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Persistent transcriptional responses show the involvement of feed-forward control in a repeated dose toxicity study

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ABSTRACT

Chemical carcinogenesis, albeit complex, often relies on modulation of transcription through activation or repression of key transcription factors. While analyzing extensive networks may hinder the biological interpretation, one may focus on dynamic network motifs, among which persistent feed-forward loops (FFLs) are known to chronically influence transcriptional programming. Here, to investigate the relevance a FFL-oriented approach in depth, we have focused on aflatoxin B1-induced transcriptomic alterations during distinct states of exposure (daily administration during 5 days followed by a non-exposed period) of human hepatocytes, by exploring known interactions in human transcription. Several TF-coding genes were persistently deregulated after washout of AFB1. Oncogene MYC was identified as the prominent regulator and driver of many FFLs, among which a FFL comprising MYC/HIF1A was the most recurrent. The MYC/HIF1A FFL was also identified and validated in an independent set as the master regulator of metabolic alterations linked to initiation and progression of carcinogenesis, *i.e.* the Warburg effect, possibly as result of persistent intracellular alterations arising from AFB1 exposure (nuclear and mitochondrial DNA damage, oxidative stress, transcriptional activation by secondary messengers). In summary, our analysis shows the involvement of FFLs as modulators of gene expression suggestive of a carcinogenic potential even after termination of exposure.

1. Introduction

Within toxicology, transcription networks play important roles as target and effectors during xenobiotic exposure. For instance, the aryl hydrocarbon receptor (AhR) is a well-known ligand of many polycyclic aromatic hydrocarbons (PAHs) and a potent inducer of Phase-I metabolizing enzymes (*e.g.*, CYPs). Other examples include networks such as Nrf2/NFE2L2, a vast array of nuclear receptors (NR1-5), tumor suppressor genes (*e.g.*, TP53), hypoxia-inducible factors (HIFs) and oncogenes (MYC) (Jennings et al., 2013). In most cases, distinct levels of induction have been associated with variable risks of toxicity and cancer (Nebert et al., 2004) and understanding the mechanisms of transcriptional activation may improve (early) toxicity prediction and development of biomarkers (Van Summeren et al., 2011).

Despite that, most investigations of human transcriptional networks are limited to a few TFs at a time due to their complexity: target genes may be redundantly regulated by numerous TFs and interactions are often rewired, yielding different outputs in response to environmental changes or cell state (Blais and Dynlacht, 2005; Vaquerizas et al., 2009). Alternatively, one may focus on network motifs, *i.e.* smaller and yet autonomous portions of complex networks that retain relevant regulatory aspects. For instance, transcriptional cascades result in sequential gene regulation; feedback loops may generate pulses or oscillations in expression (Alon, 2007). Feedforward loops (FFLs) are particularly interesting since in this case control of a target gene Z is influenced by the input (activation or repression) of two transcription factors (X and Y). While X also targets the expression of Y, both transcription factors are regulated by distinct upstream signals (small molecules, covalent modifications, binding proteins). This creates eight possible configurations (Fig. 1) in which four coherent FFLs show an overall direction of regulation equal to the path established from X to Z, while four incoherent FFLs present opposite regulation. The coherent-I (C1) is the most common type, anticipating changes in the system and sustaining expression of target genes even under transient deprivation of the activating

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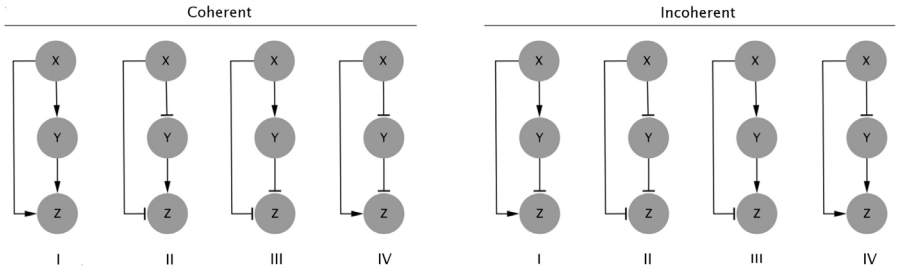

