A network based approach to understanding drug toxicity and its application to human liver disease

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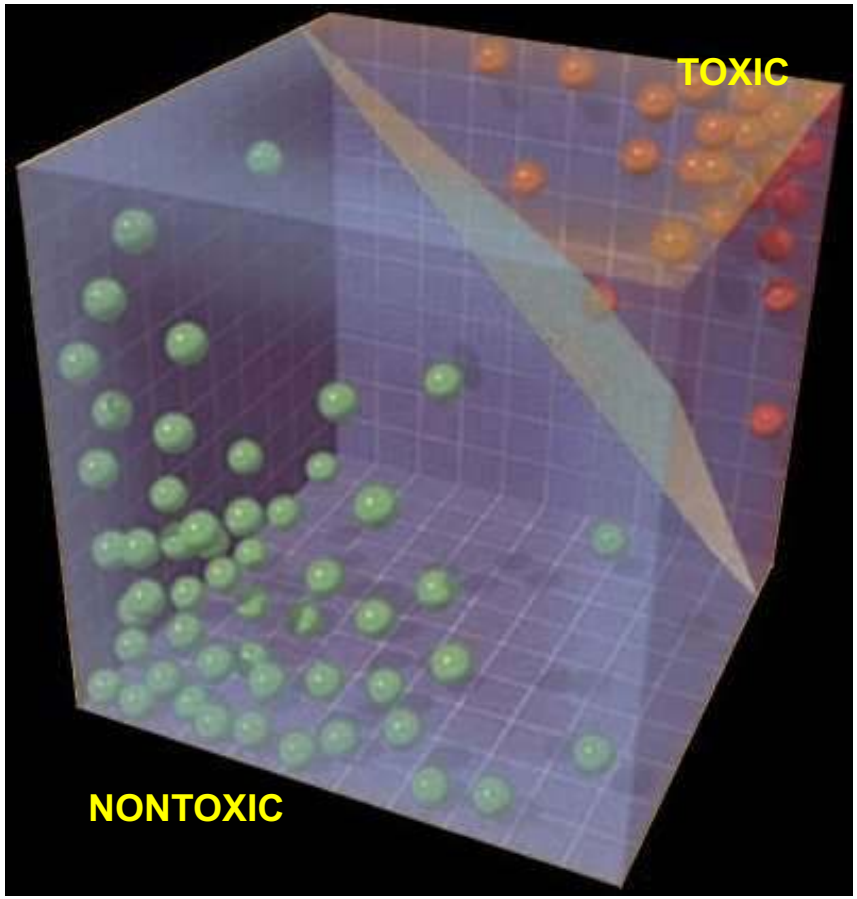
Lilly

Overview

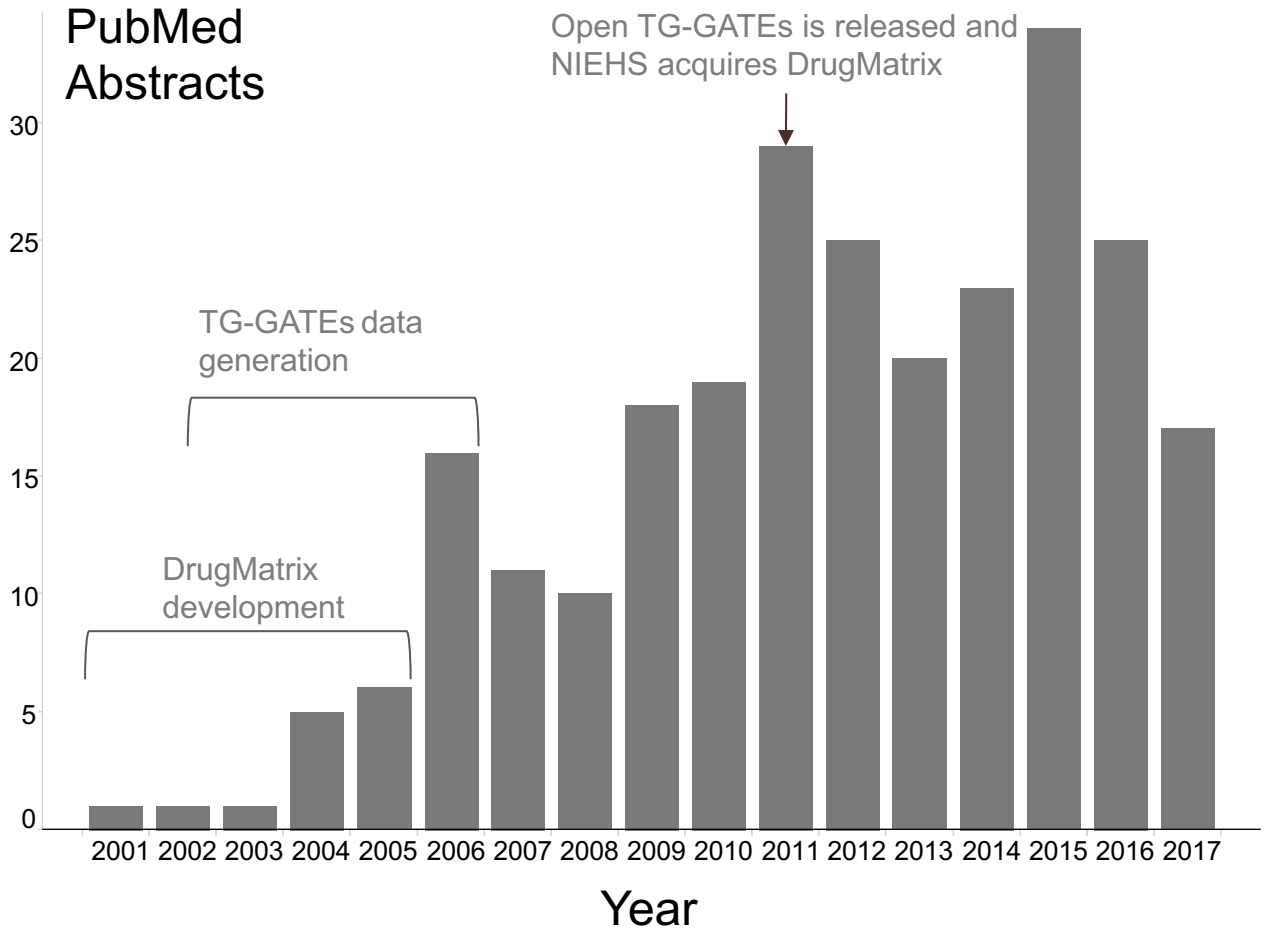
- Expression signatures and models for predicting toxicity
- The TXG-MAP: a network-based approach for understanding mechanisms of toxicity
- In vivo vs. in vitro: can we use cultured cells for MoA determination?
- Challenges with whole-tissue gene expression analysis

Overview: gene expression signatures

- Training set: expression profiling of liver tissue after treatment with ‘toxic’ (e.g. ALT inducers) and ‘non-toxic’ doses of various compounds
- Supervised learning approaches (e.g. support vector machines) identify patterns of expression that differentiate two groups
- Application of model for classifying samples with unknown toxicity outcome
- Many applications in toxicogenomics: DrugMatrix, MAQC II, etc.

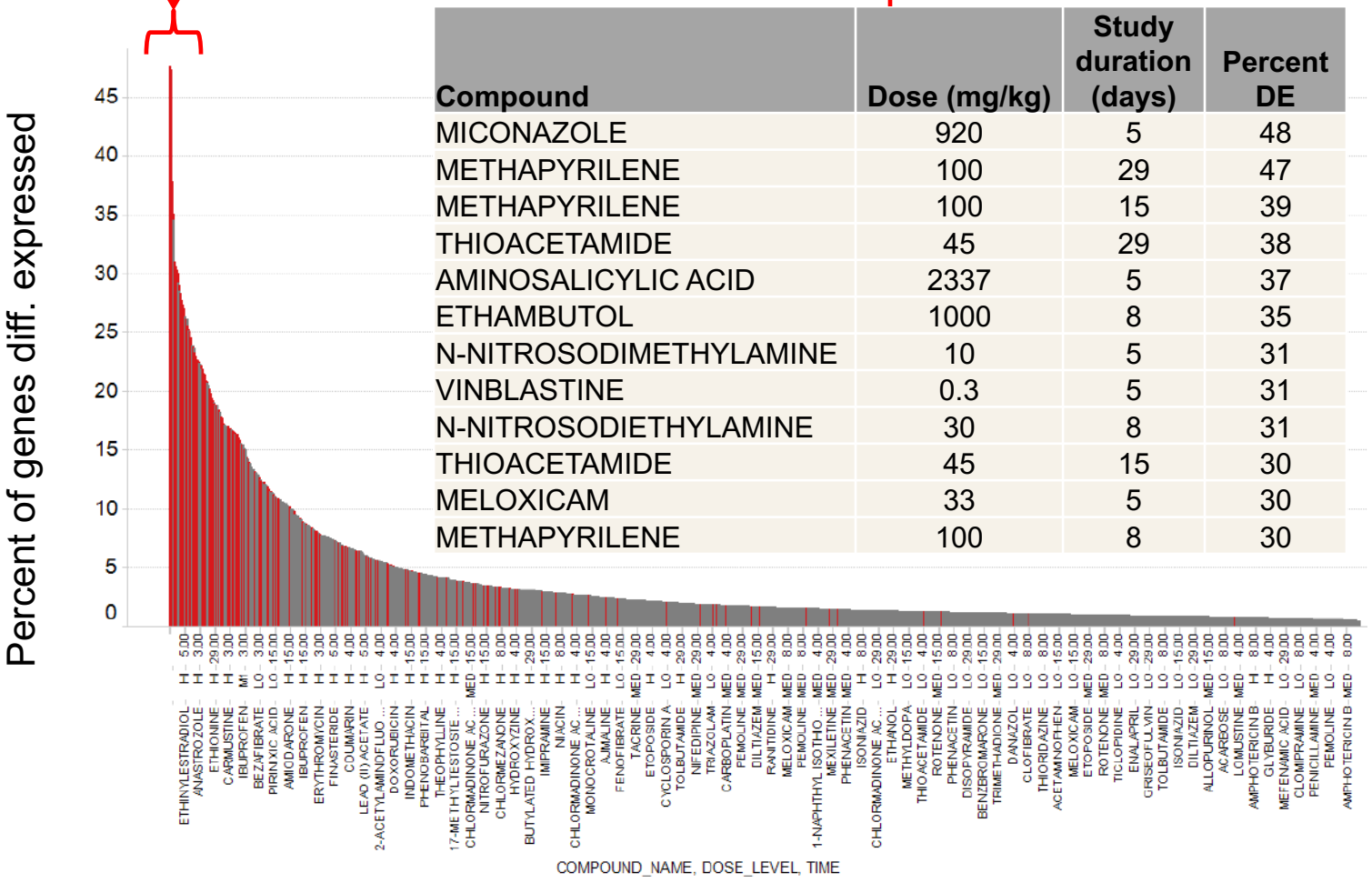


Gene signatures and other ‘omic predictors of toxicity: a well-trod path



liver AND toxicity AND (“toxicogenomic*" OR "gene signature*" OR "expression signature*" OR "systems biology" OR "molecular network*")

Overall percent of genes differentially expressed is prognostic of tissue injury



Compound	Dose (mg/kg)	Study duration (days)	Percent DE
MICONAZOLE	920	5	48
METHAPYRILENE	100	29	47
METHAPYRILENE	100	15	39
THIOACETAMIDE	45	29	38
AMINOSALICYLIC ACID	2337	5	37
ETHAMBUTOL	1000	8	35
N-NITROSODIMETHYLAMINE	10	5	31
VINBLASTINE	0.3	5	31
N-NITROSODIETHYLAMINE	30	8	31
THIOACETAMIDE	45	15	30
MELOXICAM	33	5	30
METHAPYRILENE	100	8	30

- 1895 treatments, of which 220 cause adverse liver pathology in repeat dose studies
- 85/119 treatments causing >=15% gene DE have adverse liver pathology
- gene differentially expressed when abs(FC) > 1.5 with limma p-value < 0.05 on 9074 liver expressed gene set

Assessing gene expression-derived features for adverse outcome analysis

- Expression data from 362 single dose experiments of 24 hr duration **predictive** of outcome in 29 day repeat-dose experiment (TG-GATEs data; adverse outcome = hepatocellular necrosis, bile duct hyperplasia or fibrosis)

- Evaluate whether a gene expression-derived score is a significant variable in a logistic regression model that uses overall transcriptional activity as a covariate (avg EG – average absolute eigengene): coefficient and p-value for β2

$$\ln \frac{P_{AO}}{1 - P_{AO}} = \beta_0 + \beta_1 \cdot Avg\ module\ score + \beta_2 \cdot module\ score$$

