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In vitro Perturbations of Targets in Cancer Hallmark Processes Predict Rodent Chemical Carcinogenesis

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Running Title: *In vitro* cancer hallmarks predict *in vivo* rodent carcinogenicity

Abstract

Thousands of untested chemicals in the environment require efficient characterization of carcinogenic potential in humans. A proposed solution is rapid testing of chemicals using *in vitro* high-throughput screening (HTS) assays for targets in pathways linked to disease processes to build models for priority-setting and further testing. We describe a model for predicting rodent carcinogenicity based on HTS data from 292 chemicals tested in 672 assays mapping to 455 genes. All data come from the EPA ToxCast project. The model was trained on a subset of 232 chemicals with *in vivo* rodent carcinogenicity data in the Toxicity Reference Database (ToxRefDB). Individual HTS assays strongly associated with rodent cancers in ToxRefDB were linked to genes, pathways and hallmark processes documented to be involved in tumor biology and cancer progression. Rodent liver cancer endpoints were linked to well-documented pathways such as PPAR signaling and TP53 and novel targets such as PDE5A and PLAUR. Cancer hallmark genes associated with rodent thyroid tumors were found to be linked to human thyroid tumors and autoimmune thyroid disease. A model was developed in which these genes/pathways function as hypothetical enhancers or promoters of rat thyroid tumors, acting secondary to the key initiating event of thyroid hormone disruption. A simple scoring function was generated to identify chemicals with significant *in vitro* evidence that was predictive of *in vivo* carcinogenicity in different rat tissues and organs. This scoring function was applied to an external test set of 33 compounds with carcinogenicity classifications from the EPA's Office of Pesticide Programs and successfully (p=0.024) differentiated between chemicals classified as "possible"/"probable"/"likely" carcinogens and those designated as "not likely" or with "evidence of non-carcinogenicity". This model represents a chemical carcinogenicity prioritization tool supporting targeted testing and functional validation of cancer pathways.

Introduction

Predicting the potential carcinogenicity of the thousands of chemicals to which humans are exposed presents a significant challenge, especially in the case of non-genotoxic carcinogens. Long-term animal studies are typically used to determine a chemical's carcinogenic potential, and follow-up studies to determine the mode of action. However, it is impractical to apply this testing strategy to tens of thousands of existing chemicals due to cost and time restraints. Additionally, some consider the value of rodent carcinogenicity testing to human risk assessments to be questionable, given the large number of false positives produced. *In vitro* high-throughput screening (HTS) approaches are being developed to prioritize chemicals for targeted testing programs, and to identify gene targets relevant to human cancer progression (Collins *et al.*, 2008; Dix *et al.*, 2007; Kavlock *et al.*, 2009; Martin *et al.*, 2010; NRC, 2007). Given the multi-factorial etiology of cancer and the large numbers of chemicals with unknown cancer potential that need to be evaluated, there is a need for more efficient screening beginning with predictive *in vitro* methods to build a pathway-based understanding for groups or classes of chemicals.

We demonstrate a carcinogenicity screening approach that uses a large collection of HTS assays targeting multiple genes, proteins, pathways and cancer-related processes, including targets associated with the cancer hallmarks described by Hanahan and Weinberg (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). These authors described the hallmark traits that most cancers exhibit: sustaining proliferative signaling, evading growth suppressors, evading immune destruction, enabling replicative immortality, tumor-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death and deregulating cellular energetics. These traits arise through successive mutations resulting in a mature tumor with growth independence and the capability to invade and metastasize. Some chemicals induce mutations directly (i.e. DNA-reactive mutagenic carcinogens)

activating processes supporting cancer initiation and progression; however, many chemical carcinogens do not appear to act through this direct mechanism (i.e. they are non-genotoxic carcinogens), and instead act as tumor promoters (Cohen and Arnold, 2010). Most of the test chemicals used here are pesticides that are applied to food crops, which is a registration use category that effectively excludes mutagens (Judson *et al.*, 2010). Therefore a necessary assumption of this work is that any cancers or lesions caused by chemicals in the test set arise through non-genotoxic mechanisms, and predictive assays identified in model development correspond to non-genotoxic and non-mutagenic cancer processes.

The EPA ToxCast project encompasses a growing data set of *in vitro* HTS and high-content screening (HCS) information for thousands of environmental chemicals (Martin *et al.*, 2010), many of which also have 2-year regulatory guideline *in vivo* cancer studies in rats or mice (Martin *et al.*, 2009). Analysis of the ToxCast data demonstrates that numerous environmental chemicals interact with multiple targets and perturb critical molecular pathways and cellular processes, at least in the *in vitro* assays. In the present study, we tested the hypothesis that chemicals that perturb certain cancer-linked targets or processes in human *in vitro* HTS assays will have a significantly higher likelihood of being carcinogens, as evidenced by carcinogenicity in the 2-year chronic assays in rodents. Our approach began by using a training set of chemicals with both *in vitro* assay data and *in vivo* rodent cancer data to derive a measure of the increased likelihood of carcinogenicity when a chemical is positive in an *in vitro* assay. This increased likelihood is quantified as an odds ratio. The assays with large cancer-related odds-ratios were then mapped to known cancer-related biological pathways and accompanying hallmark processes where possible. We also present a prioritization method in which chemicals were scored for possible carcinogenic potential based on the number of cancer-associated endpoints significantly perturbed in assay screening.

Methods

A collection of 292 chemicals from ToxCast Phase I was used for this analysis (Martin *et al.*, 2010) as listed in **Supplemental Table S1**. The majority of these chemicals are food-use, non-genotoxic pesticide active ingredients for which 2-year chronic cancer bioassay data are available in rat and/or mouse from the EPA Toxicity Reference Database (ToxRefDB) (Martin *et al.*, 2009). ToxRefDB provides classification data, i.e. positive or negative, for each chemical (http://actor.epa.gov/toxrefdb/) for preneoplastic or neoplastic lesions in rat and mouse for multiple tissues. For mouse and rat endpoints, there were 223 and 232 chemicals, respectively, with both *in vitro* and *in vivo* data. (Of these 200 had both rat and mouse data.) This subset of chemicals was used for our initial analysis to identify significantly associated *in vitro* assays and *in vivo* lesion endpoints. Cancer-related endpoints were only included if at least 20 chemicals out of the 223 or 232 set were positive for the endpoint. For mouse, these cancer-related endpoints were: Liver Preneoplastic, Liver Neoplastic, Lung Preneoplastic and Spleen Preneoplastic. For rat, these endpoints were: Kidney Preneoplastic, Liver Preneoplastic, Liver Neoplastic, Testes Preneoplastic, Testes Neoplastic, Thyroid Preneoplastic and Thyroid Neoplastic. In subsequent figures and tables, the endpoint severity is indicated as preneoplastic=level 2 and neoplastic=level 3. Level 1 classification includes a large set of non-cancer-related pathologies that were not examined here. The cancer-related endpoint data are provided in **Supplemental Table S2**.

For each chemical in ToxCast Phase I, 672 *in vitro* assay measurements were generated, including a broad array of biochemical and cellular assays from 7 technology platforms. All assays were run in concentration-response format, and from these data, either an AC50 value (concentration with 50% of maximal activity) or an LEC value (Lowest Effective Concentration, significant difference from averaged controls) was calculated for each chemical-assay pair. Much of the assay data has been published previously (Houck *et al.*, 2009; Knight *et al.*, 2009; Knudsen *et al.*, 2011; Martin *et al.*, 2010; Rotroff *et al.*, 2010), and is publicly available (U.S. EPA, 2011). The assay data table used in the present analysis is given in **Supplemental Table S3**. Most of the assays correspond to single genes. Assay types include direct protein interaction (binding and activity) and mRNA or protein expression level measurements. Assays included were

those for which at least 10 chemicals across the 292 tested showed a significant response. This removes the tendency to bias the results towards a small group of chemicals that were highly promiscuous across targets, and were often the only active chemicals in some assays.

Univariate associations were calculated between the *in vitro* assays and *in vivo* endpoints. Both were converted to binary values, so a chemical-assay pair was set to 1 if there was activity at any concentration, and a chemical-endpoint pair was set to 1 if there was activity at any dose. The basic association measure was an odds ratio (OR) calculated from the 1/0 (activity/inactivity) vector for an assay and the 1/0 vector for the endpoint. A large OR indicated that a chemical positive for the corresponding assay had an increased likelihood of being associated with the specified type of cancer. Assays with large ORs were considered risk factors for chemical carcinogenicity, similar to epidemiological risk factors for cancer (e.g. smoking and lung cancer). For each assay-endpoint pair, a 95% confidence interval was calculated. To correct for multiple testing, a permutation test was performed for each endpoint by permuting the endpoint and calculating the OR values for all assays. Permutation-derived 95% confidence intervals for each endpoint were calculated from the OR distribution across all assays. Assay-endpoint pairs were considered significant if the confidence interval for the pair did not include 1 (i.e. an OR of "no evidence of association"), and if the point estimate of the OR was outside of the 95% permutation test-derived CI for the endpoint. All analyses were performed using R (version 2.13.0), and software is available upon request.