Fig. 1. Overview of all possible configurations of 3-node feed-forward loops (FFLs).

stimulus, whether or not from exogenous origin (Blais and Dynlacht, 2005; Milo et al., 2002). Despite the essential role in maintenance of homeostasis, exacerbated signaling may have undesirable effects, as proposed by an investigation showing a link between activation of a NOTCH1/c-MYC C1 FFL and leukemic cell growth (Palomero et al., 2006).

From a toxicological perspective, network motifs are likely key components of toxic response, and better understanding of their roles may improve outcome of cellular responses and toxicity prediction (Bhattacharya et al., 2011). We hypothesize that toxicants, their intermediate metabolites or intracellular changes arising from exposure may therefore act as upstream signals to activate FFLs, leading to adaptive/adverse responses that may persist even after toxicant removal. To investigate that, we took advantage of our recently published datasets (GSE67005 and GSE71547) (Rieswijk et al., 2016). Both datasets contain transcriptomic measurements from primary human hepatocytes (PHHs), the former after 5 days of repeated dose exposure to a low dose (IC_{20}) of aflatoxin B1 (AFB1) and the latter after a 3-day, non-exposed period (washout). AFB1 is a known human carcinogen and an established risk factor for the development of hepatocellular carcinoma (HCC), the main type of primary liver cancer and the third most common cause of cancer-related mortality. Hepatic metabolism of AFB1 generates several metabolites, including AFB_1-8,9-epoxide which induces DNA lesions by covalently binding to guanine nucleotides (Denissenko et al., 1998). In addition, a recent study in rodents chronically fed with AFB1 (Merrick et al., 2013) showed extensive regulation of several targets from the AhR, Nrf2, E2f1 and other downstream TFs and these alterations may act as key events in the early stages of malignant transformation.

To test our hypothesis, we first constructed a map of human transcription by retrieving curated, directed interactions (activation or repression) between transcription factors and target genes from literature. This map was then used to identify individual transcription factors and/or C1 FFLs and whether they remained active upon removal of the challenging agent AFB1. Finally, the biological relevance and possible impacts were assessed within the scope of (chemical-induced) carcinogenesis.

2. Methods

2.1. Data processing

Transcriptomic measurements from primary human hepatocytes exposed to AFB1 were obtained from publicly available data deposited on Gene Expression Omnibus (GEO) under the accession numbers GSE67005 (5 days) GSE71547 (washout). Hepatocytes were cultured in a collagen-collagen sandwich layer for 48 h prior treatment. Following that period, cells were incubated with a relatively low toxic dose of AFB1 (IC_{20}, 0.3 μM), administered daily during 5 days. Thereafter cells were harvested, the RNA isolated and subsequently subjected to microarray analysis. Another batch of hepatocytes that underwent the same treatment was maintained in medium for another 3 days after terminating AFB1 exposure (washout period). RNA was isolated and analyzed as previously. Detailed protocol and procedures are described elsewhere. (Rieswijk et al., 2016)

Raw files from Affymetrix Human Genome U133 Plus 2.0 arrays were assessed for quality control on ArrayAnalysis (arrayanalysis.org) and pre-processed (background correction, probe reannotation and data filtering) using the R package affy (Gautier et al., 2004). Differentially expressed (DE) genes were selected using moderated *t*-test from LIMMA, a linear model for microarrays. Since experimental data encompassed only mRNA measurements, we worked under the assumption that mRNAs from TFs are expressed at lower levels in comparison to non-TF genes (Vaquerizas et al., 2009) and that expression levels reflect its activity (Blais and Dynlacht, 2005). Therefore, only a corrected *p*-value cutoff (FDR < 0.05) was applied to select DE features (target genes and TFs), regardless of fold change thresholds. Identification of TFs was carried out through the Animal transcription factor database (AnimalTFDB, http://www.bioguo.org/AnimalTFDB/), which contains a list of 1544 human regulatory genes including transcription and chromatin remodeling factors, complexes and co-factors.

