ILSI Health and Environmental Sciences Institute (HESI)

Opportunities for Collaboration on In Vitro Testing Proposal

February 28, 2008

Meeting with HESI Genomics Committee Representatives, and EPA, NIEHS Staff

Research Triangle Park, NC

HESI Representatives

Dr. Jiri Aubrecht *(Pfizer)*
HESI Genomics Committee Chair

Dr. Albert Fornace *(Georgetown University)*
HESI Scientific Advisor

Dr. Robert Schiestl, *(UCLA)*
HESI Scientific Advisor

Syril Pettit, M.E.M.
HESI Senior Scientific Program Manager

Why This Meeting?

- The HESI Committee on Genomics shares common goals with EPA, NIEHS, NCGC, and NTP of facilitating the best science to improve mechanism-based chemical risk assessment
- HESI Committee in Genomics is seeking partners to develop and execute a mechanistically-based in vitro testing program.
  - 2007 NRC Report on Toxicity Testing in 21st Century
  - ToxCast
  - January 2008 MOU on HTS
- HESI seeking to engage input from:
  - NTP's Biomolecular Screening Branch,
  - EPA's ToxCast Program
  - NIH Chemical Genomics Center
  - NIEHS staff
  - Others!

What Is HESI?

- Non-profit organization that stimulates and supports scientific research and education.
- A collaborative forum for government, academic, and industrial scientists to work in concert to develop innovative and high quality approaches to resolving complex scientific issues that cross all sectors.
- HESI programs result in high quality, unbiased science that is published in the peer-reviewed literature.

HESI Committee on Genomics

- Long Track Record of Successful Collaboration and Leader in the Field: (since 1999)
- Committee has developed and implemented successful, prospective experimental programs. >15 peer reviewed publications.
- Respected internationally for open, balanced approach and multi-sector, international membership. Ability to reach a well-informed consensus.
- Demonstrated ability to create partnerships.
- Committee structure provides both technical expertise, 'sweat-equity', and funding.

Participants

Private Sector

Actelion Pharmaceuticals
Amgen, Inc.
AstraZeneca
Bayer HealthCare
Biogen Idec MA, Inc.
Boehringer-Ingelheim Pharmaceuticals, Inc.
Bristol-Myers Squibb Co.
The Dow Chemical Company
Eli Lilly and Company
GlaxoSmithKline
Hoffmann-La Roche, Inc.
Institut de Recherches Int. SERVIER
Johnson & Johnson Pharmaceutical Research and Development, LLC
Novartis Pharmaceuticals Corporation
Pfizer Inc
Sankyo Co., Ltd.
sanofi-aventis
Schering-Plough Research Institute
Sumitomo Chemical Co., Ltd.
Syngenta Central Toxicology Laboratory
Tanabe Seiyaku Co., Ltd.
Taiho

Government

U.S. Environmental Protection Agency
U.S. Food and Drug Administration
U.S. National Cancer Institute
U.S. National Center for Toxicological Research
U.S. National Institute of Environmental Health Sciences – National Center for Toxicogenomics

European Agency for the Evaluation of Medicinal Products
Netherlands - RIVM National Institute of Public Health and the Environment

Academia

Harvard University
University of Surrey
Michigan State University
Georgetown University

Proposal Presentation

- We would value your input on the following:
  - Technical merit of proposal
    - Comments on the scientific rigor?
  - Relevance of the proposal to current testing/regulatory paradigms
    - Would the results of this program be valuable in addressing the needs of ToxCast, NRC report, etc.?
  - Level of engagement
    - Where would you/your organize most like to engage on this project (e.g., planning, design, oversight, execution/lab work, database, etc.)
      - ToxCast computational tox
  - Support
    - What options for financial or in-kind support might be available if there is interest?

Proposal Presentation

Short-term (mechanistically-based) testing for Genetic Toxicity and Carcinogenicity

TOXICOLOGICAL SCIENCES 99(1), 20–25 (2007)
doi:10.1093/toxsci/kfm147
Advance Access publication June 4, 2007

FORUM SERIES

Genetic Toxicity Assessment: Employing the Best Science for Human Safety Evaluation Part VII: Why Not Start with a Single Test: A Transformational Alternative to Genotoxicity Hazard and Risk Assessment

Warren W. Ku,*,[1] Jiri Aubrecht,† Robert J. Mauthe,† Robert H. Schiestl,‡ and Albert J. Fornace Jr§

*Exploratory Medicinal Sciences; †Drug Safety, Pfizer Global Research and Development, Groton, Connecticut, 06340; ‡Department of Pathology, Environmental Health and Radiation Oncology, School of Medicine and Public Health, University of California, Los Angeles, California 90095; and §Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, District of Columbia 20057 and Harvard School of Public Health, Boston, Massachusetts 02115