Assays included in this analysis were mapped to cancer hallmarks and other biological processes via their corresponding gene targets. For this purpose, the following hallmark process designations were used: Angiogenesis (inducing angiogenesis), Apoptosis (resisting cell death), Growth Factor (sustaining proliferative signaling and evading growth suppressors), Limitless Replication (enabling replicative immortality), Metastasis (activating invasion and metastasis), Immune (avoiding immune destruction and tumor-promoting inflammation) and Energy Metabolism (deregulating cellular energetics) (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). No assays

were directly mapped to the genome instability and mutation hallmark processes, but as stated previously, chemicals included in our analysis were unlikely to be direct mutagens.

Genes were mapped to hallmark processes using Gene Ontology (GO) (Ashburner *et al.*, 2000) categories whose name contained a hallmark-associated keyword. These keywords were the following: Apoptosis: ("DNA repair", "apopto"); Angiogenesis: ("blood vessel mat", "angiogen", "vasculature", "vasculog", "vascula", "blood vessel dev", "blood vessel morph"); GrowthSignal: ("growth factor", "prolifer", "transcription factor activity"); Metastasis: ("chemotaxis", "cell adhesion", "differentiation", "migration", "motility"); LimitlessReplication: ("telome"); EnergyMetabolism: ("hypoxia", "energy met", "mitochondri"); Immune: ("immun", "inflamm", "sensitization", "T cell"). A number of endpoint-associated assays were involved with an additional category, xenobiotic metabolizing enzymes (XME). All Phase I, II and III xenobiotic metabolism enzymes and related genes were mapped to XME using these keywords: ("monooxygenase", "oxidoreductase", "xenobiotic", "transporter", "glucuronosyltransferase activity"). Additional mapping of assays / genes to hallmark processes followed a review of the literature. A number of assays are not gene-based but instead measure cellular phenotypes, for example mitochondrial membrane potential. Where possible, these assays were mapped to the hallmark processes manually based on literature information. A number of genes mapped to more than one process. This mapping is provided in **Supplemental Table S4**.

A cancer hazard score for each chemical with rat carcinogenicity data (n=232) was calculated as the number of assays activated by the chemical that were involved in significant assay-endpoint pairs. The higher the score, the more likely it is that the chemical will be a carcinogen. For the external validation set of chemicals without ToxRefDB rat data (n=60), we searched for additional cancer data using the ACToR database (http://actor.epa.gov) (Judson *et al.*, 2008), the EPA document "Chemicals Evaluated for Carcinogenic Potential: Office of Pesticide Programs (OPP) August 2010" (U.S. EPA, 2010), the Carcinogenic Potency Database (Gold *et al.*, 2001), the National Library of Medicine TOXNET CCRIS (Chemical Carcinogenesis Research Information

System) database (National Library of Medicine, 2011), the National Toxicology Program database (NTP, 2011), and ToxRefDB mouse studies. An electronic library (eLibrary, **Supplemental Table S6**) was built and curated semi-automatically from the open scientific literature. Relevant articles were retrieved from PubMed using ChemoText baseline version (Baker and Hemminger, 2010) and the Medical Subject Headings (MeSH) terms for each chemical. These references were then categorized based on additional MeSH cancer disease terms ("Neoplasms", "Carcinoma", Cocarcinogenesis", etc.).

We looked for statistical trends among the 33 chemicals with EPA OPP classifications of potential carcinogenicity hazard, which are based on extensive risk assessments including rodent in vivo data. Chemicals classed as "Group B -- Probable Human Carcinogen" or "Likely to be Carcinogenic to Humans", under the 1986 EPA Cancer Risk Assessment Guidelines for pesticides, are those for which the weight of evidence of carcinogenicity based on animal studies is "sufficient." Chemicals classed as "Group C -- Possible Human Carcinogens" have limited evidence of carcinogenicity in animals in the absence of human data and chemicals classed as "Unlikely to be Human Carcinogens" or "Group E -- Evidence of Non-Carcinogenicity in Humans" have evidence of non-carcinogenicity in animals or humans respectively. Certain chemicals are still undergoing assessment, or the data obtained were unclear, and these have classifications of "Group D -- Not Classifiable as to Human Carcinogenicity", "Cannot be Determined" or "Data are Inadequate for an Assessment of Carcinogenic Potential". A limited number of assessments incorporated exposure estimates, and multiple descriptors were used when applicable. Chemicals that were "possible", "probable" or "likely" carcinogens were classified as positives (n=20) and chemicals that were "not likely" or had evidence of non-carcinogenicity were classified as negatives (n=13). A Mann-Whitney test was performed to determined statistical significance between the cancer hazard scores for positives vs. negatives.

Results

Summary of Associations between *In Vitro* Assay Data and *In Vivo* Rodent Carcinogenicity Data: Statistically significant assay-endpoint associations are presented in the form of a forest plot in **Figure 1,** which shows the mean odds ratio (OR) and confidence intervals for each association. The majority of gene targets associated with cancer endpoints in ToxRefDB are in turn associated with the cancer hallmark processes (as described in Methods) or with interactions with xenobiotic metabolizing enzymes (XME). The remaining assay targets that correlate with endpoints but could not be mapped directly to hallmark or XME-related processes assessed microtubule disruption, hepatic steatosis, upregulation of thrombomodulin and increase in nuclear size in rat primary hepatocytes. It is important to note that the *in vitro* assay results are largely for human targets, while the *in vivo* endpoints being predicted are from rodent carcinogenicity studies. The choice of using mostly human *in vitro* assays was driven by practical considerations. First, the vast majority of commercially available *in vitro* HTS assays used in the ToxCast research program were developed to support human pharmaceutical research. Although human-based assays make sense for the ultimate goal of predicting human toxicity, predictive models of rodent carcinogenicity are driven by the practical need for reliable data that is not widely available directly linking chemical exposure to cancer in humans. The results described here are for the 223 and 232 compounds with mouse and rat carcinogenicity data in ToxRefDB, respectively.

Figure 1 displays the assay, gene, and the associated hallmark or XME process for selected associations that pass the significance criteria. Processes that map to cancer hallmarks are in bold. The highest-OR associations are between the opioid receptor OPRL1 (opiate receptor-like 1) and rat thyroid proliferative lesions (rat thyroid 2, OR=16.8, 95% CI=[3.25,86.5]); and PDE5A (phosphodiesterase 5A, cGMP-specific) and rat liver neoplasia (rat liver 3, OR=11.6, 95% CI=[2.17,61.5]). These are all cell-free, protein-ligand binding or activity assays. XME-associated assay responses include inhibition of the cytochrome P450 enzymes CYP1A1 (mouse liver 2, mouse liver 3), CYP3A4 (rat liver 2, rat thyroid 2, rat thyroid 3), CYP2A2 (mouse liver 2), CYP2J2 (mouse liver 2), the monoamine oxidase MAO-A (A) (rat kidney 2), and upregulation of the membrane transporters ABCB1 (mouse liver 2), ABCG2 (mouse spleen 2, mouse

liver 2) and SLCO1B1 (rat liver 3, rat kidney 2). Another metabolically relevant assay target associated with rodent cancer (rat liver 3, OR=5.68, 95% CI=[1.76,18.3]) is upregulation of HMGCS2 (3-hydroxy-3-methylglutaryl-CoA synthase 2), which is regulated by PPARA (peroxisome proliferator-activated receptor alpha). Chemicals interacting with PPARA are known to induce or facilitate liver tumors in rodents (Corton, 2010; Klaunig *et al.*, 2003; Rusyn *et al.*, 2006).

Several "negative" associations, i.e. those that are statistically significant with OR<1, were identified through the analysis. A negative association indicates that chemicals that produce a significant response in the assay have a decreased likelihood of causing cancerous lesions in rodent bioassays. One could hypothesize that the biological pathway tested by the assay is predictive of a protective effect. Targets with negative associations were down-regulation of IL8 (interleukin 8) and PLAUR (human plasminogen activator, urokinase receptor) protein levels in BioMAP (Houck *et al.*, 2009) assays conducted using human primary cells in an induced inflammatory state. IL8 expression is a marker of inflammation, one of the hallmark processes. Increased expression of PLAUR is associated with advanced stages of papillary thyroid cancer (Ulisse *et al.*, 2011). The other displayed negative association was with inhibition of CYP2C19.

Hallmark and XME-related assays were somewhat over-represented in the set of statistically significant associations relative to assays in other categories. For the hallmark and XME linked tests, 2.6% and 2.8% were significant, versus 1.6% for the remainder. Chi-squared p-values for these comparisons are 0.21 and 0.17 respectively. However, note that the ToxCast assays were not randomly selected across the genome, but showed a selection bias to genes in pathways associated with cancer and XME. The gene-endpoint associations for the hallmark and XME classes are plotted as an interaction map in **Figure 2**. The endpoints are labeled with the species (Rat or Mouse), the organ and the severity level. The association map is sparse, indicating that the targets or pathways significantly associated with cancer endpoints differ by organ and species. The assays associated with the greatest number of endpoints measured perturbation of p53 activity

(TP53, highlighted in pink), which was positively associated with preneoplastic lesions in liver, thyroid and testes and neoplastic lesions in thyroid and testes, all in rats. The second most prevalent association was between an androgen receptor (AR, highlighted in red) antagonist transactivation assay and mouse liver preneoplastic lesions, rat liver preneoplastic lesions and rat testes neoplastic lesions. There is a clear species difference in interactions, with a higher prevalence of XME-related interactions for mouse than for rat.