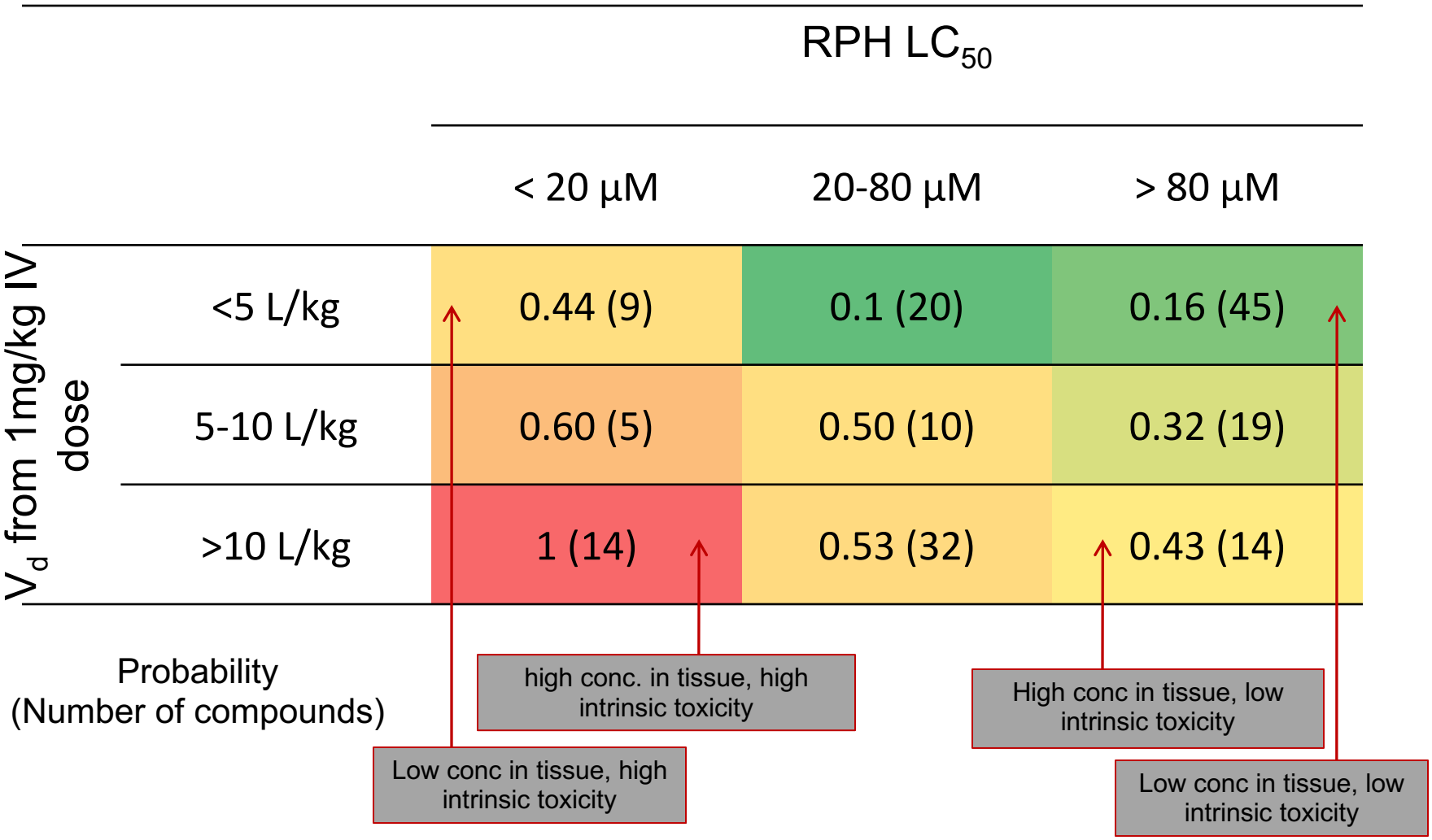
Method	p-value not adjusted for avgEG
Zhang et al 4 gene signature (Pharmacogenomics J, 2013)	7.4E-11
DrugMatrix ALT signature (ASPLP)	1.3E-05
module 69 (cell-cell junction; flotilin complex)	2.5E-11
module 320 (oxidative stress (Txnrd1)	0.74

… no need for any of those over-complicating bioinformaticians (who don't do any real work) … let's just run a qPCR panel and average 10-20 genes …

… is that any better than LDH release? …

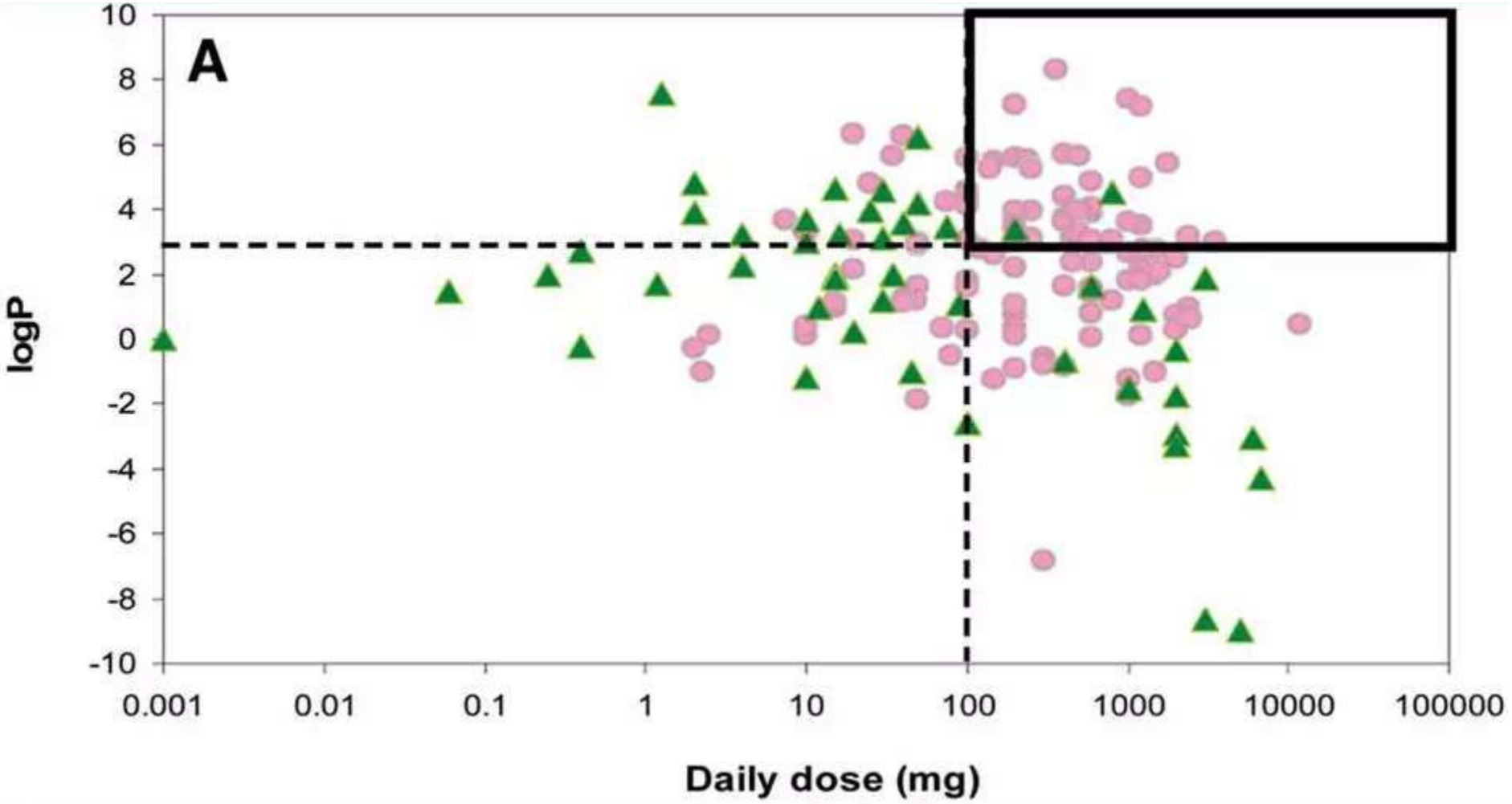
… or in silico endpoints (cLogP, QSAR) ?

Lilly risk-grid for estimating probability of adverse outcomes in 4 day rat tox studies



Sutherland et al, J Med Chem, 2012

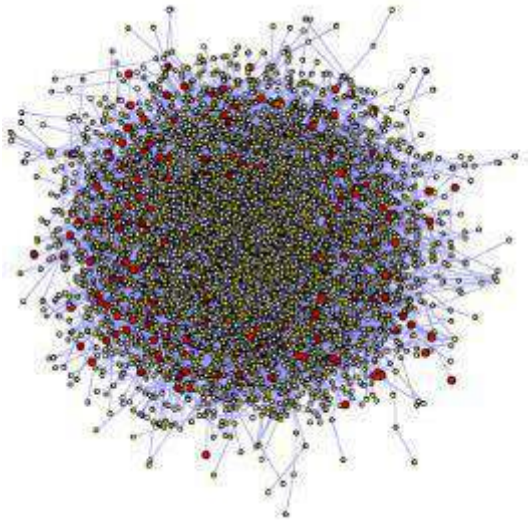
Chen / Tong “rule of two” – lipophilicity and daily dose vs. DILI



Chen et al, Hepatology 2013

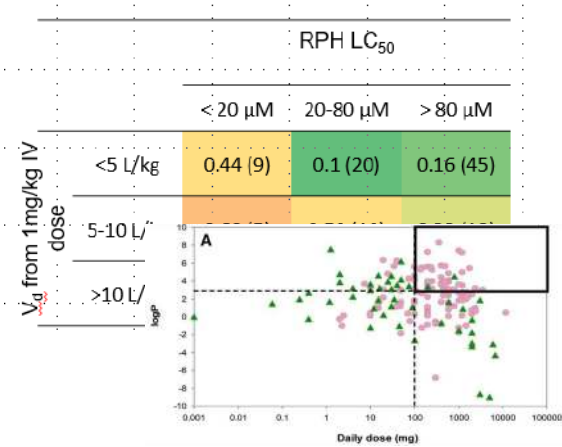
Is fancier better?

Gene signatures and systems biology



qPCR panel (transcriptional "temperature")

In-silico / in-vitro derived rules



Summary (1)

- Gene expression signatures can predict liver injury
- Counting the number of differentially expressed genes in liver is predictive of liver injury
- Simple in-silico approaches are predictive …
- Must prove the added-utility given added complexity

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When the prediction failed: understanding MOA when unexpected toxicity arises

Probability (Number of compounds)	RPH LC_{50} < 20 µM	RPH LC_{50} 20-80 µM	RPH LC_{50} > 80 µM
V_d from 1mg/kg IV dose <5 L/kg	0.44 (9)	0.1 (20)	0.16 (45)
V_d from 1mg/kg IV dose 5-10 L/kg	0.60 (5)	0.50 (10)	0.32 (19)
V_d from 1mg/kg IV dose >10 L/kg	1 (14)	0.53 (32)	0.43 (14)

22% of molecules in low-moderate risk bins produce adverse outcomes in 4 day rat tox studies

Beyond prediction: transcriptomics and safety assessment

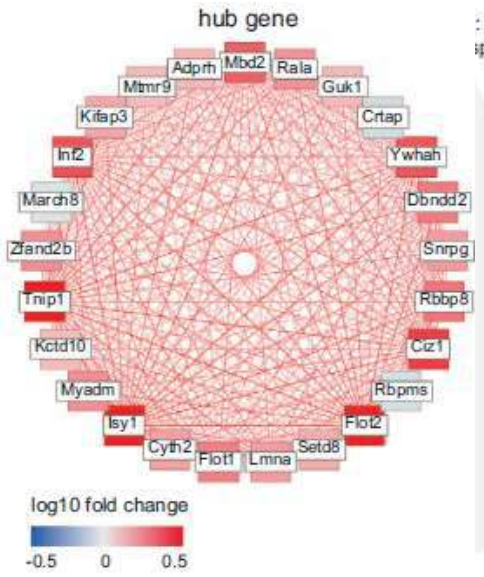
- What is the MOA leading to toxicity?
- Can we develop an a qPCR panel for SAR purposes (i.e. rationally design a better molecule)?
- Is it relevant in humans?
  - Network preservation
- Is it monitorable in humans?
  - Measurable biomarkers in the network?

