program. Buchman (2002) suggested that FHOOXODU KRPHRVWDVLV DULVHV WKURXJK WKH FRPELQDWLR feedback mechanisms and spontaneous properties of interconnected networks, making it "dynamically stable." Manke et al. (2006) used dynamic systems theory for data from large-scale protein interaction screens in yeast and *C. elegans* to demonstrate entropy as a fundamental invariant and a measure of structural and dynamic properties of networks. Tyson et al. (2003) interpreted the dynamics of regulatory and signaling pathways in the cell as "... *Strikingly similar to the wiring diagram of a modern electronic gadget. Instead of resistors, capacitors, and transistors hooked together by wires, one sees genes, proteins, and metabolites hooked together by chemical reactions and intermolecular interactions*." Some reviews of methodologies are available (Koch and Ackermann, 2012; Jack et al., 2013; Hoeng et al., 2014; Sturla et al., 2014; Sauer et al., 2015). In pharmacology, drug action is increasingly interpreted as interference with such complex networks (Hood and Perlmutter, 2004; Araujo et al., 2007; Kreeger and Lauffenburger, 2010).

A living cell is a complex, dynamic system comprised of hundreds of thousands of active genes, transcribed mRNA, proWHLQV ZLWK DOO RI WKHLU PRGLÀDWLRQV PHWDEROLWHV DQG constituents from lipids and carbohydrates, to mention only a few. All of this is undergoing (even under homeostatic conditions) continuous change and exchange regulated by complex interactions in networks resulting in rhythmic and chaotic patterns. This becomes even more complex if we see a population of cells, different cell types interacting, or then the organ functions they form and their systemic interaction in the organism. As a further complication, living organisms react to their

environments, which constantly affect all levels of organization. It is illusory to attempt to fully describe and model such a complex system. It is also naïve to take any component and HSHFWLWWRUHÁFWWKHZKROHVVWHP7KHJRDOPXVWEHWRXQ derstand enough of the system to understand the major impacts, and this is essentially what research into diseases or toxicology is about: understanding the impacts which make lasting and severe changes to biological systems.

7R XVH DQ DQDORJXQGHUVWDQGLQJ WKH WUDIÀLQ requires characterization of a system of hundreds of thousands of pedestrians, cars, bicycles, etc. But we do not need, and we cannot understand, each and every element's behavior to underVWDQGWKDWVRPHWKLQJIDIIHFWHG,WKHUHLVDWUDÀ VHH SDWWHUQV RI FKDQJHV WUDÀ PDPUHGLUHFWLRQRIÁZHHO gency forces deployed, etc.). If we take a snapshot photograph of the situation from a satellite, we might already see certain clusters or the appearance of ambulances. Even better, we can YLVXDOLJHÁHVDQGVKRZZKHUHWKHÁZLVKLQGHUHGDQDO lyze the direction of movement.

Omics technologies, in combination with WGCNA, are like these satellite photographs, often just a snapshot of the system. By comparison with the "normal" situation, we can start to identify major cellular derangements, especially when we have time series, replicates and dose-response analyses available. We do not need to monitor each and every "car" – a small number of WKHPVXIÀHWRFKDUDFWHULHZKDSSHQVRQWKHRDGV and some of them are more informative (e.g., ambulances, poOLFH FDUV DQG ÀHÀKWHUV LIHUHQW WSHV RI LQWHUHVWLQJ result in similar patterns (accident, construction work, a sport event) if taking place in the same region. The stronger the disruption, the easier it is to detect perturbation at places farther DZD RUZKHWHYHUZHPHDVXUHZKLOHDWUDÀ PDPZLOO impact on pedestrians and bicyclists, the effects of a roadblock will be substantial).

The analogy falls short, however, when we see that our omics snapshots are selective: they see either mRNA, proteins, metabolites or other cellular constituents. This would be equivalent to a camera recording only cars but missing anomalies like a marathon or a bicycle race taking place in the city. In order to understand these situations, we need to combine our monitoring.

A few lessons from our analogy:

– A dynamic system can hardly be understood from a single snapshot.
– Repeated and varied measurements, especially of different components, will give a more robust view of the system.
– The better we understand normal states and earlier perturbations, the better we know *where* and *what* to monitor and how to interpret it.
– Knowing early and stress responses (ambulances and police cars) is a good way to sense trouble even when we do not know why they are deployed.
– [2] :H FDQ VLPXODWH WUDÀ IRU SODQQLQJ ZKLOH XQGHUVWDQGLQ only the major principles of the system.
– The stronger the hit to the system and the longer lasting the effect, the more likely we will see it and interpret it correctly.