Mapping Genes Associated with Rodent Cancer Endpoints to Pathways and Hallmark Processes: All of the statistically significantly associated genes were mapped to curated pathways from KEGG (Kanehisa, 2002), REACTOME (Matthews *et al.*, 2009), WikiPathways (Pico *et al.*, 2008) and Pathway Interaction Database (Schaefer *et al.*, 2009). This mapping is summarized in **Figure 3**, and the corresponding references are provided in **Supplemental Table S5**. While some of these assays measure protein levels or interactions with proteins, the corresponding gene and gene symbol is used in the discussion.

In **Figure 3** genes annotated in red and green were identified using data from BioMAP assays (Berg *et al.*, 2006; Houck *et al.*, 2009), which measure target protein levels in human primary cells primed with factors such as TNF-α, IFN-γ and IL-1β to simulate states of vascular inflammation or immune activation. A gene highlighted in green indicates that an increase in protein levels is associated with the cancer endpoint and red indicates that a decrease in protein levels is associated. For many of the chemokines and growth factors with significant associations, one can rationalize the sign of the interaction. Suppressing the angiostatic actions of CXCL9 and CXCL10 (chemokine (C-X-C motif) ligand 9 and 10) could contribute to an environment favorable for new blood vessel growth (Romagnani *et al.*, 2004). Similarly, up-regulation of CCL2, a pro-angiogenic chemokine associated with spleen and liver endpoints, could provide proliferative and migratory signals to endothelial cells forming new vessels to feed a tumor (Vicari and Caux, 2002). Cellular adhesion molecules such as VCAM1 and ICAM1, both necessary for new blood vessel growth and stabilization, also show

significant association with multiple cancer endpoints. Androgen receptor (AR) signaling has a number of physiological roles, and from **Figure 3,** appears to affect apoptosis, proliferation, cell invasion, cell migration and angiogenesis. In this case, the chemicals in question are directly interacting with AR as antagonists.

Associations with Rodent Liver Endpoints: We previously published a preliminary analysis of associations between assays and liver preneoplastic and neoplastic lesions, focusing on AR, PPARs (including HMGCS2), CCL2, THBD, PLAT, CYP3A4 and IL1A (Judson *et al.*, 2010). Since that analysis, additional data have been generated and reanalyzed to yield additional associations between TP53, H2AFX, MMP1, PDE5A, PLAUR and SLCO1B1 and either preneoplastic or neoplastic rat liver lesions (see **Figure 2**). Anti-TP53 activity disrupts apoptosis machinery, and it is associated with carcinogenicity in multiple organs. TP53 is also linked to redox sensitive pathways. H2AFX is a marker for oxidative stress. MMP1, like HMGCS2, is regulated by PPARA, and it is well-documented that perturbation of the PPARA pathway may lead to rodent liver pathologies. We were not able to identify any published links between PDE5A and liver carcinogenicity, but several other cancer-related findings have been reported. The anti-cancer drug Sulindac inhibits PDE5A and leads to growth inhibition and increased apoptosis in breast (Tinsley *et al.*, 2009) and colon tumors (Tinsley *et al.*, 2010). On the other hand, down-regulation of PDE5A leads to increased cell invasion in melanoma (Arozarena *et al.*, 2011). PDE5A activity is also androgen-dependent in certain tissues (Mancina *et al.*, 2005), providing a further possible link back to the direct AR association with liver carcinogenicity. The interaction of PLAUR (uPAR) and its ligand uPA drive angiogenesis-dependent tumor growth, and antagonism of this interaction is a target of liver, lung and colon chemotherapy (Li *et al.*, 1998). Finally, SLCO1B1 is regulated by PXR, and sustained PXR activity is associated with rodent liver tumorigenicity (Goetz and Dix, 2009).

Associations with Rodent Thyroid Endpoints: **Figure 2** shows that there are significant associations between rat thyroid lesion endpoints and assays for the human genes CXCL9, CXCL10, ICAM1, IL1A, TP53, H2AFX, OPRL1 and CYP3A4. These genes

are linked to a variety of cancer hallmark processes (**Figure 3**), including cancer cell adhesion, metastasis, leukocyte endothelial interaction in angiogenesis, apoptosis and oxidative stress signaling. **Figure 4** shows a heatmap of the chemicals causing rat follicular thyroid cancer (FTC, the predominant thyroid endpoint in ToxRefDB) and the associated assays. This is typical of the interactions for other endpoints, in that the matrix is sparse, indicating that the pattern of markers of hazard tends to be chemical specific. Additionally, several of the rodent thyroid carcinogens show no activity in any of the assays, indicating a need to expand the set of assays to screen for multiple modes of action for thyroid hormone disruption.

The molecular basis for chemical-induced human and rat thyroid tumors is thought to differ (IARC, 1999), which needs to be accounted for in our linkage of perturbation of human *in vitro* targets with rat *in vivo* thyroid cancer. We propose a model, illustrated in **Figure 5**, in which the genes associated with rodent thyroid tumors act as thyroid tumor enhancers or promoters in humans. This model includes two major sets of initiating events that lead to changes in thyroid biology. The first is disruption of immune/inflammatory signaling. The second is disruption of thyroid hormone concentrations via one or more modes of action (Crofton, 2008). CYP3A4, while not an enzyme responsible for altered thyroid hormone concentrations, is used here as a bioindicator for activation of PXR (results in transcriptional upregulation of CYP3A4 as well as glucuronyltransferase and sulfotransferase enzymes that catabolize thyroid hormones in humans and rats). An assumption inherent to this model is that inhibition of CYP3A4 might correspond to activation of PXR by the chemical, shown previously for certain compounds.(Luo *et al.*, 2002) A necessary condition for rat thyroid follicular cell tumors is disruption of thyroid hormones (TH) levels (branch 1), but such a disruption in humans is not believed to lead to thyroid tumors, but instead to neurodevelopmental toxicity (branch 2) (Crofton and Zoeller, 2005). Interestingly, many of the rat thyroid associations seen here also match genetic or pathway associations documented in human thyroid tumors and other thyroid disease states (branches 3, 5 and 7). In rodents the perturbation of these targets (branches 4 and 6) is likely secondary to thyroid hormone

disruption, which may be also caused by the chemicals under study but is not necessarily captured by any of the available assays.

For most of the *in vivo* 2-year chronic/cancer ToxRefDB studies used in our training set, TH levels were not measured or reported, so direct correlation of hormone levels with gene targets is not possible. Rodent FTC is caused by excessive thyroid stimulating hormone (TSH) stimulation of the thyroid gland and is not considered predictive of human thyroid tumor development, as TSH is thought to be less inducible in humans due to a longer systemic half-life and higher concentration of THs in circulation and in the thyroid gland itself (Capen, 1994; Hill *et al.*, 1998; McClain, 1999). However, a chemical that causes thyroid tumors in rats is still of concern in humans primarily because it signifies that the chemical may decrease TH concentrations across species, or may be related to an uncharacterized mechanism for the development of other thyroid disorders in humans.

The most common genetic lesion seen in human thyroid carcinoma is the PAX8-PPARG rearrangement (Kroll *et al.*, 2000), which presumably disrupts the functioning of both of these genes. From **Figure 3**, one can see that PPARG is associated with regulation of CXCL10, the levels of which are associated with thyroid proliferative lesions. CXCL9 and CXCL10 responses to IFN-γ are modulated by PPARG agonists in humans (Antonelli *et al.*, 2010a; Antonelli *et al.*, 2010b). NFkB regulation has been shown to be associated with human thyroid carcinoma (Pacifico and Leonardi, 2010), and this gene is downstream from the thyroid carcinoma-associated IL1A and upstream of the collection of chemokines CXCL9, CXCL10, CCL2 and the cell-adhesion molecules ICAM1 and VCAM1. IL1A, NFkB and IFN-γ regulate levels of TH in cultured human cells (Sato *et al.*, 1990). Rasmussen (Rasmussen, 2000) and Gerard et al. (Gerard *et al.*, 2006) have demonstrated that IL1 / TNFα / IFN-γ disrupt thyroid cell function and TH levels in human thyrocytes, mediated through NO signaling. Lu et al. have used a mouse model to show that altered TH levels are not in themselves sufficient to cause murine FTC, and find that activity associated with p38 / TGFβ is required (Lu *et al.*, 2010). These facts indicate a possible association between the chemokine dysregulation

measured in *in vitro* assays and TH disruption. This would suggest a connection between the cancer-associated assays and the required key event of TH disruption in rat follicular cell thyroid tumors. Finally, TP53 mutations are often found in advanced human thyroid carcinomas, but not early stage tumors (Ito *et al.*, 1993).