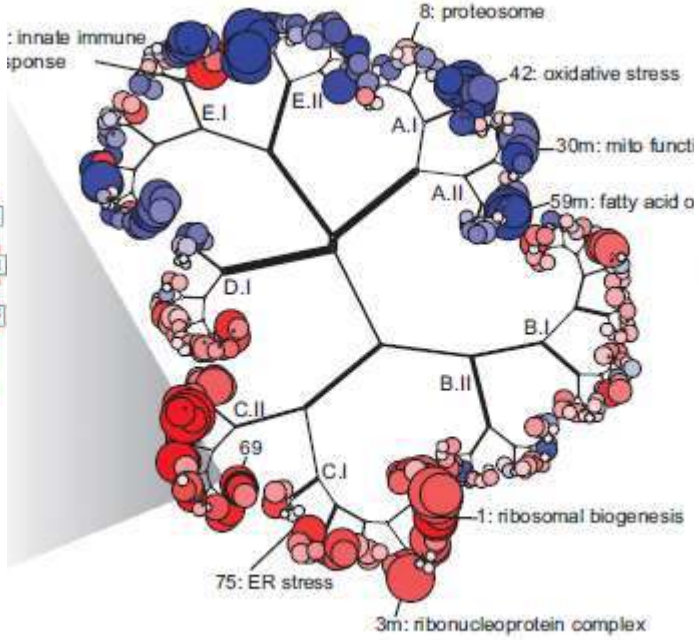
The TXG-Map in a nutshell

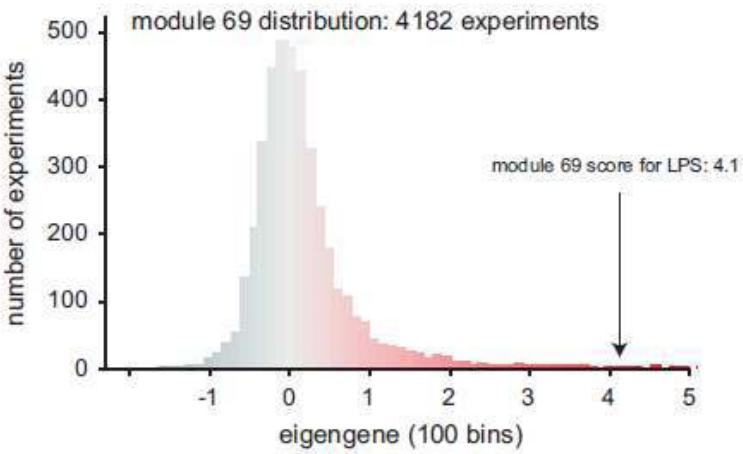
co-expression networks using WGCNA and DM liver data

organized in phylogenetic-tree like map to analyze individual treatments (here: LPS in rat liver)

Understand treatment effect in context of 4182 DM and TG rat liver experiments

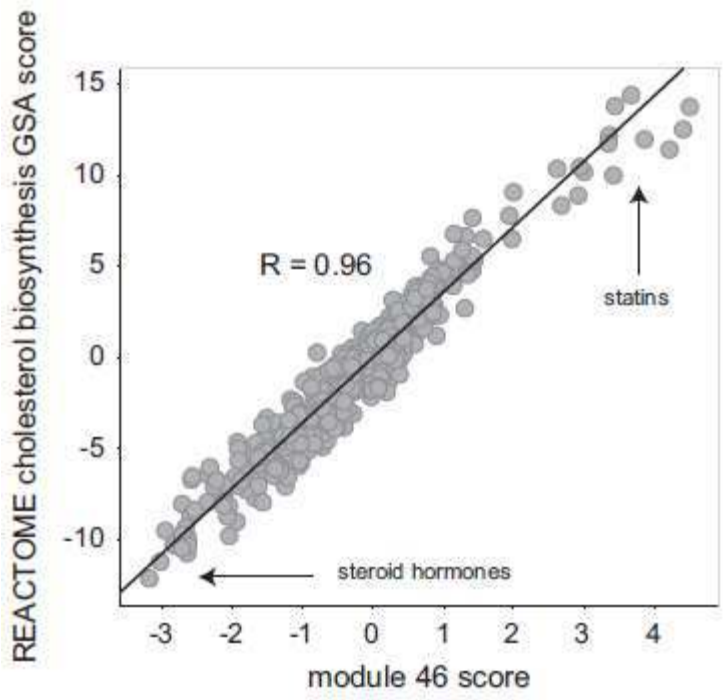


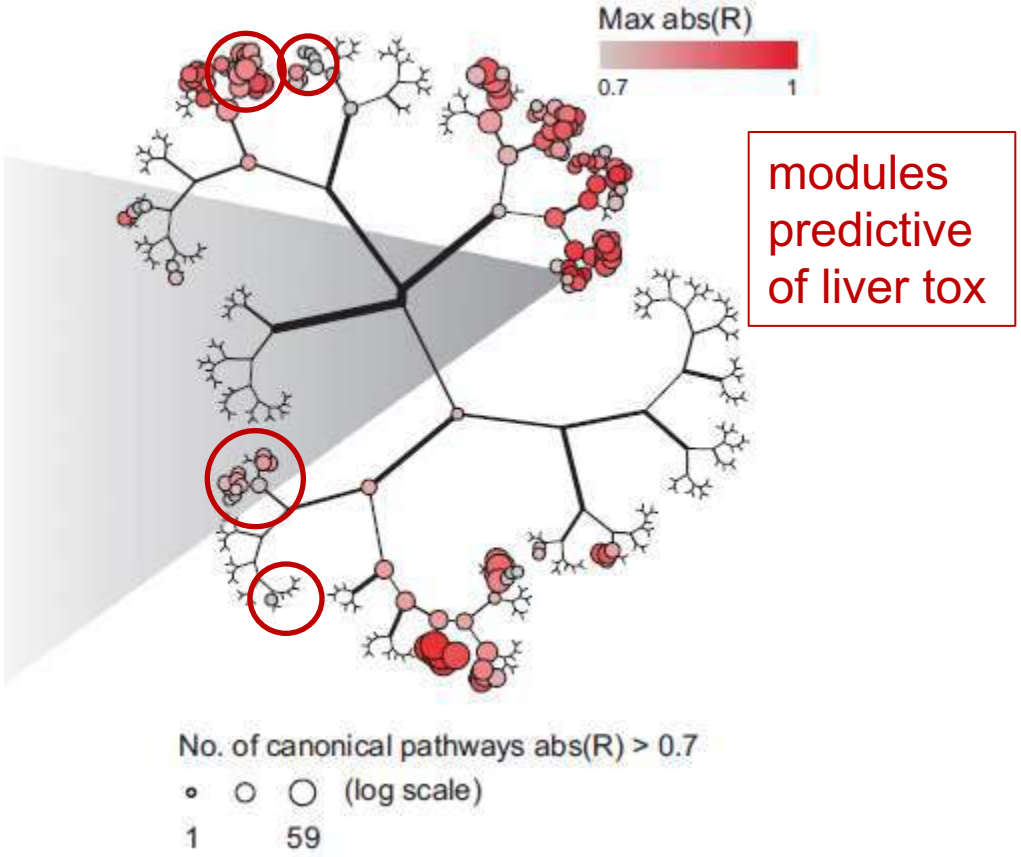




Sutherland et al, Pharmacogenomics J, 2017

What's wrong with pathway analysis?

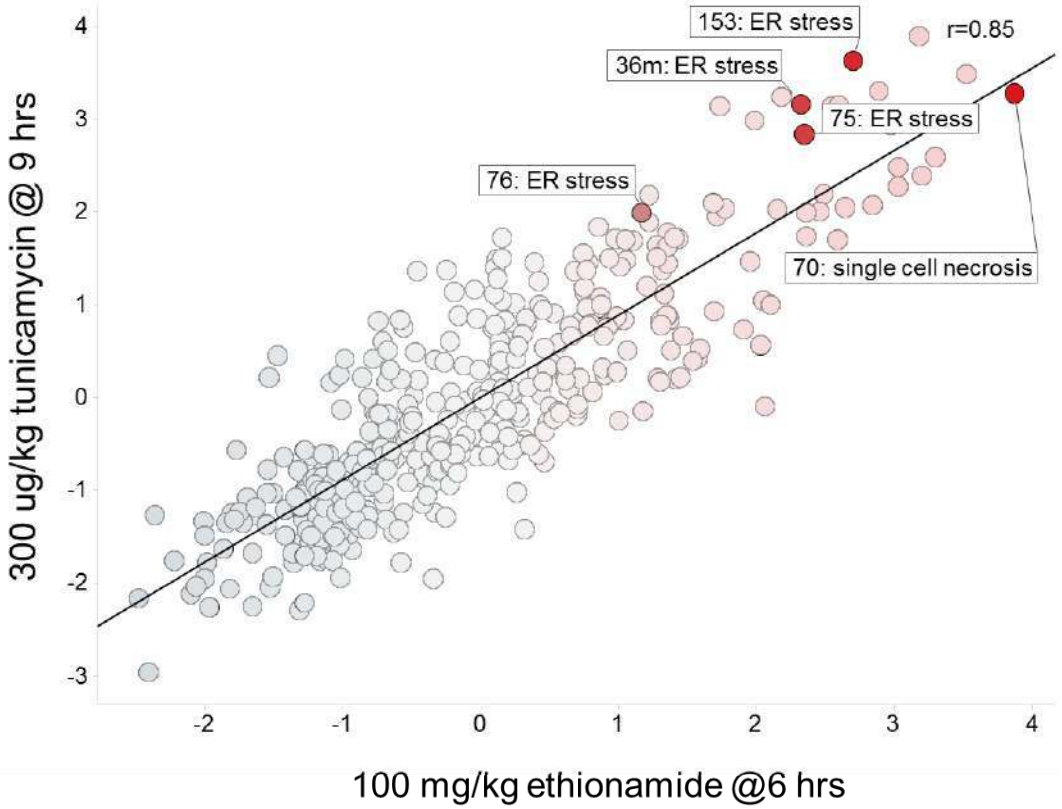


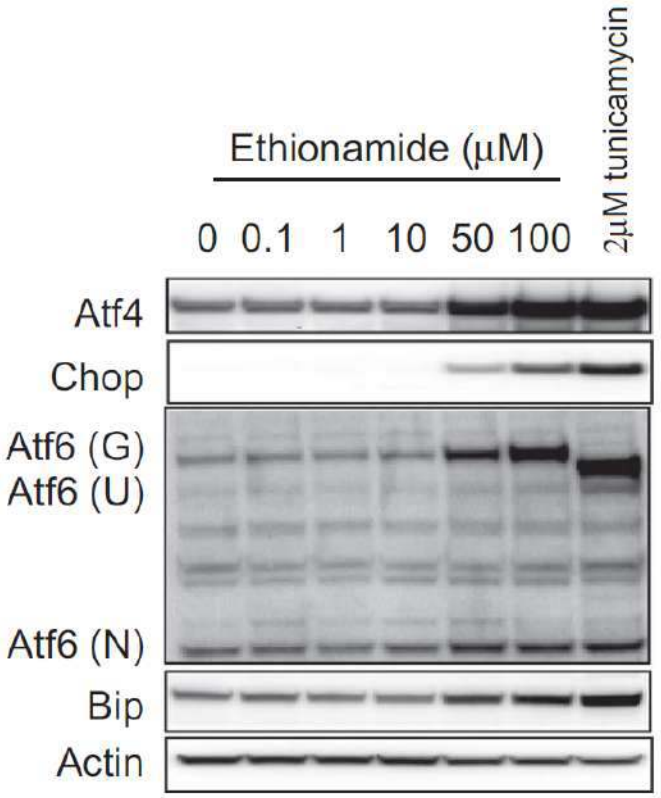


modules predictive of liver tox

- GSEA on canonical pathways sometimes gives similar results as module analysis (e.g. module 46 and cholesterol biosynthesis)
- Large areas of co-expression biology are not represented by pathways

MoA of ethionamide toxicity

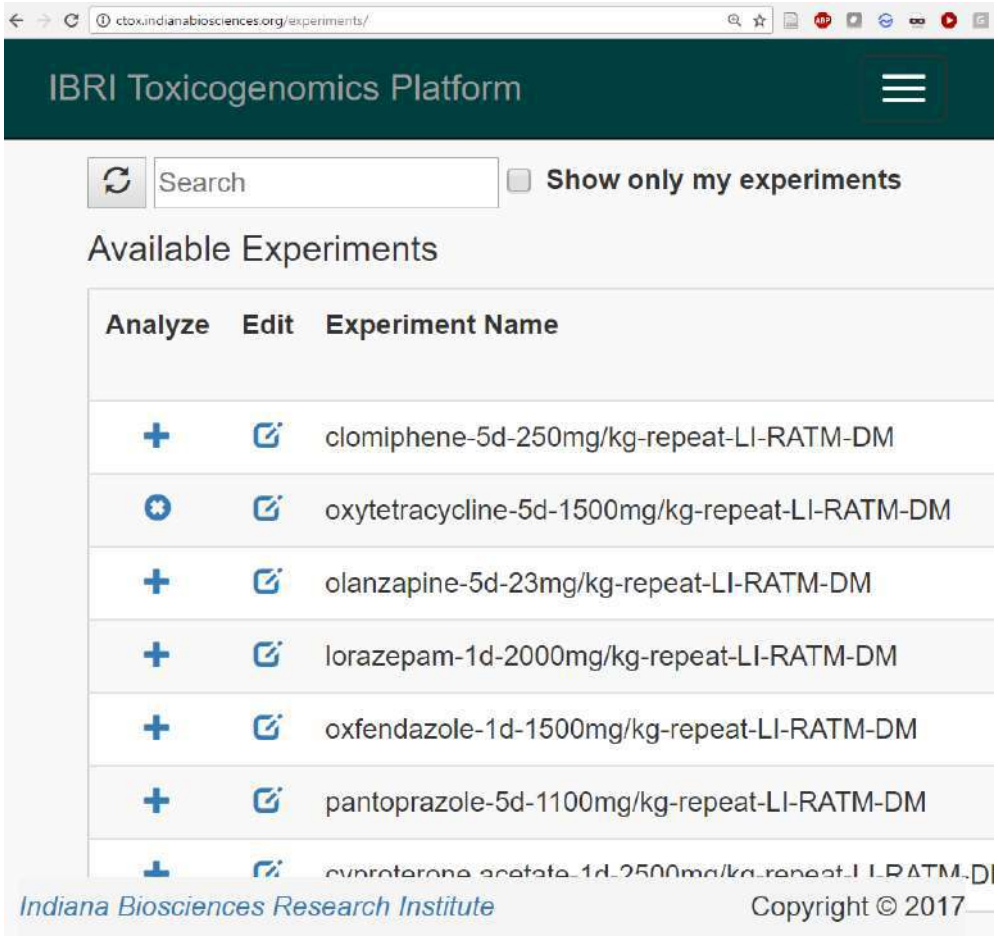




- When using module scores as ‘expression phenotype’, ethionamide strongly resembles tunicamycin
- Modules associated with ER stress and single cell necrosis highly induced