For toxicology, however, such systems approaches (Hartung et al., 2012) are still "pie in the sky." Virtual experiments will at some point show how these networked systems achieve their stability and resilience when exposed to toxicants.

Consideration 7: Resilience is not always just the return to the prior state

There are four ways cells respond to a hit/stress (Fig. 1): What does not (1) kill them makes them either (2) stronger or (3) unaffected, or (4) overtly evident, leaves a scar for later hazard encounters or susceptibilities (4). The challenge of a cell by a toxicant induces defense mechanisms (discussed above) and this can, in the long run, result in protective effects. This phenomenon has been termed among others "hormesis" (Calabrese and Baldwin, 2001; Calabrese and Blain, 2005) in toxicology and radiation biology. It describes the phenomenon that cell viDELOLW RU ELRORJLFDO Ì QHVV LQJHQHUDO LQFUHDVH ZKH is exposed to low concentrations of a stressor. Hormesis, in this sense, is the result of resilience, i.e., the cell induces a stress-and-defense program.

Nicolas Taleb has addressed permutations of this concept in his book *Antifragility* (2012): "*Antifragility is beyond resilience or robustness. The resilient resists shocks and stays the same; the antifragile gets better ... Some things benefit from shocks; they thrive and grow when exposed to volatility, randomness, disorder, and stressors, and love adventure, risk, and uncertainty*." Interestingly, he notes "*Complex systems are weakened, even killed, when deprived of stressors*," which resembles very much an earlier article in this series suggesting that cell culture "bores" cells to death (Hartung, 2007). In that article, we argued that cell mass and functionality is not maintained in cells pampered with nutrients with no demand on metabolism and cell function.

Environmental stress continuously compromises biological systems (proper development, cell cycle, signaling pathways, etc.). Robustness of the biological systems against environmental stressors is crucial for many aspects of their proper functionality, including development programs. Robustness can be seen as part of the resilience concept: certain regulatory molecular mechanisms work against the stressors to maintain proper functioning.

Taleb (2012) addresses natural systems several times: "*It is all about redundancy. Nature likes to overinsure itself. Layers of redundancy are the central risk management property of natural systems.*" This is quite in line with genetics (two alleles plus many gene copies and variants) and the lack of effect of many gene knock-outs. Macia and Sole (2009) pointed out that it is not only redundancy but degeneracy, i.e., the ability of elements that are structurally different to perform the same function or yield the same output such as alternative metabolic pathways (Tagore and De, 2011), which results in the robustness of cellular networks. Unraveling the cellular signaling networks begins to explain how a cell can exhibit an apparent

paradox of robustness to toxic perturbations while responding specifically and sensitively to relevant inputs (Araujo and Liotta, 2006). One of these cellular signaling networks regulating robustness is posttranscriptional regulation of gene expression by microRNA through positive and negative feedback loops (Herranz and Cohen, 2010; Ebert and Sharp, 2012). Several studies have shown how microRNA may buffer the altered "noisy" gene expression and thus maintain the steady state of the system. The most important aspect of this type of regulation is the genetic and functional redundancy of microRNAs that makes them stable against environmental stress. This explains the small number or absence of phenotypes in individual microRNA knockout experiments (Miska et al., 2007) and the appearance of the phenotype only when stressed (summarized in Ebert and Sharp, 2012). Some microRNAs were shown to stimulate cellular resistance to environmental stress conditions, e.g., hypoxia (e.g., mir-210, mir-424, Chan et al., 2012; Loscalzo, 2010), temperature changes (e.g., mir-34, mir-83, Burke et al., 2015), pathogenic stress (e.g., let-7 family, Ren and Ambros, 2015), whereas others were shown to have protective properties against toxicant exposure (e.g., mir-7, mir-153, Fragkouli and Doxakis, 2014; Choi et al., 2014). These make microRNA a good candidate as a contributor to cellular resilience.