2.2. Construction of transcription factor networks

MetaCore™ is a tool comprising several methods for analyses of genome-wide data, including pathways, gene ontology and transcription regulation, the latter encompassing a large curated list resulting from diverse experimental approaches. In a comprehensive study, MetaCore has been shown to outperform several databases for retrieving meaningful, validated transcriptional interactions (Shmelkov et al., 2011). Therefore, in order to retrieve regulatory aspects of human transcription, we selected all targets from interactions specified as “activation” or “repression” from each of the 1544 regulatory genes obtained in the previous step. Networks were reconstructed from this human transcription map by selecting the AFB1-induced DEGs for each time point and by adding interactions with all potential TFs that were also identified as differentially expressed.

2.3. Motif mining

To identify network motifs, we first visualized the interaction in Cytoscape v. 2.8.3, where a node represented a gene and an edge depicted an interaction connecting two nodes. We used CentiScaPe v. 1.2.1 (Scardoni et al., 2009) to compute (among other centralities) the degree of every node in order to identify top scoring genes, or relevant regulatory hubs, for each condition. Then, another Cytoscape plugin, NetMatch (http://ferrolab.dmi.unict.it/netmatch.html), was used to examine the occurrence of C1 FFLs: the directed pattern of a generic C1 FFL (where TF X activates TF Y and both transcriptionally activate target gene Z) was matched against the target networks. Among all triads returned by

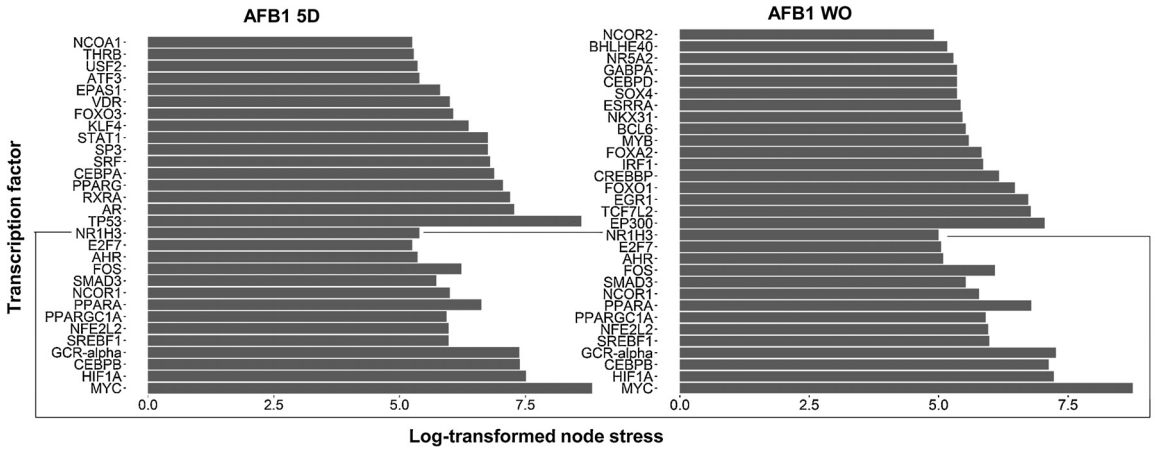

Fig. 2. Node degree scores (logarithmic scale) of differentially expressed transcription factors (TFs) in hepatocytes exposed to AFB1 after 5 days (left) of repeated exposure and during washout period (right). Each TF selected showed a score at least ten times higher than the overall stress mean of all transcription factors from the group. Entities within the box indicate common transcription factors regulated in both datasets.

the analysis, we further selected those motifs in which all genes were upregulated, thus representing persistently and consistently activated FFLs.