Developing an open-source platform for toxicogenomics research

- Cloud-hosted platform to access data and computational methods to increase reproducibility and ease of use for all scientists (not just the bioinformatics nerds)
- Collaboration between Indiana Biosciences Research Institute, Dow Agrosciences and Eli Lilly; additional participants welcome
- Current status: proof-of-concept website allowing access to DM, TG data and various analysis methods
- Contact Dan Robertson at IBRI, drobertson@indianabiosciences.org
- Access at http://ctox.indianabiosciences.org



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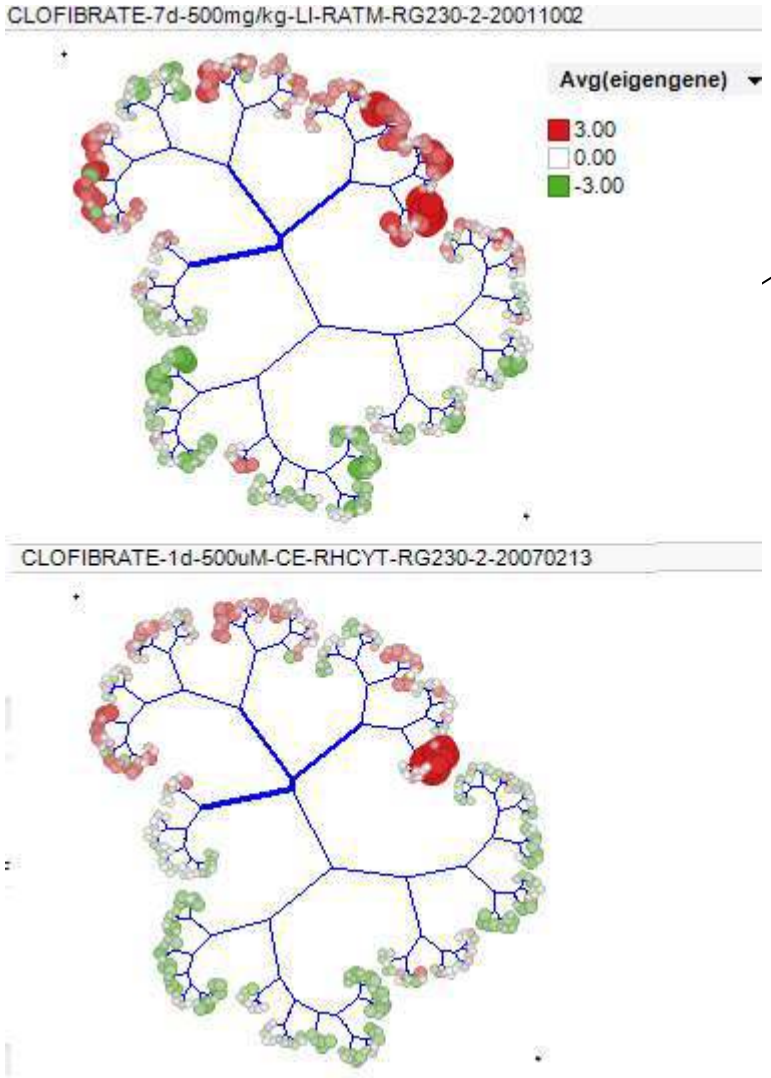
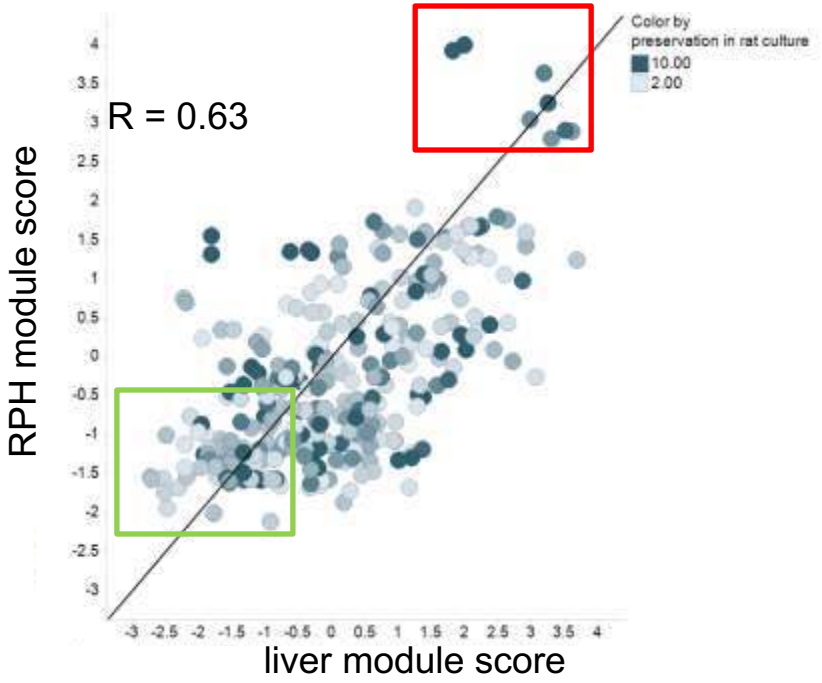
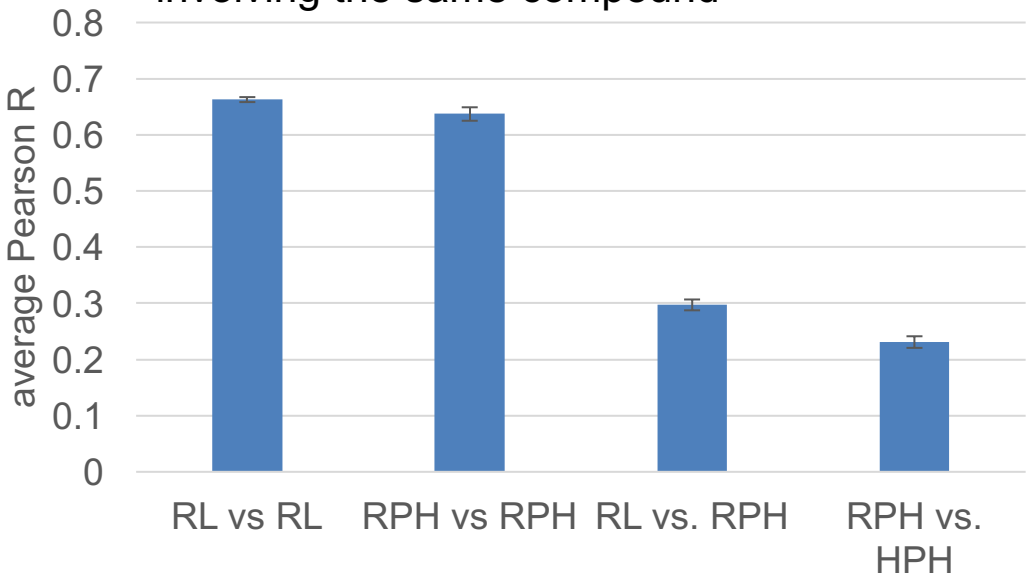
Rat Liver (RL) vs rat primary hepatocyte (RPH) Correlation



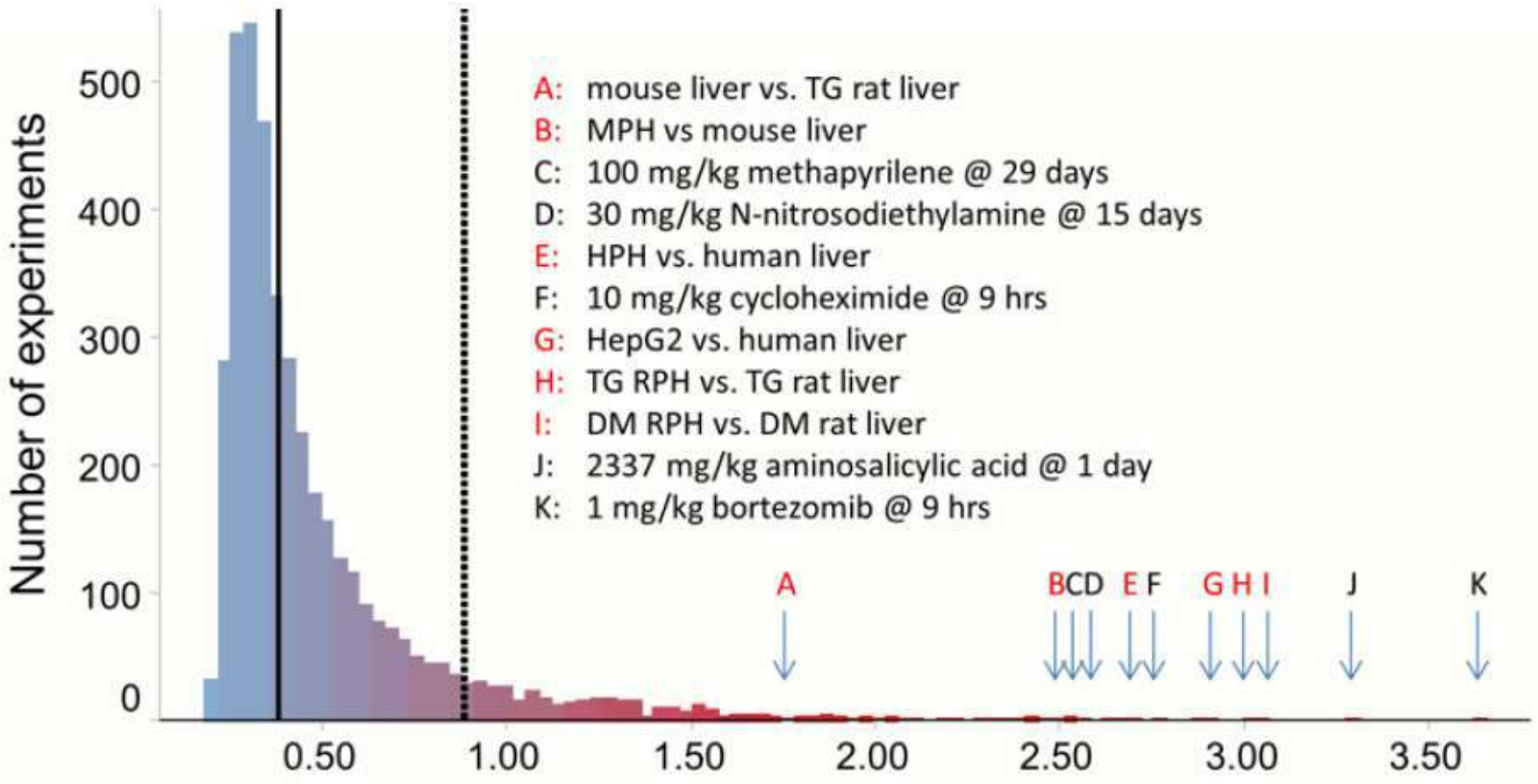


Comparison of TG-GATEs experiments involving the same compound



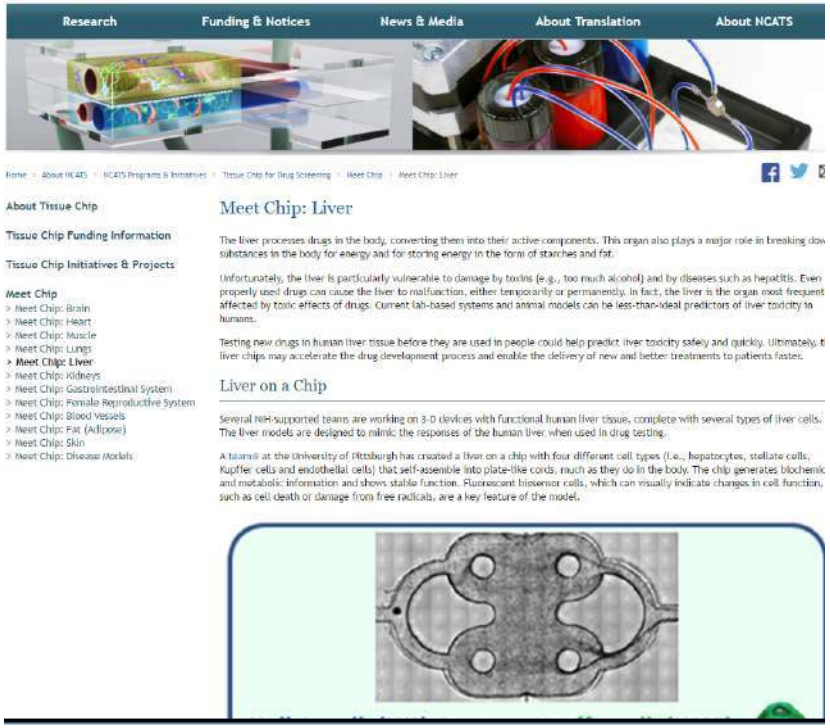
Sutherland et al, PLOS Comput Biol, 2016

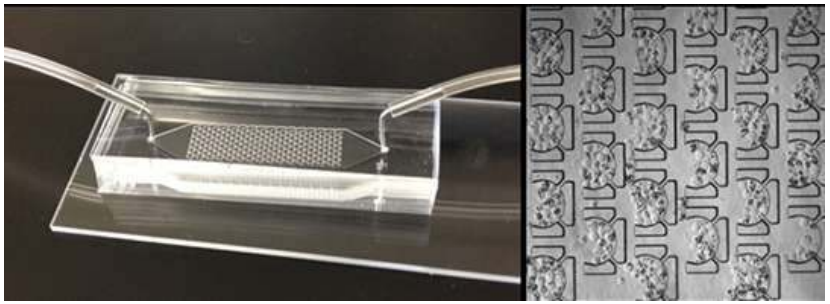
Effect of placing hepatocytes in culture in the context of ~4000 rat liver experiments



Average module score
(degree of transcriptional perturbation)

Flat culture is crude … what about other approaches?