Although this setup appears to explain why the system is robust and can take individual hits, it does not explain how it learns and becomes better. Can other epigenetic mechanisms, such as DNA methylation answer this question? The epigenome may drive response mechanisms to environmental stress on the interface between the dynamic environment and the inherited genome, possibly allowing an "epigenotoxic effect" (Szyf, 2007). Alterations in DNA methylation and histone modifications have been associated with errors [illegible] function, nervous development, and diseases such as cancer and neurodegeneration (Qureshi and Mehler, 2011; Meda et al., Esteller). DNA methylation and histone modifications are extensively regulated by different factors (e.g., translocation (TET) oxygenase family, DNA methyltransferases, methyl-CpG- binding proteins, histone acetylases, and histone deacetylases), which, themselves are (post)-transcriptionally regulated. Environmental exposures can lead to changes in the activity of those factors and perturb cellular DNA methylation and histone modification (Smirnova et al., Szyf). Epigenetic modifications are coming more into play when we talk about low-dose, long-term exposures. The study by Fraga et al. (2005) on monozygotic twins revealed moderate or no differences in epigenetic profiles in three-year old twins while those profiles drifted apart with the increase in age of volunteers, suggesting environmental and lifestyle contributions to the epigenome. Environmental stressors may cause a permanent change in the epigenome (so-called epigenetic memory, scar or foot-print). Epigenetic memory in the form of changes to the DNA methylation pattern could protect against, or contribute to, long-term pathogenesis or cellular vulnerability to subsequent hazards (Tyagi et al., 2015). Thus, the epigenome serves as the adaptation to stress, plasticity or resilience. Since it is evident that epigenetic alterations maintain a memory of the obtained signal to make the system robust and tolerant against the environment, it is possible that the epigenome may make the system "antifragile." There are few examples of stress tolerance development in plant biology and ecotoxicology, for example epigenetic silencing of flowering locus C under prolonged exposure to cold temperature that results in coordination of the flowering in *Arabidopsis* (He et al., 2003; Kim et al., 2005). Further, earthworms developed a tolerance against low-dose arsenic by epeginetic adaption mechanisms (Vandegehuchte and Janssen, 2014). It is suggested that the increased stress tolerance can even be transmitted in the form of altered DNA methylation patterns to the next generation, which was not exposed to the stress factor (reviewed in Vandegehuchte et al., 2014). For this reason, it is important to study epigenetic mechanisms in toxicology to further understand the mode of action regarding low-dose exposures (Mirbahai and Chipman, 2014).

This is how the experience imprints and changes future responses. Epigenetics might serve antifragility – the system is not restored, but improved. Taleb again: "*Antifragility has a singular property of allowing us to deal with the unknown, to do things without understanding them – and do them well*." Is this not exactly what a cell exposed to unknown toxicants should do? Perhaps we should not stretch the analogy of society and cells too far, but the parallels are stimulating food for thought. Friedrich Nietzsche wrote, "*That which does not destroy, strengthens*," but that is not always correct. Sometimes the results of stressors are "bad memories" such as epigenetic scars (Balmer et al., 2014a,b), mutations or other functional impairments that may predispose to disease or lead to adverse lifetime or even transgenerational outcomes. The fi[illegible] between resilience and maladaptation may need to be according to the situation.

Conclusions

Nicolas Taleb was quoted several times in this article. With his books, *The Black Swan* and *Antifragility* he has popularized ideas central to some phenomena in toxicology. Earlier in this series of articles we referenced the notion (Bottini and Hartung, 2009) that rare events (black swans) are typical in safety sciences. We force our testing strategies (high-dose, oversensitive models), however, into the "Gaussian" part of probabilities, which we can handle. Taleb's follow-up book on antifragility resonates well with some of our thoughts here: "*Fragility is quite measurable, risk not so at all, particularly risk associated with rare events*." This is good guidance and a description of what toxicology is all about: we assess the fragility of our systems with high-dose experiments to be prepared for the rare event of a low-dose risk. But antifragility adds a new dimension to our approach. Evolution has to favor anti-fragile constructions. This elasticity affords protection against the majority of (small) hits. We need to understand this to appreciate the limits of what we can stand and how we can

reinforce defenses. We need to understand where this system fails, potentially leaving scars and maladaptations leading to hazard manifestations. It appears the tools to address this are within reach, especially long-term cultures and high-content characterizations of responses, which may change our views on the origin of organ selectivity of toxic actions and chronic manifestations of toxicities.

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Conflict of interest

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