3. Results

3.1. (Re)Construction of transcription networks

In order to obtain a snapshot of the human transcription network, data from transcription factors and target genes mined from MetaCore was merged into a single network. This map comprised 41,754 interactions among 1035 TFs/TF complexes and 10,422 distinct target genes, including those coding for TFs – resulting in a network too large to display.

AFB1-induced transcription, analysis after the 5-day repeated exposure and 3-day washout period yielded networks with 4380 and 3602 interactions, respectively. Since several genes encoding for TFs were significantly expressed (217 at 5 days and 192 during washout), network centrality analyses allowed the identification of the most important nodes at both time points. For that, the top scoring TFs (log-transformed node degree score higher than the overall group mean) were selected (Fig. 2). After 5 days of repeated exposure to AFB1, we detected *TP53*, nuclear receptor TFs (*AR*,*PPARA* and *RXRA*), *CEBPA*, *KLF4*, *STAT1*, *SP3* and *SRF* as the most important TFs. Persistent (under)expression was detected during the washout period: *MYC*, *HIF1A*, *CEBPB*, *GCRA*, *PPARGC1A* were among TFs persistently upregulated, while *SREBF1*, *PPARA* and *NR1H3* featured among downregulated TFs.

3.2. Network motifs in AFB1-induced networks

In total, we identified 240 unique, putatively activated C1 FFLs during repeated AFB1 exposure. The main regulators included high stress nodes such as MYC, TP53, CEBPB and PPARG; the pairs MYC-TP53, TP53-SMAD3, CEBPB-FOS and MYC-HIF1A regulated 33% of all gene targets included into FFLs. During washout, 117 C1 FFLs were detected and EP300-MYC appeared as an important regulatory duo. Importantly 25 C1 FFLs were identified in both sets thus representing persistent expression despite the fact that the activating AFB1 impulse was terminated; approximately 60% of these C1 FFLs resulted from MYC-HIF1A and CEBPB-PPARGC1A joined interactions (Fig. 3).

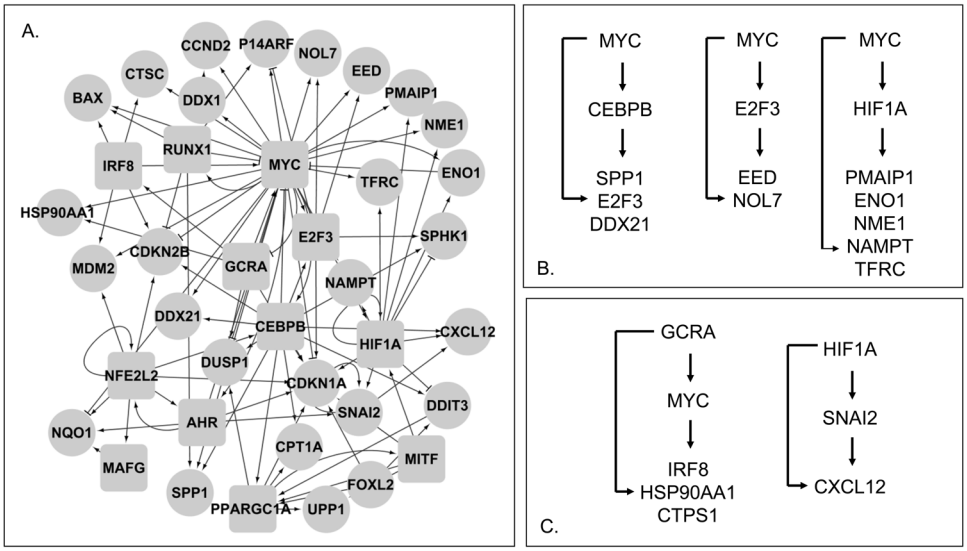

Fig. 3. Type 1 coherent FFLs and additional interactions among genes persistently upregulated after removal of aflatoxin B1 from the media of cultured hepatocytes (A). Some FFLs are detailed in B (all myc-driven) and C (driven by other TFs).