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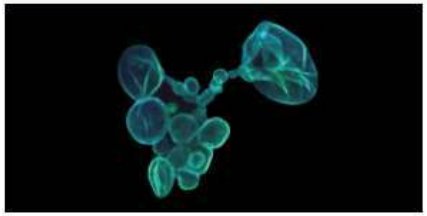

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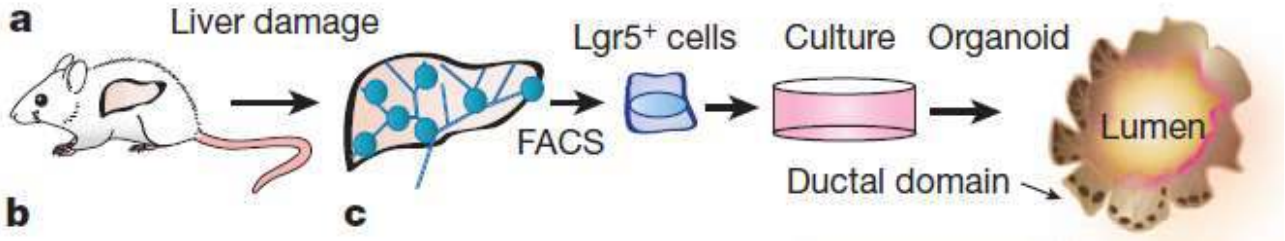
Rat liver organoids from Huch et al.

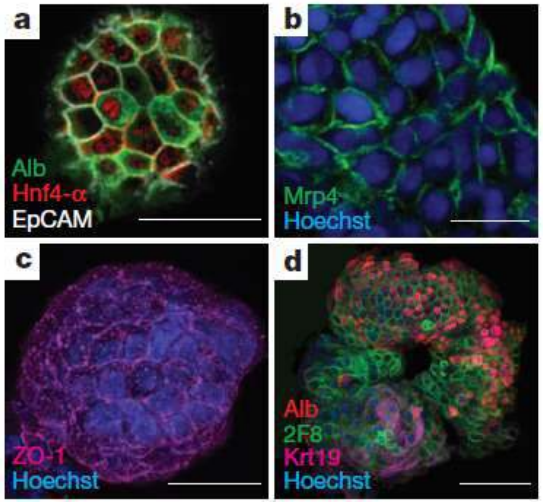
NATURE | LETTER

日本語要約

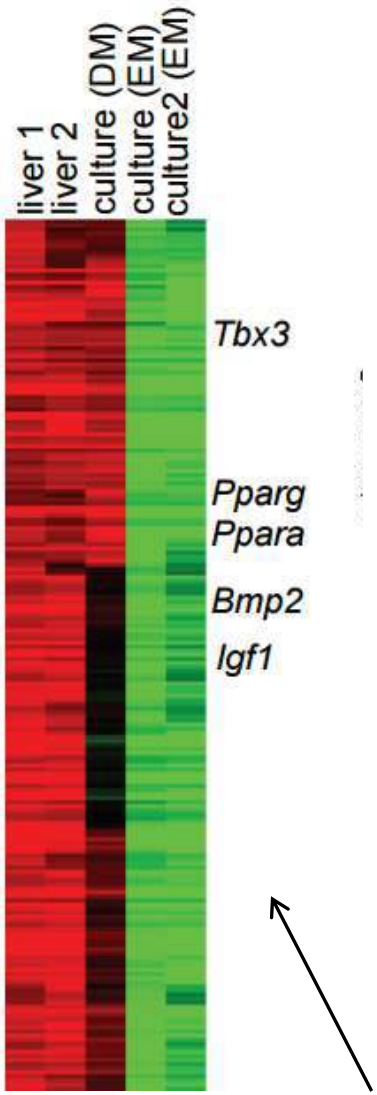
In vitro expansion of single Lgr5$^+$ liver stem cells induced by Wnt-driven regeneration

Meritxell Huch, Craig Dorrell, Sylvia F. Boj, Johan H. van Es, Vivian S. W. Li, Marc van de Wetering, Toshiro Sato, Karien Hamer, Nobuo Sasaki, Milton J. Finegold, Annelise Haft, Robert G. Vries, Markus Grompe & Hans Clevers



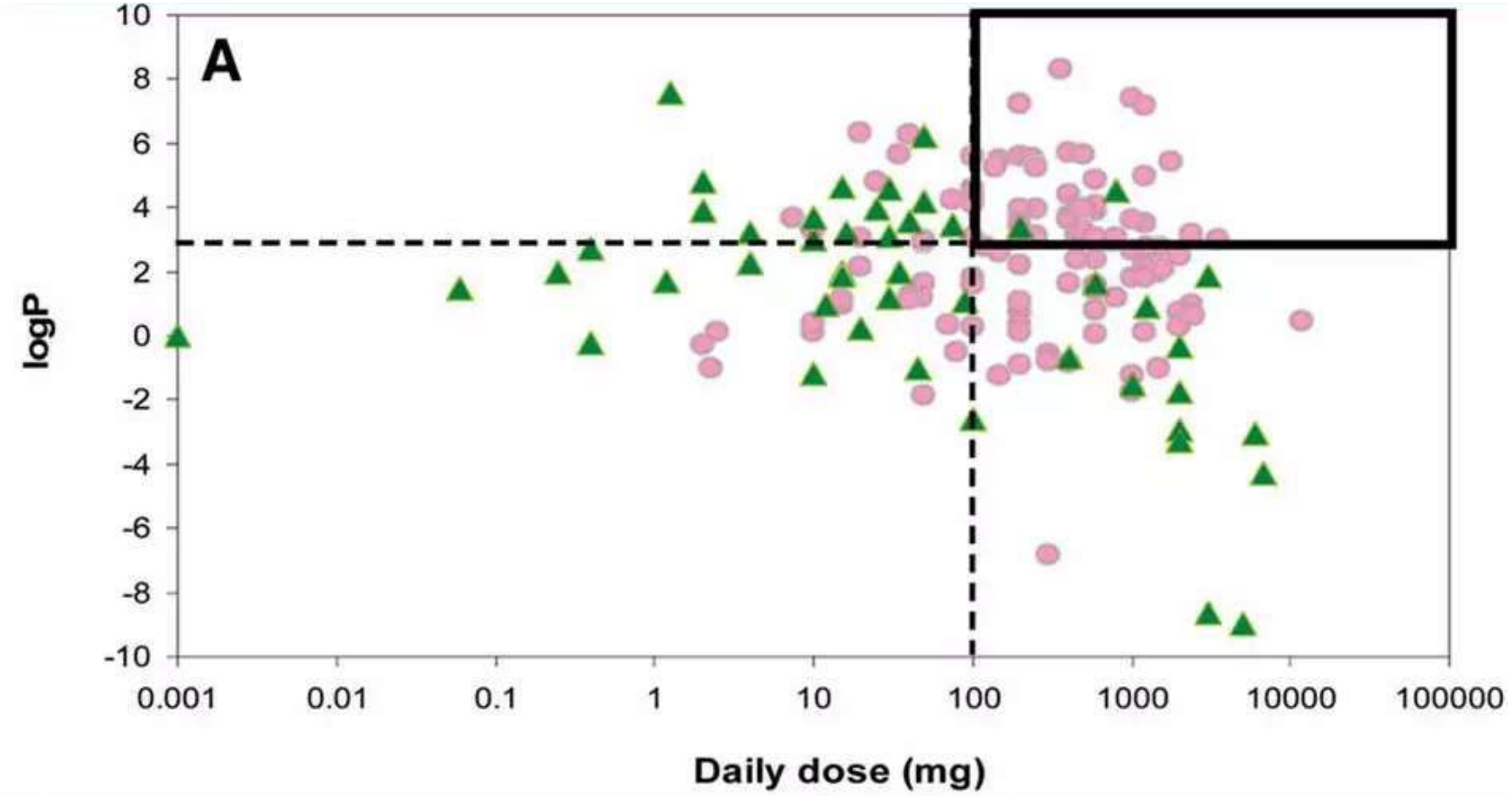


Liver organoids in culture with differentiation media



View in the paper

Classifying human DILI compounds is not enough …



Chen et al, Hepatology 2013

Summary

- Culture model evaluation using rodent cells: ~5000 rat liver treatments and dozens of models with expression data, vs. 0 treatments and ~10 diseases for human liver
- Viability for 60 days isn't enough (HepG2 cells are viable forever)
- Evaluate the extend to which culture models return cells to baseline transcriptional state observed in intact liver
- Which culture models recapitulate known MoA for same well-studied toxicants?