Predicting Carcinogenicity Potential of Untested Chemicals: We used the cancer-associated assays in a prioritization model to identify possible carcinogens within the set of chemicals with *in vitro* ToxCast data but without *in vivo* data in ToxRefDB. This model (described in Methods) is based on the hypothesis that the more cancer-hazard processes perturbed by a chemical, the more likely it is that chemical is carcinogenic. This is a simple model that neglects relative impact of different perturbations and particular sequences of perturbations that may be required for causing cancer. Nonetheless, we believe that this is a useful approach for prioritizing chemicals for further study. There were 60 chemicals tested in the *in vitro* assays for which there were no corresponding rat *in vivo* cancer data. From these, the external validation set consisted of 33 chemicals with EPA Office of Pesticide Programs (OPP) human carcinogenicity classifications (last column of **Table 1**, excluding those 8 with indeterminate information). (As noted in the methods section and discussed below, these "human" classifications are in reality a summary of data from (largely) rodent studies and so are comparable to the data used in developing the model.) These classifications summarize a review of multiple studies. Seven of the top scoring 8 chemicals (cancer hazard score ≥ 7) with OPP classifications were "possible", "probable" or "likely" human carcinogens. (Recall that the higher the score, the more likely it is that the chemical will be a carcinogen.) The remaining compound, Captan, was classed as "likely" at prolonged, high level exposures. Overall, there were 20 chemicals with scores ranging from 0 to 23 that were "possible", "probable" or "likely" human carcinogens and 13 chemicals with scores ranging from 0 to 6 that were "not likely" or had evidence of non-carcinogenicity in humans. There are still a number of false negatives, in particular 2 chemicals (Ethylenethiourea and Pirimicarb) that were classed as "probable" or "likely" and that show evidence of causing tumors in a variety of organs including thyroid, liver, pituitary, mammary gland and testes but yield a cancer hazard score of 0 or 1. Performing a Mann-

Whitney test showed that the *in vitro*-derived cancer hazard score was significantly predictive of OPP carcinogenicity classification (p=0.024).

Discussion

We have demonstrated an approach to identify and test molecular pathways or processes that, when perturbed by a chemical, raise the likelihood that the chemical will be a carcinogen. These predicted pathways can then in turn be used to prioritize environmental chemicals for targeted cancer testing. Our method combines large sets of *in vitro* activity data from HTS assays and *in vivo* rodent carcinogenicity data. The approach starts by finding significant associations between genes, proteins, and cancer hallmark processes and *in vivo* cancer-related endpoints. This step is followed by mining the literature for supporting evidence for the statistical associations. The majority of the gene and protein targets that were associated with chemical-induced carcinogenicity can be mapped to either the cancer hallmark processes, or genes involved with xenobiotic-sensing or metabolism. For many of these genes, there is support in the literature for involvement in cancer progression or severity, although not always in the same organs for which we found associations. A simple scoring function built from these associated genes was significantly predictive of cancer hazard classifications for an external test set.

An important point to note involves the linkage between the training set used in this study (guideline rodent cancer studies) and the external text set ("human cancer potential"). The nomenclature implies that we are making a potentially unjustified leap from rodents to humans. However, the "human cancer potential" determination is in almost all cases actually a weight of evidence summary of information derived from rodent studies, and could just as easily have been termed "rodent cancer potential". So the difference between the training and external validation data sets is that the former used a single guideline study in rat to determine the cancer call, while the latter may have used data from multiple (rat and/or mouse) studies in a weight of evidence approach.

Significant associations were shown between a variety of rodent cancer endpoints (preneoplastic and neoplastic lesions) and assay gene targets. The highest OR was for rat thyroid lesions and OPRL1, a gene which is primarily expressed in the nervous system. A recent study (Kaminsky and Rogers, 2008) showed that OPRL1 is also associated with immune response, and in particular with regulating CCL2 (chemokine (C-C motif) ligand 2), which is significantly associated here with rat liver preneoplastic and neoplastic lesions (rat liver 2 and rat liver 3 in **Figure 1**) and with mouse spleen preneoplastic lesions (mouse spleen 2). CCL2 activity has both inflammatory and pro-angiogenic roles (Kuroda *et al.*, 2005), and it is associated with progression of several tumor types (Roca *et al.*, 2008). This association with rat liver endpoints was described previously (Martin *et al.*, 2010). PDE5A, the gene producing the second highest OR, codes for a cGMP-specific phosphodiesterase involved with smooth muscle relaxation in the cardiovascular system. Down-regulation of PDE5A may be involved in cell invasion in melanoma (Arozarena *et al.*, 2011). The assay used in this analysis measured inhibition of PDE5A activity, consistent with what was seen previously and indicating an indirect link with the cancer hallmark of tissue invasion or metastasis. MMP1 (matrix metallopeptidase 1), like many MMPs, is involved with angiogenesis by controlling the invasive capability of endothelial cells (Blackburn *et al.*, 2007; Guenzi *et al.*, 2003b). Our analysis demonstrates strong statistical associations between the identified genes/proteins and cancer-related endpoints, and in most cases there are documented biological linkages between the two. We note that the implicated proteins may not mediate direct carcinogenic action, and it is plausible that several intermediate steps, not understood or well-characterized, participate in induction of carcinogenicity. Other observations, such as higher prevalence of XME-related associations for mouse than for rat, point toward the need for additional research to guide the interpretation of species-specific cancer outcomes. These correlations provide a variety of testable hypotheses for future research.

One interesting set of associations, and one that warrants further hypothesis-based investigation, is between rodent tumors and differential regulation of a series of inflammatory chemokines. Hanahan and Weinberg (Hanahan and Weinberg, 2011) discuss the conflicting roles of immune cells and signals in tumor progression,

emphasizing the complex network of positive and negative controls. They have noted that the relationship between inflammation and immune processes and cancer is unclear; increased inflammation can lead to cancer, but inflammation and immune processes can also serve to clear the body of cancer cells. In the current study, the pattern of associations with several protein assays (increased vs. decreased protein expression) is consistent with the observation that carcinogenesis is associated with a pro-angiogenic program facilitated by chemokines (CCL2, CXCL10, IL1a, etc.), cellular adhesion molecules (VCAM1, ICAM1, etc.) and elements of the plasminogen activating system (PAS) (THBD, MMP1, PLAT, PLAUR) involved in extra-cellular matrix interactions, migration and proliferation. A separate analysis of this dataset (Kleinstreuer *et al.*, 2011) identified a signature for disruption of vascular processes correlating with *in vivo* developmental toxicity data from prenatal guideline studies in rats and rabbits. Several components of the vascular disruptive compound (VDC) signature, such as CCL2 and CXCL10, are common to the group of cancer-hallmark associated genes. However, their directional regulation is exactly opposite, where CCL2↑/CXCL0↓ is associated with the cancer signature and CCL2↓/CXCL10↑ with the VDC signature, as one might expect. Invasion of endothelial cells into the extracellular matrix (ECM) is also a key feature of angiogenesis, and it is regulated in large part by endothelial expression of MMP1 and other proteases (Guenzi *et al.*, 2003a). Changes in elements of the PAS that control vascular growth factor release and ECM interactions may point to a shift from a quiescent state to a pro-angiogenic state as lesion progression evolves. The directional regulation of certain inflammatory chemokines, when combined with perturbation of vascular cell adhesion molecules and proteases controlling the breakdown of the ECM and release of critical growth factors, strongly supports the notion that at some point in cancer progression, the angiogenic switch is turned “ON”, facilitating tumor growth.

The cancer hallmark pathway associated genes CXCL9, CXCL10, IL1A, ICAM1, OPRL1 and TP53 are also implicated in the literature with thyroid disease in general or with thyroid tumors in humans, indicating they are all active in the thyroid axis and are important as modulators or indicators of thyroid health and disease. It is plausible that they play similar roles in the rat thyroid, but there is insufficient evidence that any of

these genes directly act in TH disruption. Thus it is unlikely that disruption of these genes is the molecular initiating event for rat thyroid carcinogenesis. However, the evidence suggests that they may act as enhancers or promoters in the development of rodent thyroid tumors, possibly secondary to the necessary TH disruption. One identified marker with a plausible connection to TH disruption is inhibition of CYP3A4. The association of hepatic CYP3A4 with rat thyroid preneoplastic and neoplastic thyroid lesions may signify the importance of a well-known mode of action for disruption of thyroid hormones in rats (Barter and Klaassen, 1994; Capen, 1994; Crofton, 2008; Vansell and Klaassen, 2001) that is thought to be plausible in humans. Xenobiotics that interact with the pregnane X receptor (PXR) and up-regulate CYP3A4 (or Cyp3a1/23 in rodents) may also transcriptionally upregulate hepatic glucuronyltransferases, sulfotransferases, and transporters, leading to an increased rate of catabolism and excretion of thyroid hormones and a subsequent decrease in circulating thyroid hormone concentrations (Barter and Klaassen, 1994; Crofton, 2008; Miller *et al.*, 2009)). While this series of events is known to occur in humans based on adverse effects from antiepileptic drugs (Gittoes and Franklyn, 1995; Simko and Horacek, 2007), it is only in the rodent that these decreases in circulating thyroid hormones have been shown to lead to neoplastic lesions of the thyroid gland (Hill *et al.*, 1998). Several rodent thyroid carcinogens had no *in vitro* activity, indicating a dual need to expand the assay set and to critically review the body of literature related to findings of thyroid tumors in rats. For instance it would be useful to determine whether tumors are being found only at (high) dose ranges that would not be reflected in the HTS assays or may not be relevant to human exposures.