4. Discussion

Transcriptional programming is an ever-changing, crucial determinant of cell fate. Here, we analyzed the effects of a potent hepatocarcinogen, AFB1, on the modulation of transcription networks, with emphasis on C1 FFLs, in human hepatocytes *in vitro*. The investigation during distinct states of exposure allowed the identification of features persistently expressed after removal of the stressor, some of which appeared embedded in feedforward loops (FFLs).

Repeated challenging with AFB1 over 5 days induced the expression of several TFs, among which *TP53* and some of its direct targets involved in cell cycle arrest (*CDKN1A*), DNA repair (*GADD45A*, *GADD45B*) and ultimately apoptosis (*BAX*) were identified as overexpressed, corroborating previous finding of *TP53* induction as a marker of direct DNA damage (Yang and Duerksen-Hughes, 1998). Although *TP53* mRNA was unchanged during washout, "classical" targets of the protein (*i.e.*, *MDM2*, *CDKN1A* and *BAX*), were determined as the main targets of these FFLs. This is consistent with post-transcriptional regulation of p53 protein (*e.g.* increased protein half-life after genotoxic insult) (Kruse and Gu, 2009). While the precise roles played by many of the identified TFs remain unclear, overexpression of *CEBPA*, *KLF4*, *STAT1* as well as repression of *AR* have also been previously reported as outcomes of p53-dependent mechanisms arising from genotoxic insult (Mantoni et al., 2006; Townsend et al., 2005; Yoon et al., 2003; Yoon and Smart, 2004). Furthermore, cessation of expression following removal of AFB1 may suggest an immediate response to sudden, extreme injury (Denissenko et al., 1998). Conversely, TF-coding genes *MYC*, *HIF1A*, *CEBPB,* known to control broader aspects of a cell's physiology (growth, metabolism, regeneration), not only prevailed during and after AFB1 exposure but also staged an intricate web of C1 FFLs.

From the larger number of C1 FFLs in networks after five days of continuous exposure, 25 persisted during washout, featuring MYC and HIF1A as main conjoined regulators. Literature mining confirmed the collaborative aspect of these transcription factors (Dang, 2007; Dang et al., 2008; Doe et al., 2012; Lin et al., 2010), and since several target genes have been described as to their functional roles, henceforward we will focus on this particular interaction to exemplify our initial motivation. *MYC* is a potent oncogene that orchestrates the expression of 10–15% of cellular genes and microRNAs involved in proliferation, differentiation, cell cycle and apoptosis. HIF1a is a transcription factor that modulates genes related to energetic metabolism, being particularly active in hypoxic conditions (Dang et al., 2008). In a cooperative scenario, it was previously shown that when *MYC* is overexpressed a collaboration with HIF1a protein takes place, favoring lactate production over oxidative phosphorylation as energy source (aerobic glycolysis, also known as the Warburg effect) (Dang et al., 2008) even in normoxic conditions (Doe et al., 2012) (Fig. 4, A and B). In our study, three of five targets of joined MYC/HIF1A regulation are involved in energetic metabolism (*NAMPT*, *ENO1* and *NME1*) (Fig. 4C). PMAIP1 (also known as NOXA), has been shown to lead to mitochondrial dysfunction during genotoxic stress (Seo et al., 2003) and the transferrin receptor (*TFRC*), an important mediator of iron uptake, further contributes to the Warburg effect and the proliferative potential of myc-enriched cells (Dang et al., 2008; O'Donnell et al., 2006). A series of MYC/HIF1A targets exclusively upregulated during washout suggests amplification – a consequence from feed-forward control – of aerobic glycolysis, including *HK2* and *ALDOA*, pivotal enzymes for maintaining glycolytic flux. Interestingly, a recent publication has shown that *ALDOA* is an important driver of glycolysis, which in turn increases HIF1A activity and cancer cell survival (Grandjean et al., 2016).