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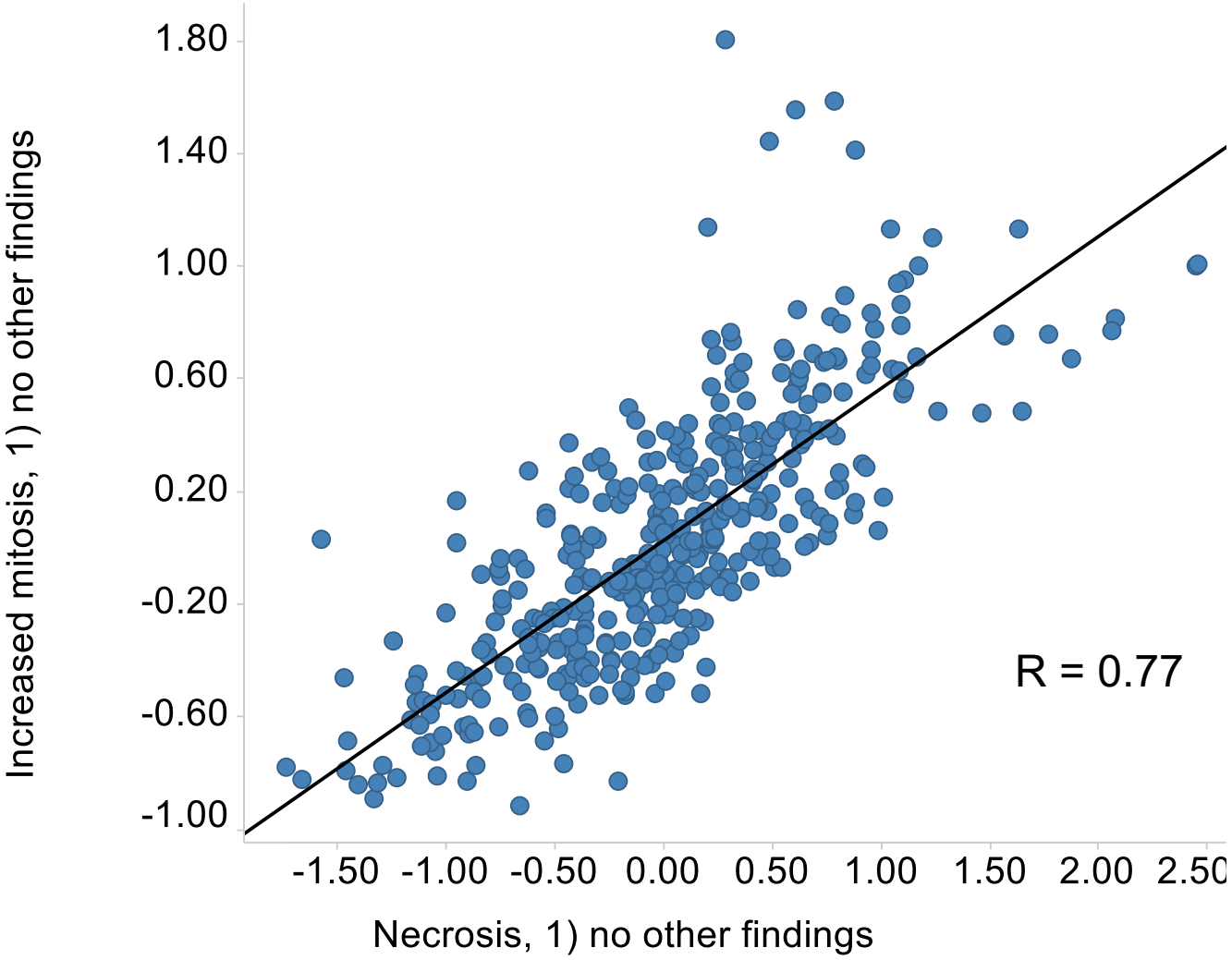
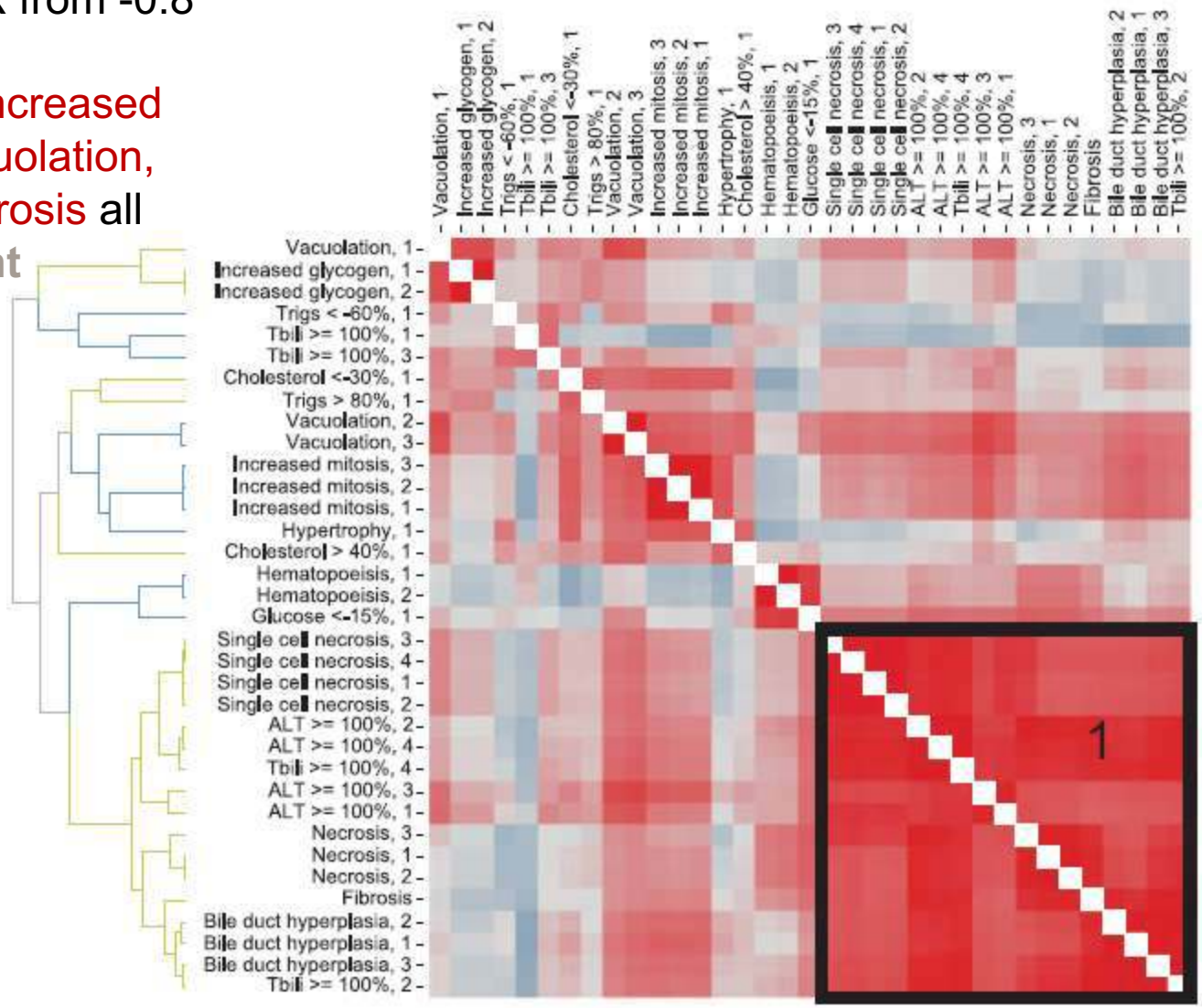
Module effect sizes for increased mitosis and necrosis are correlated



Effect size (Cohen's d) = (<score for livers with phenotype> - <score livers without phenotype>) / pooled score stdev

Pairwise comparisons of 36 tox phenotypes on effect size

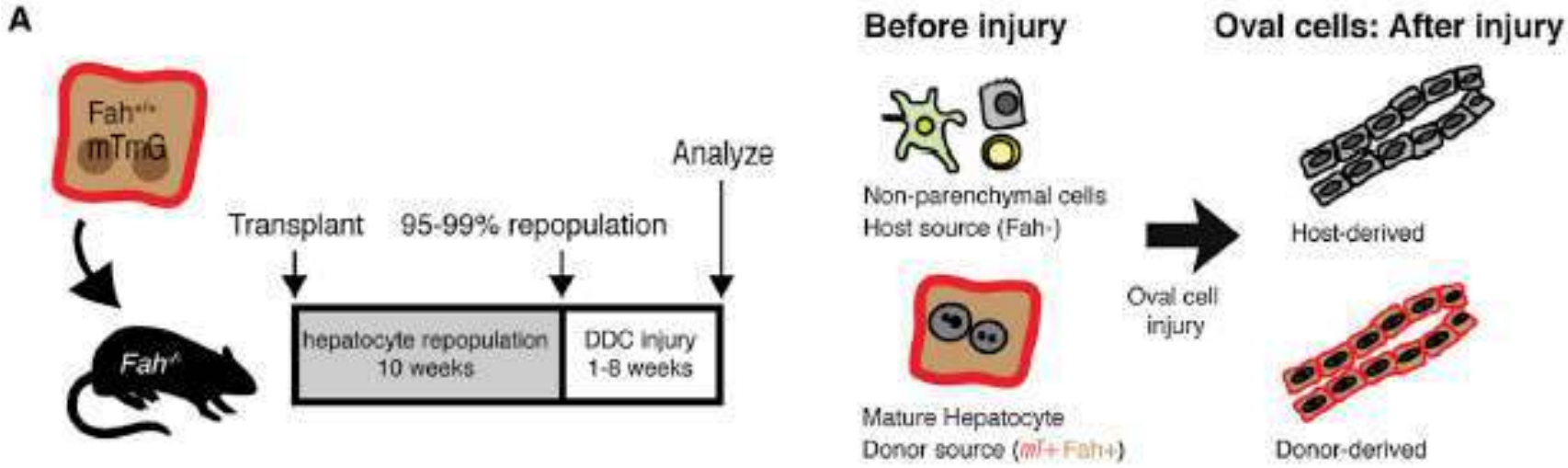
- Colored on Pearson R from -0.8 (blue) to 0.8 (red)
- Single cell necrosis, increased mitosis, necrosis, vacuolation, biliary hyperplasia, fibrosis all cluster in **bottom-right**



Sutherland et al,
Pharmacogenomics J, 2017

Lineage tracing and FACS sorting in liver injury models

- 78% of liver volume is hepatocytes, 15% empty space, 3% endothelial cells, 2% Kuppfer cells, 1% fat-storing cells, 1% hepatic stellate cells
- Transcript number is proportional to cell volume (Kempe et al, Mol Biol Cell 2015, 15:797)
- But hepatocytes are increasingly recognized as plastic cells …



"Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes", Tarlow et al. Cell Stem Cell 2014, 15: 605

Comparing gene expression of various hepatic cell types

1) Calculate a “fold change” for each gene that would arise upon conversion of one cell type to another
2) Score fold change data with WGCNA modules
3) Evaluate the extent to which expression change in whole liver can be explained by changing stoichiometry of cell types

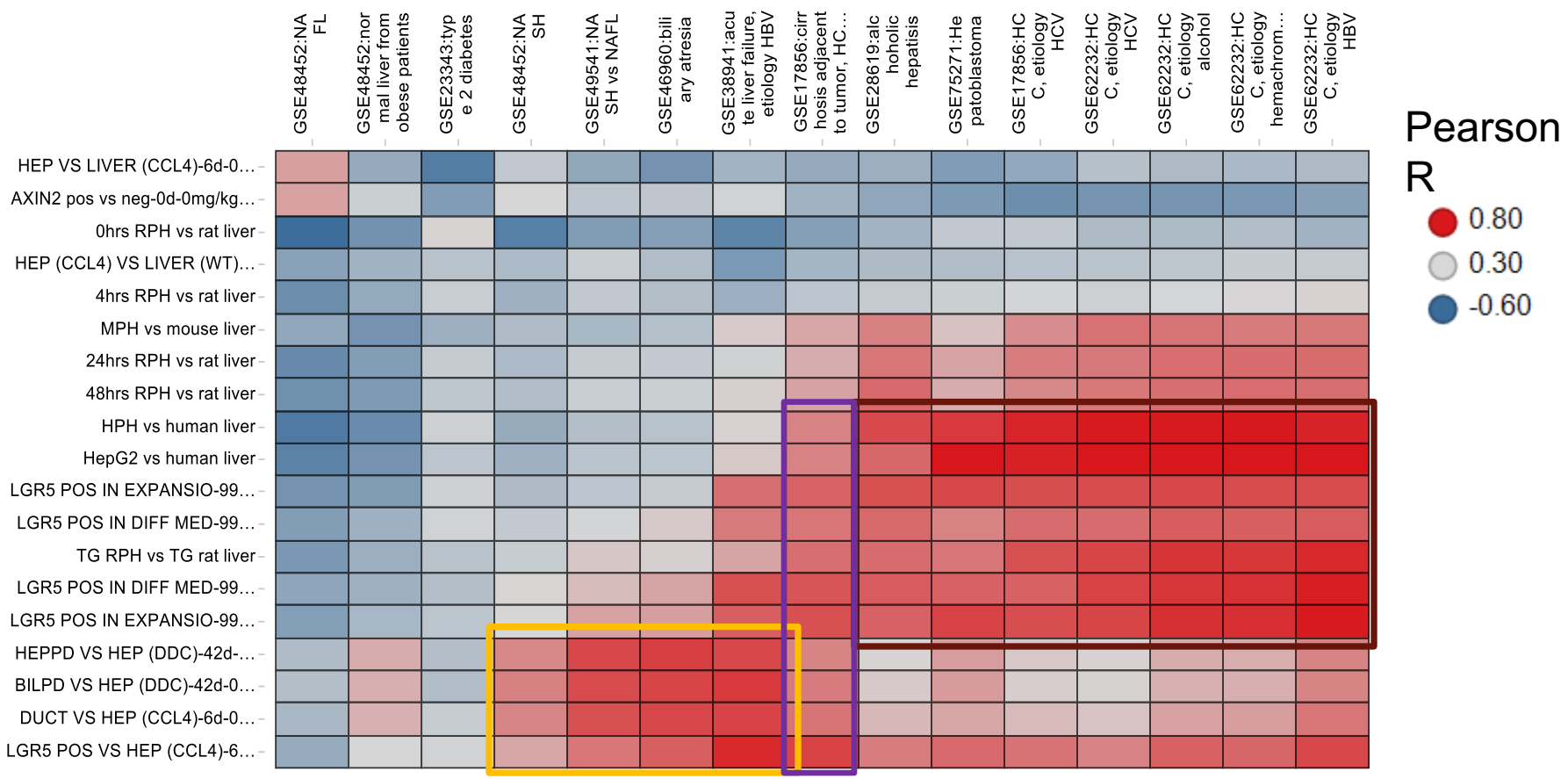
Data sources:

- Axin2+ heps vs Axin2- heps (untreated; GSE68806)
- duct vs hep (CCl4-treated; GSE32210)
- duct vs liver (CCl4-treated; GSE32210)
- hep vs liver (CCl4-treated; GSE32210)
- Lgr5+ vs hep (CCl4-treated; GSE32210)
- Lgr5+ vs liver (CCl4-treated; GSE32210)
- bilPD vs hep (DDC-treated; GSE55552)
- bilPD vs hepPD (DDC-treated; GSE55552)
- hepPD vs hep (DDC-treated; GSE55552)