For the cancer hazard prioritization model, we tested the hypothesis that a chemical that perturbs multiple cancer-associated assay targets *in vitro* will have an increased likelihood of being an *in vivo* carcinogen. This was tested by examining a set of chemicals for which *in vitro* HTS data were available, but for which there were no corresponding *in vivo* data in the model development database. The model performed well, successfully differentiating between expert-derived OPP carcinogenicity classifications in the external validation set. There were several false negatives (chemicals with a low *in vitro* test score that are likely or possible human carcinogens –

see **Table 1**.) Although not ideal, some false negatives were expected. First, the number of chemicals and pathways tested are somewhat limited. We started with a set of fewer than 300 chemicals, which are largely pesticide active ingredients tested in rats and mice and thus do not adequately represent all environmental chemicals. Given a limited set of chemicals, and a limited number of positive examples for most endpoints, our power to discover true associations was limited. This means that there will be cases where a biological target probed by an assay in the battery is a key event in chemical-induced carcinogenesis, but because there are few examples of this link in the available data set, the association may not be statistically significant. Other considerations are the correlated nature of some of the in vitro assays, which may skew the cancer hazard score, and the fact that certain associations between assays and tumor endpoints may be a result of co-correlated variables that are not measured (for example, CYP3A4 as a surrogate for PXR activation). Further, despite the large number of HTS assays (relative to many other studies), the assay battery covers only a small region of the genome, although this battery has been enriched for those related to cancer processes. Because of the small (relative to the whole genome) and non-randomly selected set of assays, we cannot make strong quantitative conclusions about what fraction of pathways (targets) linked to chemical carcinogenicity are also linked to hallmark processes, but there is a suggestive trend (see Figure 1 and related text in results). It is worth noting that the two false negatives (ETU and Pirimicarb) are carbamates that have been shown to be weakly genotoxic (Dearfield, 1994; Ündeğer and Başaran, 2005) and due to the lack of assay coverage specific to mutagenicity, would not necessarily be identified by this approach.

Completion of Phase II of the ToxCast project will extend the chemical collection to a total of 960 unique compounds (U.S. EPA, 2011) and will also add additional assays. This data set will also allow us to further test the present results, and to further refine our models of chemical carcinogenesis and other toxicities. We envision using scoring schemes like this as a prioritization tool for screening large numbers of untested chemicals in the relevant *in vitro* assays to identify those with increased likelihood of being able to induce preneoplastic and neoplastic lesions through the non-genotoxic mechanisms identified here. Such prioritized chemicals would be considered for targeted,

traditional toxicity testing. We have recently published examples of such scoring methods for signatures of reproductive (Martin *et al.*, 2011) and developmental (Kleinstreuer *et al.*, 2011; Sipes *et al.*, 2011) toxicity. Additionally, this approach could be used to identify significantly associated cellular and molecular signaling targets for follow-up functional validation studies to determine their role in cancer progression.

There are two dominant theories of chemical carcinogenesis: genotoxicity / mutagenicity and initiation / promotion (Cohen and Arnold, 2010). Both theories state that acquisition of DNA mutations is required for cells to develop malignant properties and ultimately acquire the hallmark traits. In the initiation / promotion model (Berenblum and Shubik, 1947), one chemical exposure induces DNA mutations (Dragan *et al.*, 1993) and another chemical exposure promotes proliferation, which can in turn allow further mutations to occur following increased cell division. The promoter chemical exposure could be the sole carcinogen if other sources of mutations (initiation) were available, e.g. increasing background level of mutations with age, as described in multi-stage models (Armitage and Doll, 1954; Greenfield *et al.*, 1984; Moolgavkar and Knudson, 1981). An initiator can itself be a complete carcinogen because it can directly kill cells and lead to regenerative proliferation as a mechanism of promotion. Initiators that cause mutations in tumor suppressor genes such as TP53 can have a multiplicative effect because this makes it more likely that further mutations will survive in succeeding cell generations (Hahn *et al.*, 1999; Knudson, 1993; Knudson, 1971). One consequence of these multi-stage cancer models is that a chemical can increase the hazard of cancer by increasing the probability of a mutation in a critical gene during each cell replication and/or by increasing the number of replications (Cohen and Arnold, 2010). This concept is consistent with the proposed exposure-driven functional model of carcinogenesis in which chemical exposure is the dynamic force driving changes in gene regulation and proliferation rates and yielding functional mutations that ultimately may increase genetic mutations (Lund, 2011). These results add complexity to the multi-stage cancer model by suggesting that chemical exposure may drive changes in multiple hallmark traits that facilitate cancer progression and provide enhanced opportunities for the tumor to acquire critical, heritable mutations. Further, multiple pathways can contribute to each of the

hallmark processes, and these pathways will be chemical, organ, cell-type and life-stage dependent. The current study demonstrates one approach, using a large battery of *in vitro* HTS assays, to incorporate multiple lines of evidence about pathways leading to chemical carcinogenesis.

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Table 1:

Chemical	Kidney 2	Liver 2	Liver 3	Testes 2	Testes 3	Thyroid 2	Thyroid 3	Rat Sum	Evidence Rat	Evidence Mouse	EPA Carcinogenic Potential Classification
Chlorothalonil	1	6	3	2	1	7	3	23	CPDB (kidney, liver, mammary)	ToxRefDB (negative) OPP (kidney, stomach) NTP (negative)	Likely To Be Carcinogenic
Diniconazole	0	5	3	0	1	5	4	18	Lit (thyroid)	No data	
Niclosamide	0	4	2	1	1	7	2	17	No data	No data	
HPTE (metabolite of Methoxychlor, so use this data)	0	4	1	1	1	7	3	17	CPDB (negative)	CPDB (negative) CCRIS (testes)	
Methylene bis(thiocyanate)	0	3	2	1	0	8	2	16	OPP (hemangiosarcomas)	ToxRefDB (negative)	Likely To Be Carcinogenic --Based on Metam Sodium Data
TCMTB(2-(Thiocyanomethylthio) benzothiazole)	1	3	1	0	1	4	2	12	OPP (testes, thyroid)	ToxRefDB (negative)	Group C--Possible Carcinogen
Maneb	0	2	2	0	2	3	1	10	CPDB (positive) Lit (thyroid, skin, lymphoma)	ToxRefDB (liver) OPP (liver)	Group B--Probable Carcinogen
Captafol	0	1	2	0	0	4	2	9	CPDB (kidney, liver, mammary) Lit (heart, spleen, stomach, intestinal)	CPDB (blood, liver, intestines, stomach, vascular)	Group B--Probable Carcinogen
Oxyfluorfen	0	2	1	0	1	3	2	9	No data	ToxRefDB (liver) OPP (liver)	Likely To Be Carcinogenic
Methoxychlor	0	2	1	0	1	3	1	8	CPDB (negative)	CPDB (negative) CCRIS (Testes)	

Captan	0	2	1	0	0	4	1	8	CPDB (uterus, kidney) Lit (mammary, liver, uterine, testes)	ToxRefDB (small intestines, stomach) CPDB (small intestines) NTP (small intestines)	Multiple Descriptors: Likely at prolonged, high level exposures, but not likely at dose levels that do not cause cytotoxicity and regenerative cell hyperplasia
Oxytetracycline dihydrate	1	1	3	0	0	1	1	7	No data	No data	Group D--Not Classifiable
Perfluorooctane sulfonic acid	1	1	1	0	1	2	1	7	No data	No data	
Perfluorooctanoic acid	0	2	1	0	1	2	1	7	No data	No data	
Benomyl	0	1	1	1	0	3	1	7	Lit (bone marrow)	ToxRefDB (liver), CCRIS (liver)	Group C--Possible Carcinogen
Pirimiphos-methyl	1	2	0	0	1	2	1	7	No data	ToxRefDB (negative)	Cannot Be Determined
Naled	0	1	1	0	0	3	1	6	No data	No data	Group E--Evidence of Non-carcinogenicity
Fluazifop-P-butyl	1	0	2	0	1	1	1	6	No data	No data	Not Likely To Be Carcinogenic
Thiophanate-methyl	0	1	1	0	0	2	2	6	OPP (thyroid)	ToxRefDB (liver), OPP (liver)	Likely to be Carcinogenic
Fluroxypyr-meptyl	0	0	0	0	0	5	0	5	No data	No data	Not Likely To Be Carcinogenic --Based on Fluroxypyr Data
Metam-sodium hydrate	0	0	0	0	1	3	1	5	CDPR (angiosarcoma)	CDPR (angiosarcoma)	Likely To Be Carcinogenic
Tri-allate	0	1	0	0	0	3	1	5	ToxRefDB (kidney)	ToxRefDB (liver)	Group C--Possible Carcinogen
Metolachlor	0	1	1	0	0	2	1	5	OPP (Liver)	ToxRefDB (negative)	Group C--Possible Carcinogen
Acifluorfen	1	1	2	0	0	0	0	4	No data	CPDB (liver, stomach)	Multiple Descriptors: Likely to be Carcinogenic at High