The Warburg effect, however, represents a causality dilemma. When it was initially proposed (more than 80 years ago), the author pointed towards enhanced glycolysis as a turning point which would eventually lead to selective advantage of a malignant

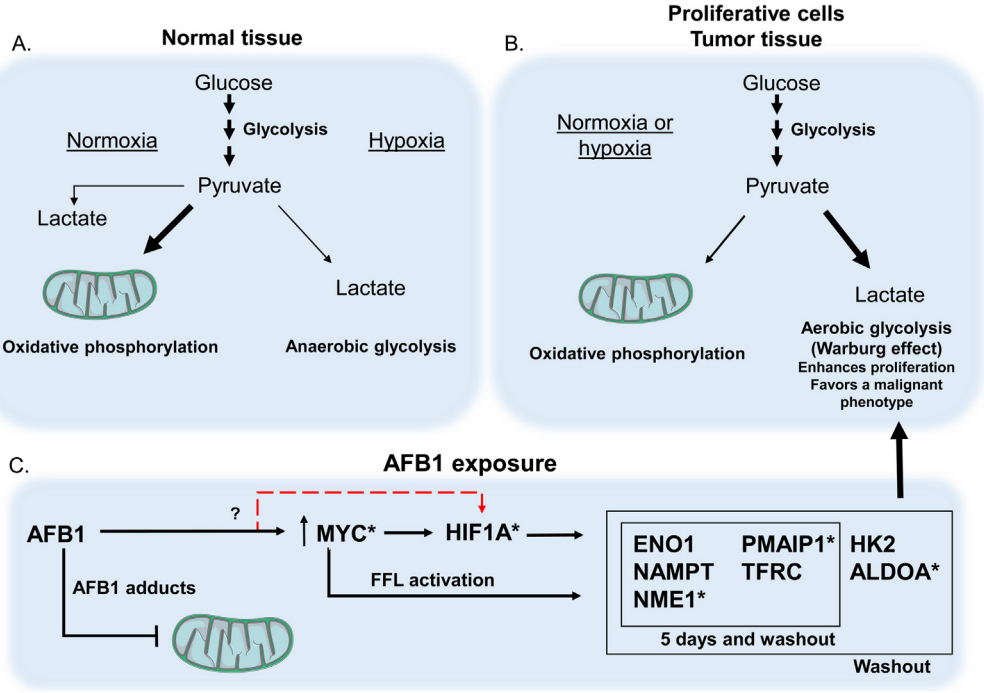

Fig. 4. Comparison of glucose metabolism in normal physiological situation (A) and in proliferative or tumor tissues (B). (C) illustrates how AFB1 exposure may activate a MYC/HIF1A feed-forward loop and generate a Warburg-type scenario: enhanced MYC expression induces HIF1A, and both activate common target genes. Diminished mitochondrial activity due to AFB1 adducts may have an additive effect by favoring an energetic shunt in normoxic conditions. Arrow width (A and B) holds a positive correlation to the amount of energy produced by that path. Asterisk (*) indicates genes that were also found in an independent dataset of AFB1-treated HepaRG cells after 72 h of single exposure (expression values are detailed in Supplementary data).

phenotype (Warburg, 1930). Although evidence has shown that enhanced glycolysis predisposes cells to malignant transformation by aiding cell immortalization (Kim and Dang, 2006; Kondoh, 2008; Kondoh et al., 2005), it is now generally accepted that metabolic changes are intermediate steps (Hanahan and Weinberg, 2011), or even a resulting phenotype, of the current multi-stage model of oncogenesis. Nonetheless, even after a short exposure period, AFB1 datasets evidenced some of the proposed mechanisms culminating in the Warburg effect (Kroemer, 2006) including upregulation of glycolytic genes (*NAMPT*, *ENO1*, *NME1*), hypoxic switch (*HIF1A* overexpression) and mitochondrial dysfunction (represented by increased *NOXA* and supported by *in vivo* rodent studies showing preferential inhibition of mitochondrial activity in comparison to other organelles) (Niranjan et al., 1982).