Comparing tox phenotypes to sorted cell comparisons

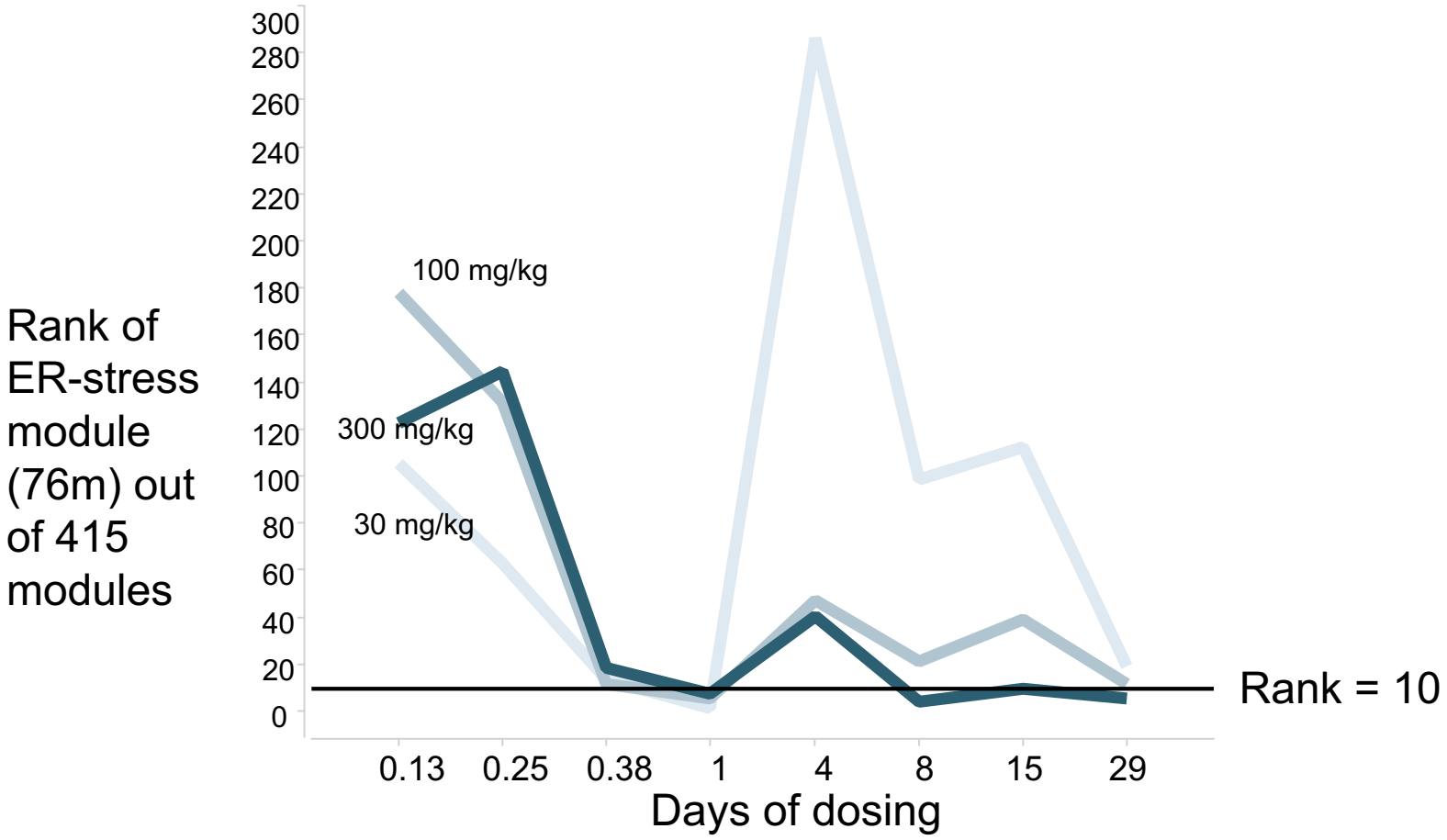
Tox phenotype or model	Most similar sorted cell comparison	R-most similar sorted cell comparison
Tbili >= 100%, 2) with hyperplasia at any grade	LGR5 POS VS LIVER (CCL4)	0.73
Fibrosis at any grade with any other pathology at any grade	LGR5 POS VS LIVER (CCL4)	0.70
Bile duct hyperplasia, 1) no other findings	LGR5 POS VS HEP (CCL4)	0.62
Single cell necrosis, 1) no other findings	DUCT VS HEP (CCL4)	0.61
Glucose <-15%, 1) no path findings and FC >-15%	DUCT VS LIVER (CCL4)	0.39
Hematopoeisis, 2) any other finding	DUCT VS LIVER (CCL4)	0.37
Necrosis, 1) no other findings	LGR5 POS VS HEP (CCL4)	0.33
Increased mitosis, 1) no other findings	LGR5 POS VS HEP (CCL4)	0.27
Tbili >= 100%, 1) no path findings	HEP VS LIVER (CCL4)	0.24
Cholesterol > 40%, 1) no path findings	HEP VS LIVER (CCL4)	0.21
Vacuolation, 2) allowing hypertrophy at any grade	LGR5 POS VS LIVER (CCL4)	0.14
Trigs > 80%, 1) no path findings	HEP VS LIVER (CCL4)	0.01
Hypertrophy >= 1.33, 2) no other finding	HEP VS LIVER (CCL4)	-0.01
Trigs < -60%, 1) no path findings and FC >-15%	HEP VS LIVER (CCL4)	-0.02
Cholesterol <-30%, 1) no path findings and FC >-15%	LGR5 POS VS HEP (CCL4)	-0.04
Vacuolation, 1) no other findings	LGR5 POS VS HEP (CCL4)	-0.04
Increased glycogen, 2) any other finding	LGR5 POS VS HEP (CCL4)	-0.09

Comparing human liver disease (change from normal) to sorted cell comparisons



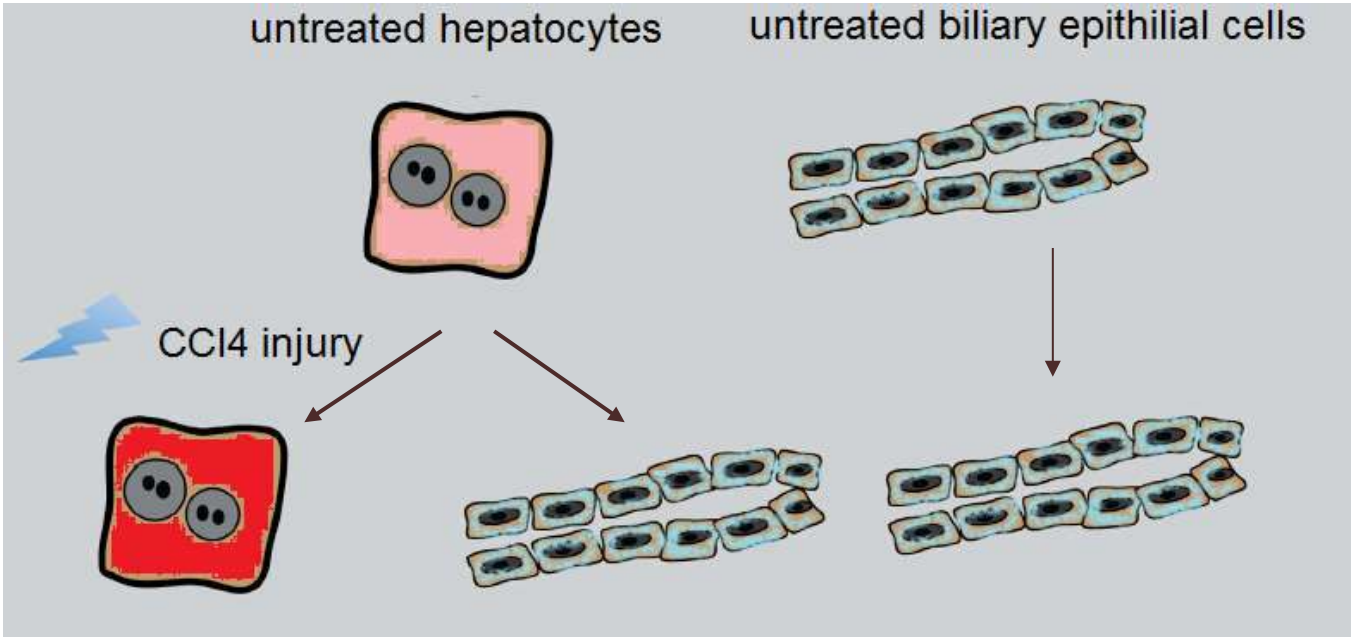
- NASH and biliary atresia expression changes explainable by **increased "duct-like" cells**
- HCC of various etiologies and hepatoblastoma explainable by changes seen in **cultured cells, including Lgr5+ cells**
- Non-tumor cirrhotic tissue from HCC patients **is intermediate**

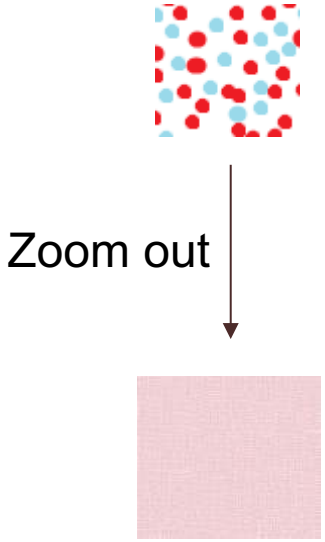
Whole liver gene expression analysis of CCl4 treatment: mostly the wrong answer



- rank of module 76m in top 10 only for 6 out of 24 TG rat liver experiments using CCl4 treatment (3 doses x 8 time points)

CCl4-treated rodent FACS sorted hepatocytes





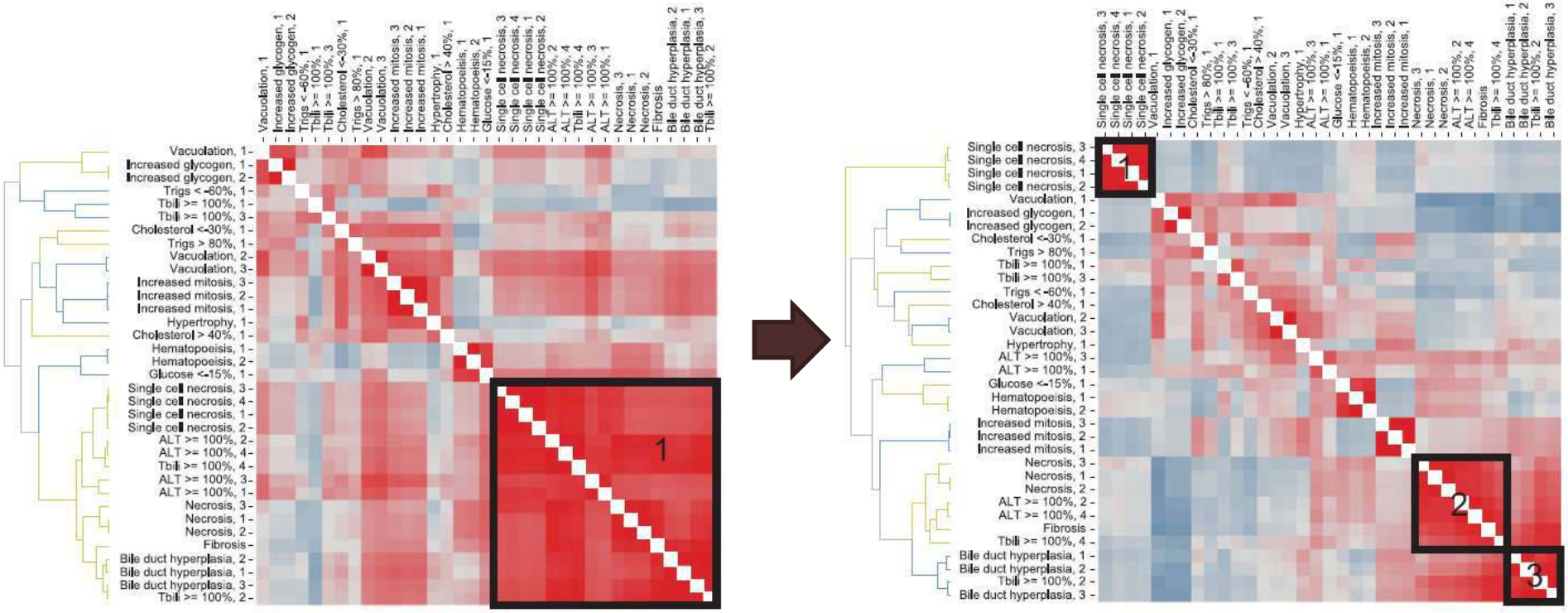
Adapted from Tarlow et al., Cell Stem Cell 2014, 15: 605

Amount of transcript for endoplasmic reticulum proteins

Rank of ER-stress module when analyzing expression of sorted heps: 3 out of 415

data from GSE32210 comparing sorted heps @ 6 days of CCl4 treatment vs. untreated liver

Role of average module score in uniquely associating modules with pathology



- Treating average module score as a covariate in associating module behavior with pathology resolves several histologically distinct phenotypes into separate clusters
- Hypothesis: average module score is an approximate surrogate for extent of ductular reaction (and hence liver injury)

Summary

- Several histologically-distinct injured states (“tox phenotypes”) of liver resemble each other when using whole liver gene expression data
- Several human liver diseases resemble each other when using whole liver gene expression
- The resemblance can be largely explained by increasing proportions of “hepPD”, “Lgr5+” of biliary epithelial cells in the liver
- Changing proportions of cell types may obscure underlying changes within each population
- Analyzing selected animal models via FACS-sorted cells may be worth considering

What does this mean for whole-organ expression profiling?