											Doses, Not Likely to be Carcinogenic at Low Doses
Methyl isothiocyanate	0	0	0	0	1	2	1	4	No data	No data	There are insufficient data to characterize the cancer risk
Napropamide	0	1	0	0	0	2	1	4	No data	No data	Not Likely To Be Carcinogenic
Chlorpyrifos oxon (metabolite of chlopyrifos)	0	0	1	0	0	3	0	4	ToxRefDB (negative) CPDB (negative)	CCRIS (negative)	Group E--Evidence of Non-carcinogenicity
Dibutyl phthalate	0	1	0	0	0	2	1	4	Lit (Leydig Cell tumor)	No data	
2-Phenylphenol	0	0	1	0	1	1	1	4	CPDB (bladder)	ToxRefDB (Liver)	Not Likely To Be Carcinogenic
Benfluralin	0	0	0	0	0	2	2	4	CCRIS (liver, thyroid)	ToxRefDB (liver)	Suggestive Evidence of Carcinogenicity, but Not Sufficient to Assess Carcinogenic Potential
Tetramethrin	0	0	1	0	0	2	1	4	OPP (testes)	ToxRefDB (negative)	Group C--Possible Carcinogen
Butralin	0	1	0	0	0	1	1	3	No data	No data	
Phthalic acid, mono-2-ethylhexyl ester	1	0	1	0	0	1	0	3	No data	No data	
Methidathion	1	0	0	0	1	1	0	3	Lit (liver)	ToxRefDB (liver) CPDB (liver)	Group C--Possible Carcinogen
Terbacil	0	0	1	0	0	1	1	3	No data	ToxRefDB (liver)	Group E--Evidence of Non-carcinogenicity
Diazoxon (metabolite of diazinon)	0	0	0	0	0	2	0	2	ToxRefDB (negative) CPDB (negative) CCRIS (negative)	CCRIS (negative)	Not Likely to be Carcinogenic
Dimethyl phthalate	0	0	0	0	0	2	0	2	No data	No data	
Methyl hydrogen phthalate	0	0	0	0	0	2	0	2	No data	No data	

Thiobencarb	0	0	0	0	0	1	1	2	No data	No data	Group D--Not Classifiable
EPTC (*S*-ethyl dipropyl(thiocarbamate))	0	0	0	0	0	2	0	2	No data	ToxRefDB (negative)	Not Likely to be Carcinogenic
Norflurazon	0	0	0	0	0	1	1	2	No data	ToxRefDB (liver) OPP (liver)	Group C--Possible Carcinogen
Clopyralid-olamine	0	0	0	0	0	1	0	1	No data	No data	Not Likely to be Carcinogenic
Cyanazine	0	0	1	0	0	0	0	1	CPDB (mammary gland) CCRIS (negative) Lit (adenocarcinoma, carcinosarcoma)	CCRIS (no cancer)	Group C--Possible Carcinogen
Pirimicarb	1	0	0	0	0	0	0	1	Lit(liver)	OPP (liver, lung, ovary, mammary gland)	Likely to be Carcinogenic
Symclosene	0	0	0	0	0	1	0	1	No data	No data	
Fluometuron	0	0	0	0	0	1	0	1	CBDB (negative) CCRIS (negative)	ToxRefDB (lung) OPP (lung) CBDB (negative) CCRIS (negative)	Group C--Possible Carcinogen
Imazapyr	0	0	1	0	0	0	0	1	No data	ToxRefDB (negative)	Group E--Evidence of Non-carcinogenicity
Maleic hydrazide	0	0	0	0	1	0	0	1	CPDB (negative)	ToxRefDB (negative) CPDB (negative) CCRIS (liver)	Group E--Evidence of Non-carcinogenicity
6-Deisopropylatrazine	0	0	0	0	0	0	0	0	No data	No data	
Chloroneb	0	0	0	0	0	0	0	0	No data	No data	Data Are Inadequate for an Assessment of Carcinogenic Potential

Ethylenethiourea	0	0	0	0	0	0	0	0	CPDB (thyroid) OPP (thyroid) CCRIS (thyroid, liver, pituitary) Lit (thyroid)	CPDB (liver) OPP (thyroid, pituitary, liver) CCRIS (liver)	Group B--Probable Carcinogen
Etridiazole	0	0	0	0	0	0	0	0	OPP (liver, bile duct, mammary gland, thyroid, testes)	No data	
Methyl cellusolve	0	0	0	0	0	0	0	0	No data	No data	
Monocrotophos	0	0	0	0	0	0	0	0	No data	No data	
Phenoxyethanol	0	0	0	0	0	0	0	0	No data	No data	
Tebuthiuron	0	0	0	0	0	0	0	0	No data	No data	Group D--Not Classifiable as to Carcinogenicity
Monobutyl phthalate	0	0	0	0	0	0	0	0	No data	No data	
Cloprop	0	0	0	0	0	0	0	0	No data	ToxRefDB (liver)	
Imazethapyr	0	0	0	0	0	0	0	0	No data	ToxRefDB (negative)	Not Likely to be Carcinogenic
Triclopyr	0	0	0	0	0	0	0	0	No data	ToxRefDB (negative)	Group D--Not Classifiable as to Carcinogenicity

Table and Figure Legends:

Table 1: Summary of cancer hazard model for chemicals not included in the training set for rat endpoints. The columns "Kidney 2" through "Thyroid 3" indicate the number of genes associated with those tissue-specific preneoplastic (2) or neoplastic (3) lesions in the cancer hazard model that were perturbed in one of our assays for each chemical. Rat sum is the sum of the previous 7 columns. Chemicals are sorted in decreasing value of this column. The Evidence Rat column gives any evidence from ToxRefDB (data entered into the database after the training data set was extracted), CDPR, CPDB, NTP, TOXNET CCRIS, or the PubMed-based eLibrary (Lit). The Evidence Mouse column is the same for mouse. EPA Carcinogenic Potential Classification is provided by the EPA Office of Pesticide Programs. (U.S. EPA, 2010)

Figure 1: Forest plot showing the mean odds ratio (OR) and confidence intervals (CI) for each significant association between *in vitro* assay and *in vivo* endpoint. Only associations with 3 or more true positives are shown. The colored circles give the point estimate of the OR and whiskers give the 95% CI. The gray bars indicate the endpoint-specific permutation-test 95% CI. The linkage to types of processes is indicated by the color of the OR circle: red is cancer hallmark-related, cyan is XME-related and white is other. The assay name is listed at the far left. The associated gene, gene-related process, species, cancer type and cancer severity level (2 = preneoplastic lesions and 3 = neoplastic lesions) are indicated to the right. A darker line indicates overlap of the assay-specific and the endpoint confidence intervals. Supplemental Figure S1 is high-resolution pdf of this figure.

Figure 2: Interaction map showing links between certain rodent cancer endpoints, gene expression changes associated with those endpoints, and *in vitro* assays. Endpoints are shown in white (2=preneoplastic lesions, 3=neoplastic). Assays are shown as small green circles connecting to target genes. Each green dot represents a single assay. Hallmark-associated genes are shown in red and XME-associated genes in cyan. TP53 and its associations are highlighted in pink. Lines connect genes and their associated assay(s), and assays and their associated endpoints.

Figure 3: Map of carcinogenicity-related genes in the context of canonical pathways. Genes associated with increased cancer hazard are indicated by bold-underlined text. Green indicates that the assay detected an increase in protein levels, red a decrease. Black indicates an assay not measuring the direction of protein level changes. Genes surrounded by a double box (e.g. AR) are receptors. A bulls-eye indicates transcriptional regulation. A red line indicates repression. Linkages were derived from the literature and published pathway maps. The numbers correspond to references, provided in **Supplemental Table S5**.

Figure 4: Heatmap illustrating rat thyroid tumorigens and their activity ($-\log_{10}$(AC50)) in assays associated with rat thyroid tumors. Darker colors indicate more potent interactions. Targets are shown following ToxCast assay name. Abbreviations: CLM: Cellumen, NVS: Novascreen, GPCR: G protein coupled receptor, BSK: Bioseek,

KF3CT: keratinocyte cell system, SM3C: smooth muscle cell system, hDFCGF: fibroblast cell system.

Figure 5: Conceptual diagram linking pathways to rodent and human thyroid outcomes. Disruption of thyroid hormones (TH) levels (branch 1) leads to thyroid follicular cell tumors in rats and to neurodevelopmental toxicity in humans (branch 2). Other rat thyroid associations observed here match genetic or pathway associations documented in human thyroid tumors and other thyroid disease states (branches 3, 5 and 7), while in rodents the perturbation of these targets (branches 4 and 6) is likely secondary to TH disruption.