Although these initial alterations may explain a pre-neoplastic state as long as there is exposure to the chemical, it does not explain how such changes can be maintained beyond exposure time. To this date, only *E. coli* has been used to model and validate kinetic models proposed for FFL behavior based on the relationships between the transcription factors and the promoter(s) of the target gene – "gates" – leading to enhanced expression (Kalir et al., 2005; Mangan et al., 2003). Supported by HIF1A and MYC integration (Dang et al., 2008) and the influence of *MYC* overexpression on modulation of HIF1A itself and its targets (Doe et al., 2012), we conclude that MYC/HIF1A fits the model proposed for a so-called "AND" gate. This type of gate requires the presence of both TFs at the promoter(s) – especially the driving node (*MYC*) – and constant, persistent input signaling (in this case, repeated exposure) to initiate the loop, which remains active as long as upstream signals are present. AFB1 not only has been shown to strongly induce *MYC* expression (at least 10-fold compared to controls) in livers of exposed rats, but also appears to do so without genetic alterations (*i.e.*, gene amplification or rearrangement) – most likely associated to increased expression or stabilization of the mRNA (Larson et al., 1993). Furthermore, intracellular alterations (DNA damage, molecule adduction, oxidative stress and consecutive secondary messengers) may remain for a long period after ending the exposure. For instance, previous investigations have shown that AFB1-induced damage to mitochondria is fairly persistent: while nuclear transcription and nucleus-dependent translation processes are recovered after 12 h of exposure, these mitochondrial activities remain inhibited for at least 24 h (Niranjan et al., 1982). We hypothesize that such alterations, persistent after termination of exposure, may serve as input signals to FFLs – in this particular scenario contributing to prolongation and even exacerbation of the Warburg effect (Fig. 4C).

To validate these findings, we investigated independent datasets of AFB1-exposed liver cells for the presence of MYC/HIF1A FFL. We selected two datasets (GSE40117 and GSE28878), each containing transcriptomic measurements from single dose exposures and all generated by the same platform (for details refer to Supplementary data). For the first dataset, conducted with HepaRG cell line, we found that *MYC*, *HIF1A*, *NME1*, *PMAIP1* and *ALDOA* were all significantly upregulated after 72 h. In contrast, the remaining set, containing measurements from HepG2 cells, showed repression of *MYC* in all time points (12, 24, 48 and 72 h), while all other genes apart from *PMAIP1* (upregulated) and *HK2* (downregulated) did not significantly change. This is remarkable since these results not only corroborate the proposed persistent "AND" loop (*MYC* should be present to induce *HIF1A* and target genes), but also indicates that these changes may be more critical to healthy hepatocytes, since HepaRG has been shown to superiorly mimic primary hepatocytes and liver in comparison to HepG2 (Jennen et al., 2010).

In summary, our analysis shows that feed-forward control, on the molecular (C1 FFLs) and even pathway level may be important players in toxicity and subsequent carcinogenesis: cell instability arising from frequent exposure and/or non-lethal but long-lasting alterations generate mediators that may act as input signals for (typically inactive) critical FFLs. Our results suggest that even a relatively short-lasting impulse by a chemical carcinogen may induce changes in pivotal cancer-related signaling pathways which are persistent after exposure has stopped. However, further experiments are needed to validate these findings and assess the relevance of unknown transcriptional interactions – especially from insufficiently investigated transcription factors – and the significance of the biological processes being regulated. The identification of stimul(i)us upstream to the FFLs, its persistence following termination of exposure may also aid risk assessment of chemicals by identifying common molecular events.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

LR conducted the experimental work and pre-processed the data; TMS constructed the networks; TMS, TvdB, JK and DJ wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2016.10.009.

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