- No impact for ‘signature’ applications –“barcode” doesn’t look like the product but represents it uniquely
- If we care about mechanism however …
  - Statistics can help dissect a population of profiles but less useful for individual cases
  - Short duration studies (<12 hours) likely minimize effects of population changes
  - Long duration studies (and therefore analysis of human samples) may require single cell RNA-seq or FACS + conventional analysis to derive useful insights

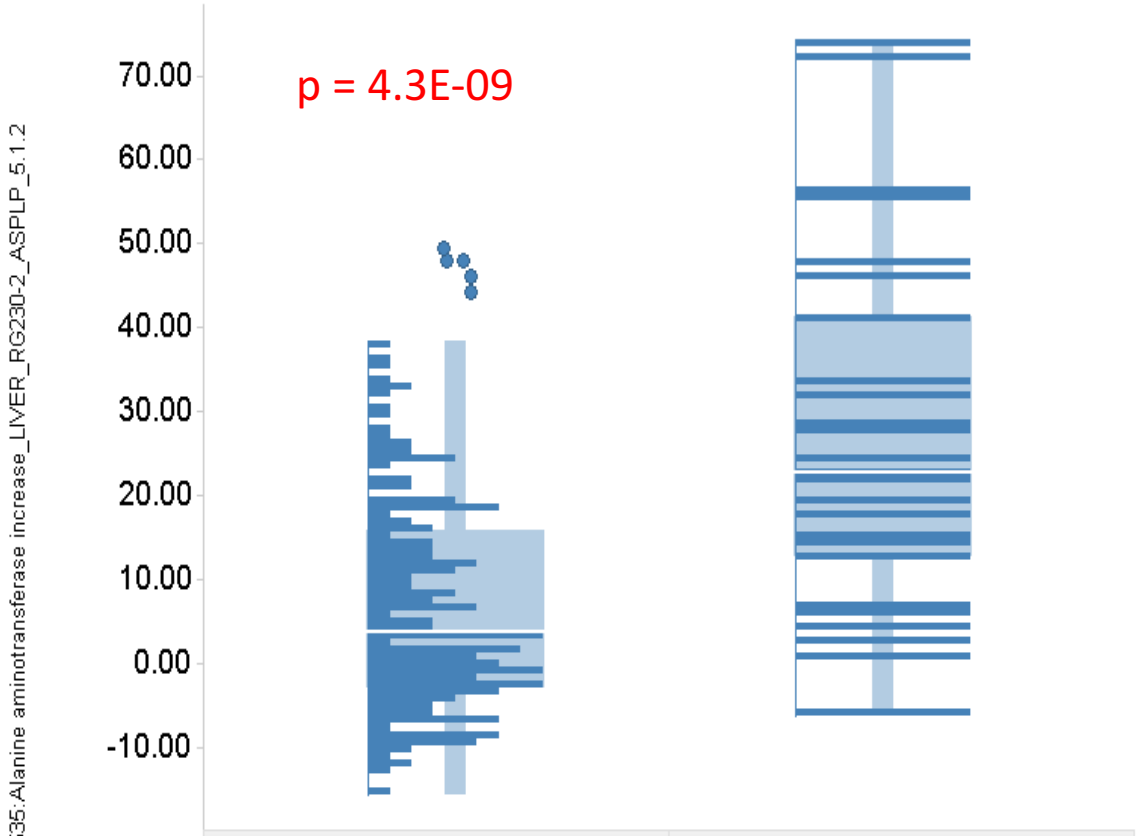
Gene signatures based on DM experiments "predict" adverse Lilly pathology outcomes

- forward-validation: signatures from DM data, validation on Lilly expression and tox outcomes

- matched histology vs. gene expression results for 201 treatment groups annotated with adverse (25) or non-adverse (176) histopath findings

- ANOVA on:
  - X: adverse/non-adverse
  - Y: DM signature score

- report p-value

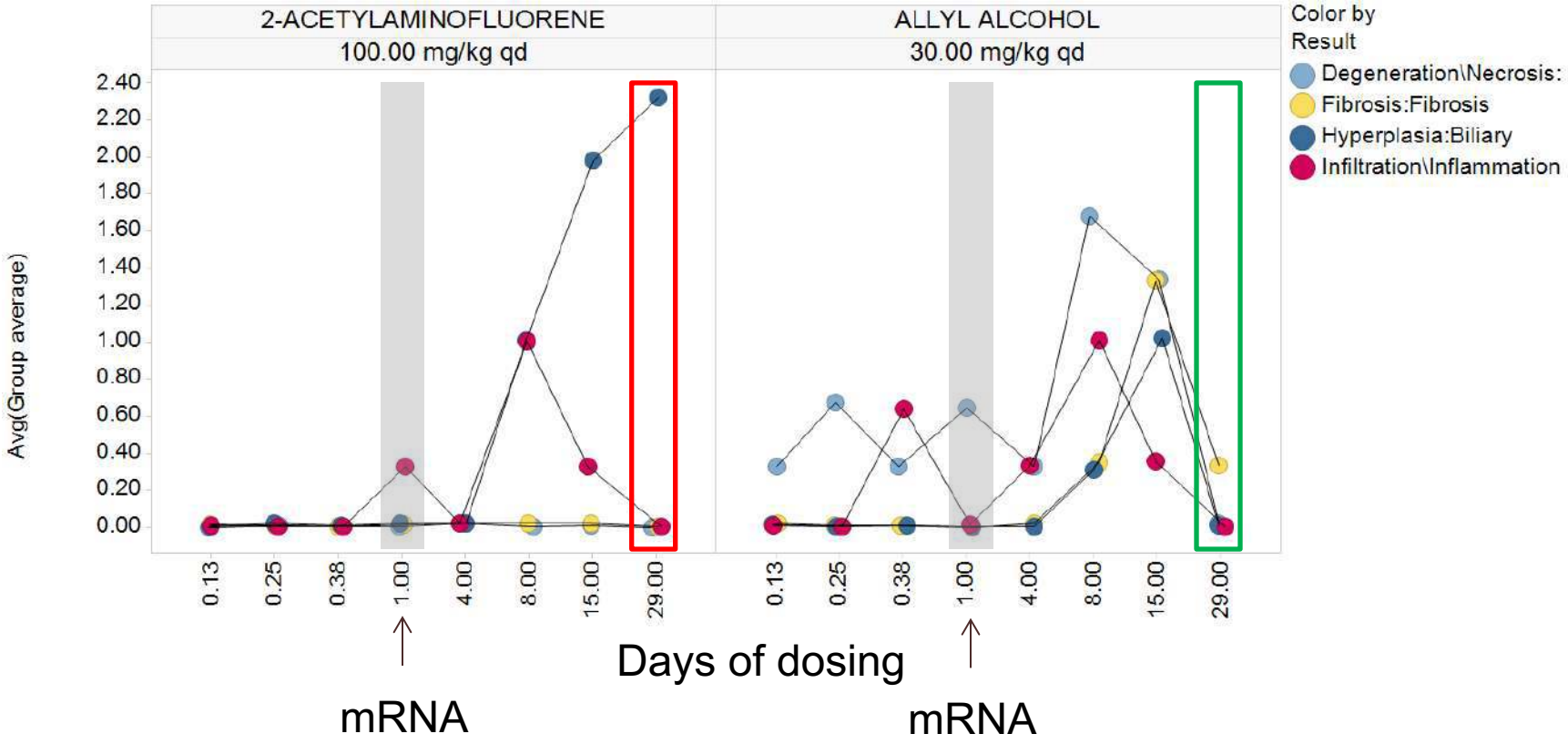


	No	Yes
Count	176	25
Median	3.748	22.762
Outliers	5	0

Prediction: "a statement about what will happen or might happen in the future"

Outcome at 29 days:
adverse

Outcome at 29 days:
non-adverse



- Predictive: mRNA from earlier tissue sample where pathology not present
- Concurrent: mRNA from tissue where the pathology is present

Impact of cell culture on hepatocyte expression

Typical drug-treatment experiment: What happens to hepatocytes inside a liver when exposed to drug

- Liver from drug treated animals (3) vs. liver from vehicle-treated animals (3)
- Calculate fold change for each gene: $\log\left(\frac{avg\ expression\ in\ treated\ animals}{avg\ expression\ in\ control\ animals}\right)$

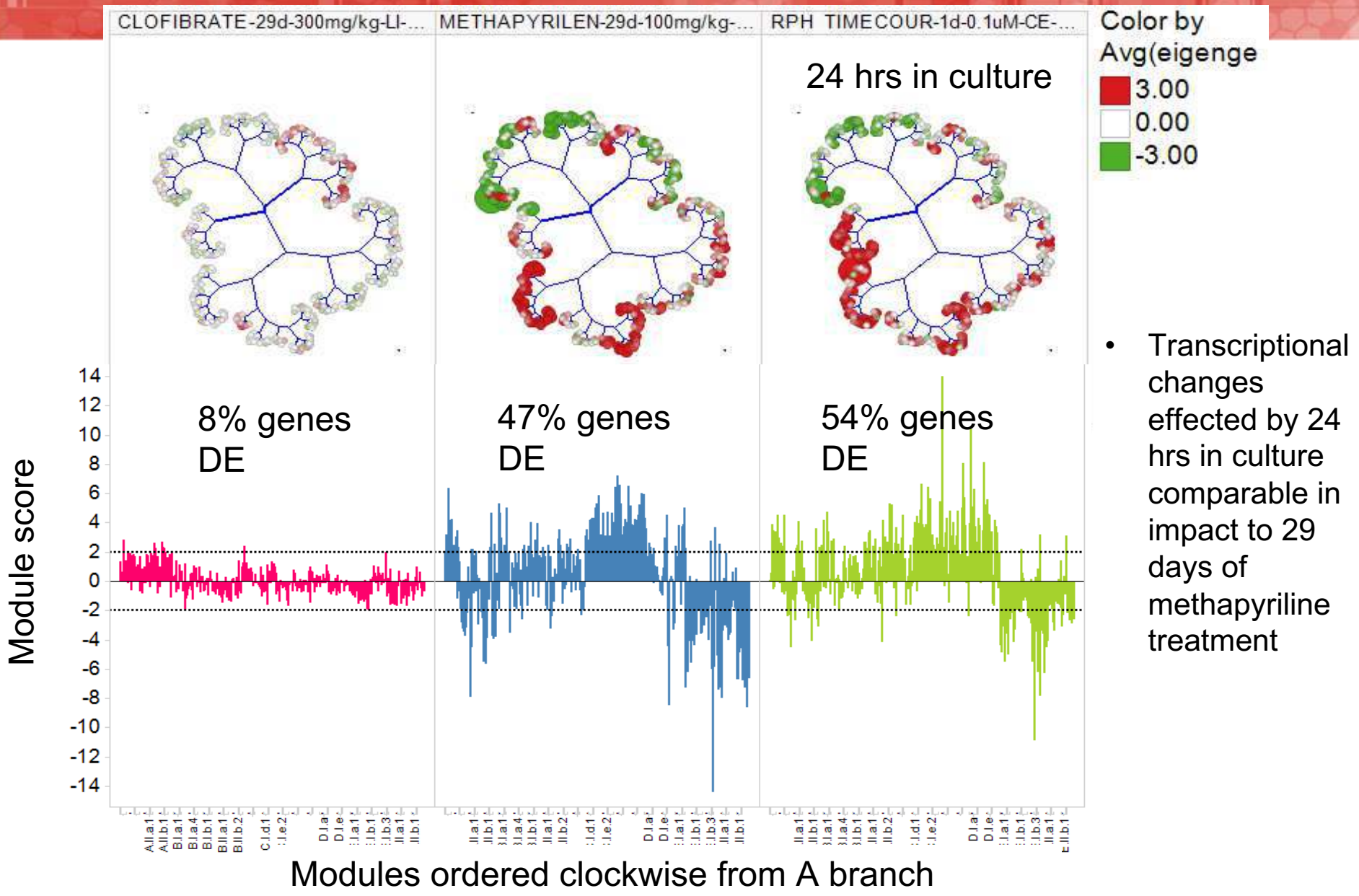
What happens to hepatocytes in culture, when compared back to their state in liver?

- Isolate hepatocytes with standard perfusion procedure
- Perform expression profiling at 0 hrs (immediately after isolation; no exposure to culture medium), 4, 24 and 48 hours in culture
- Calculate fold change for each gene:

- $\log\left(\frac{avg\ expression\ in\ culture\ at\ 4,24\ or\ 48\ hrs}{avg\ expression\ in\ untreated\ liver}\right)$ or
- $\log\left(\frac{avg\ expression\ in\ culture\ at\ 4,24\ or\ 48\ hrs}{avg\ expression\ at\ 0\ hrs}\right)$

Similar results; Sutherland et. al., PLOS Comput Biol 2016

Comparing transcriptional effects of clofibrate, methapyrilene and 24 hrs cell culture



- Transcriptional changes effected by 24 hrs in culture comparable in impact to 29 days of methapyriline treatment