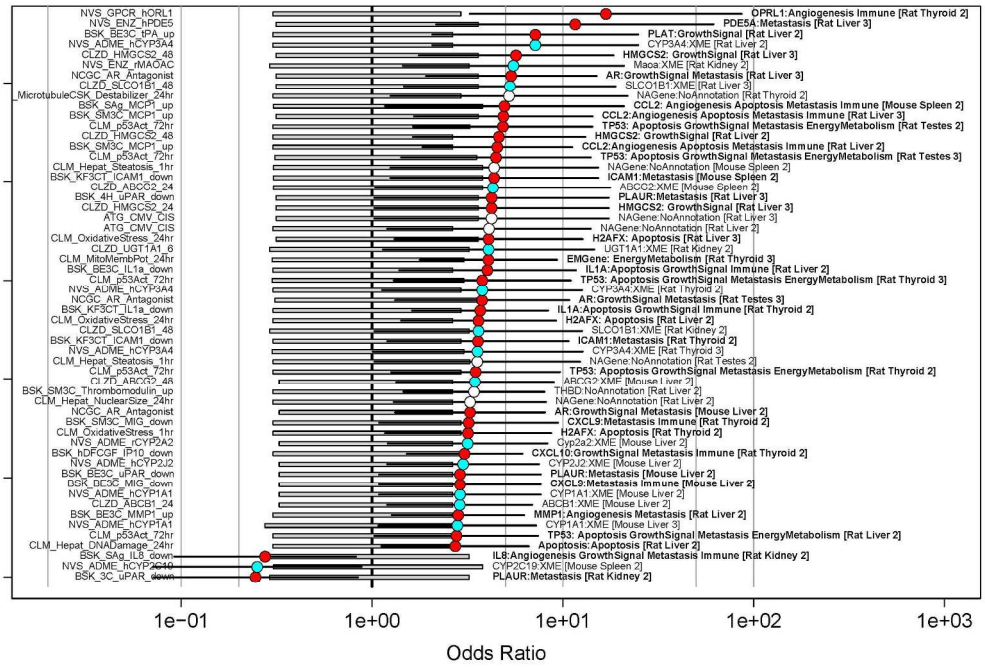


Figure 1: Forest plot showing the mean odds ratio (OR) and confidence intervals (CI) for each significant association between in vitro assay and in vivo endpoint. Only associations with 3 or more true positives are shown. The colored circles give the point estimate of the OR and whiskers give the 95% CI. The gray bars indicate the endpoint-specific permutation-test 95% CI. The linkage to types of processes is indicated by the color of the OR circle: red is cancer hallmark-related, cyan is XME-related and white is other. The assay name is listed at the far left. The associated gene, gene-related process, species, cancer type and cancer severity level (2 = preneoplastic lesions and 3 = neoplastic lesions) are indicated to the right. A darker line indicates overlap of the assay-specific and the endpoint confidence intervals.
316x217mm (200 x 200 DPI)

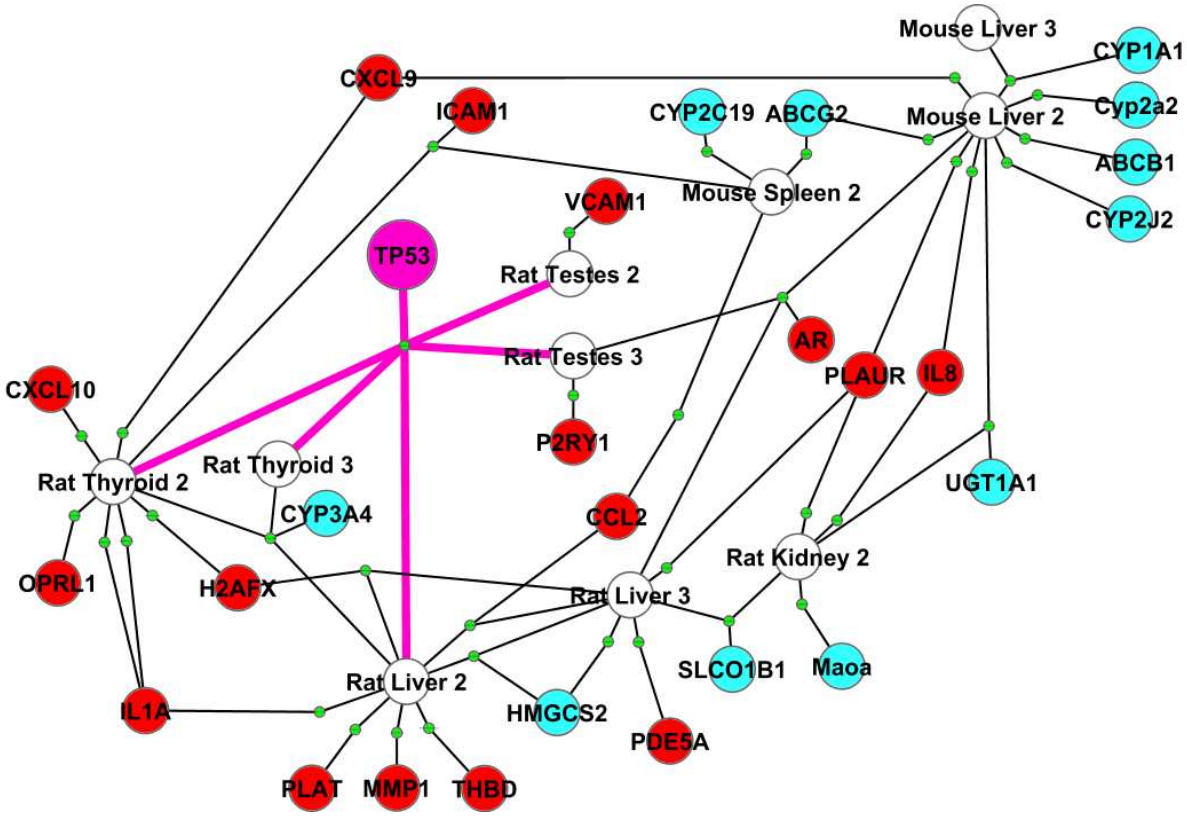
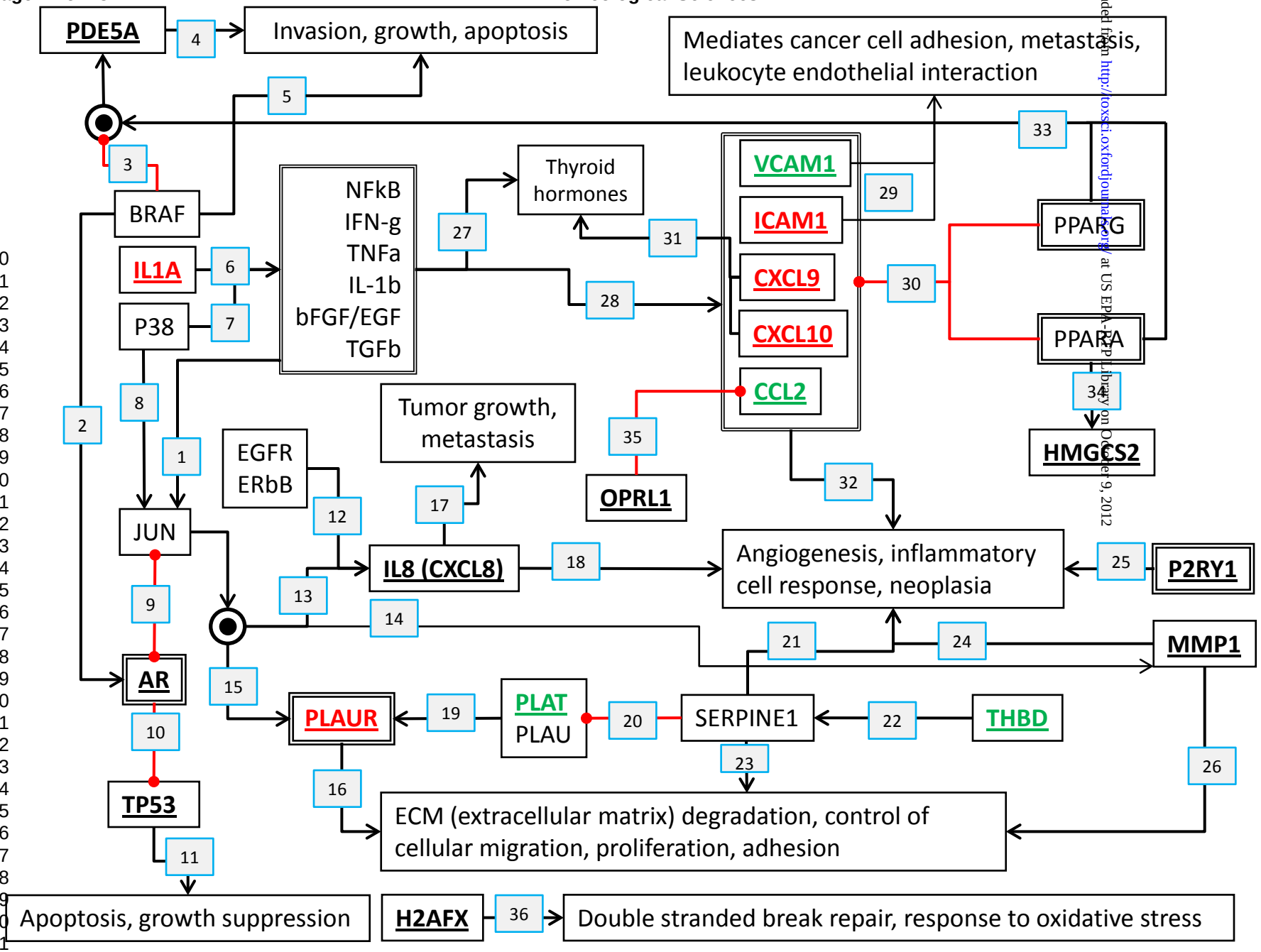
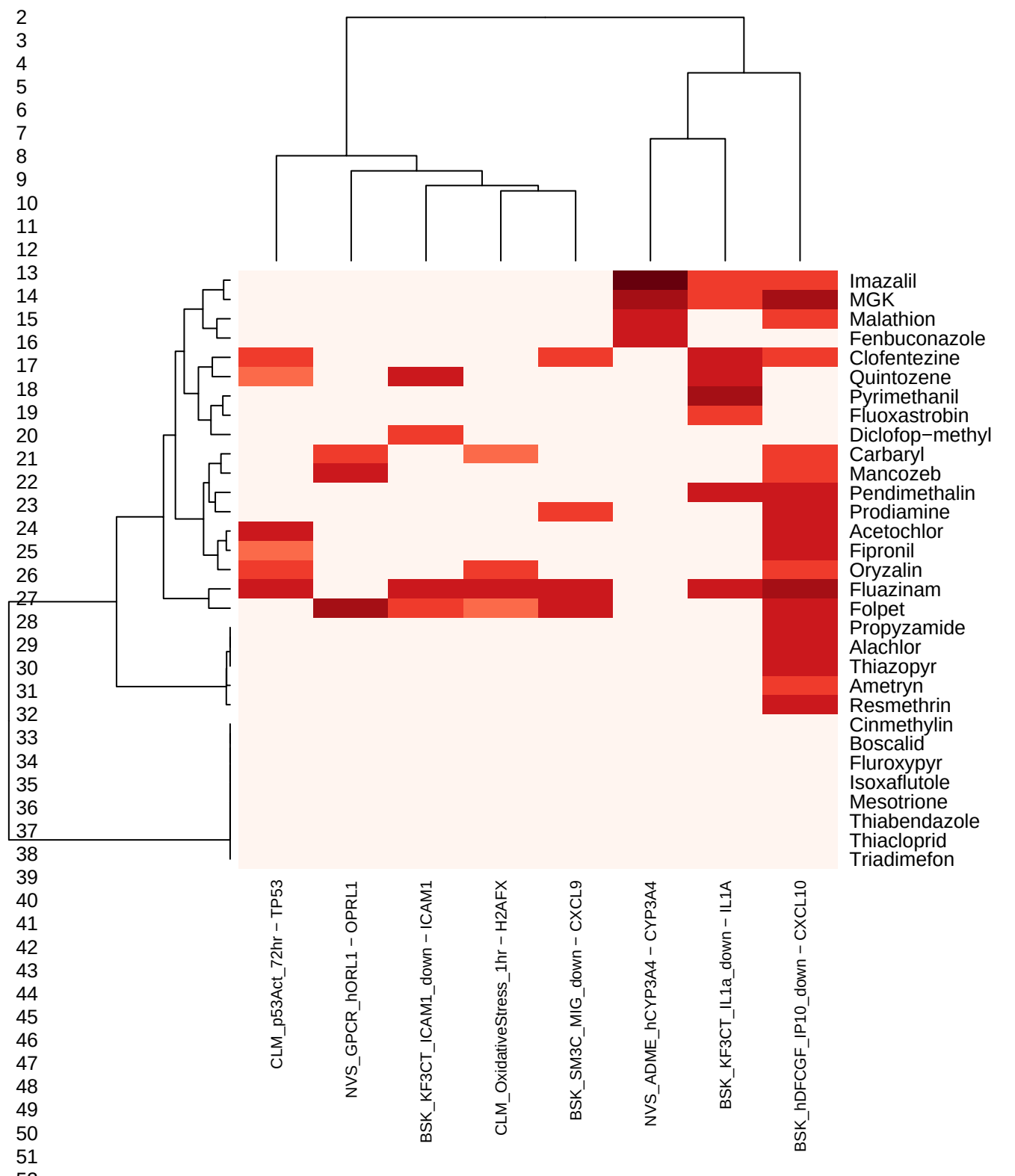


Figure 2




Imazalil
MGK
Malathion
Fenbuconazole
Clofentezine
Quintozene
Pyrimethanil
Fluoxastrobin
Diclofop−methyl
Carbaryl
Mancozeb
Pendimethalin
Prodiamine
Acetochlor
Fipronil
Oryzalin
Fluazinam
Folpet
Propyzamide
Alachlor
Thiazopyr
Ametryn
Resmethrin
Cinmethylin
Boscalid
Fluroxypyr
Isoxaflutole
Mesotrione
Thiabendazole
Thiacloprid
Triadimefon
CLM_p53Act_72hr – TP53
NVS_GPCR_hORL1 – OPRL1
BSK_KF3CT_ICAM1_down – ICAM1
CLM_OxidativeStress_1hr – H2AFX
BSK_SM3C_MIG_down – CXCL9
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BSK_KF3CT_IL1a_down – IL1A
BSK_hDFCGF_IP10_down – CXCL10

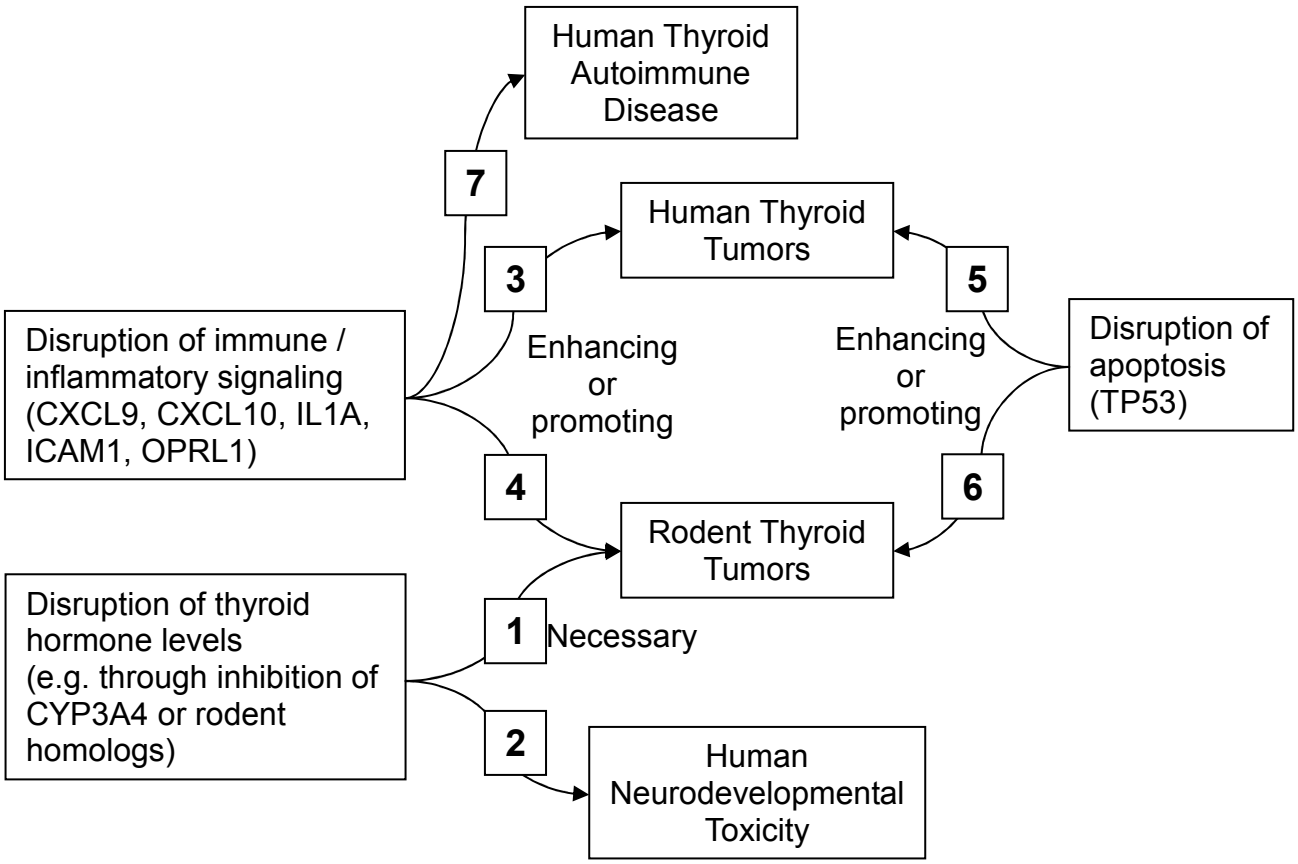

Figure 5