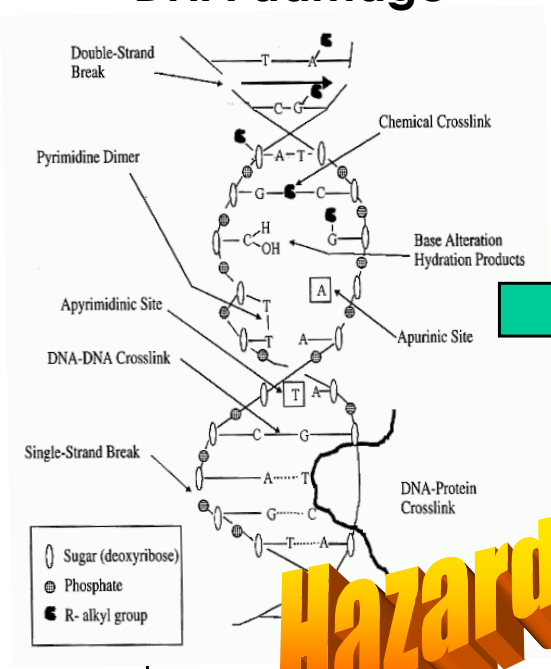
Received March 5, 2007; accepted May 30, 2007

Outline

- Gaps and challenges for predicting chemical carcinogenicity
- Proposed testing paradigm
- Proof of concept studies
  - DEL
  - Toxicogenomics
- Proposed research and partnership

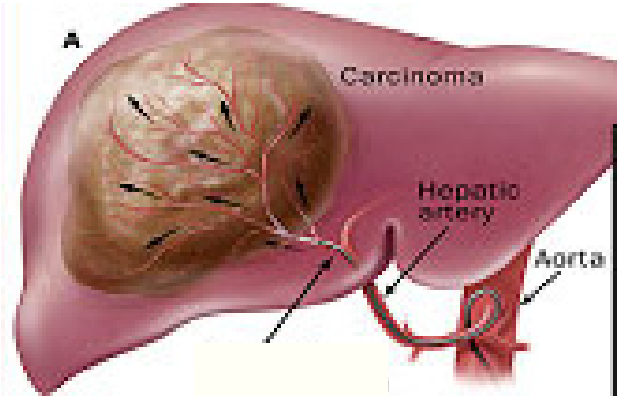
Genetic Toxicity and Carcinogenesis

DNA damage



Carcinogenesis
Multistage process

Cancer



Hazard

- **Required for IND**
- **Genetox battery**
- **Cost: $60K/cmpd**
- **Time: 1-3 month**

Non-genotoxic mechanisms

- Proliferation
  - Direct/regenerative hyperplasia
  - Hormone changes
- Nuclear hormone receptor activation
- Epigenetics
  - DNA Methylation
  - Histone modifications

Risk

- **Required for NDA**
- **2-year bioassay**
- **Cost: $3M/cmpd**
- **Time: 3 years**

Gaps and challenges

- **Bridge genetox and carci testing**
  - Hazard ID and risk assessment
  - Relevance of positive genetox findings
- **Providing insights into mechanisms of genotoxicity and their to risk assessment, relevance to cancer**
  - Relevance of mechanisms of action to cancer development in vivo
    - Relation to genotoxic vs. non-genotoxic carcinogenesis
  - Biomarkers
- **Speed and throughput**
  - REACH etc., EPA – ToxCast, NTP…
- **Animal usage - 3R!!!**
- **Funding for translational research**
  - Opportunity to make a difference via partnership

Considerations for in vitro testing

- **Hallmarks of cancer**
  - Genome instability
    - LOH, mutation, chromosome aberration, deletion, translocation, micro-satellite instability
  - **Malfunctioning checkpoints for proliferation**
    - Cell signaling, apoptosis, differentiation…
- **Challenge**
  - Predicting long term and complex nature of chemical carcinogenesis using short term vitro testing

Current testing paradigm

Genetic damage
Genome instability

Relevance and Risk
Malfunctioning of checkpoints

Genetox battery

- **AMES**
- **Chromosome damage**
  - **HLA, MLA, IVMN**
  - **In vivo MN**

Follow-up assays

- **Other genetox endpoints in vitro or in vivo**
  - **Comet, UDS, DNA adducts,...**
  - **Second in vivo test (transgenic)**
- **Mechanistic carcinogenicity studies in vivo**
  - **Hormonal analysis, proliferation,……**
  - **Transgenics**

- **Current testing battery addresses mainly genetic damage**
  - Relevance of findings in absence of understanding underlying mechanisms is difficult
- **Follow-up assays**
  - Some use similar endpoint lack f mechanistic information
  - Available mechanistic carcinogenity studies restricted by current knowledge
  - Low throughput and high cost

Proposed testing paradigm

Ku et al.: Why not start with a single test. Tox Sci, 2007

Genetic damage
Genome instability

DEL recombination assay in yeast

- Addresses all spectrum of genetic lesions
- High throughput
- Initial mechanistic insights

HT screen

Relevance and Risk
Malfunctioning of checkpoints

System biology approach

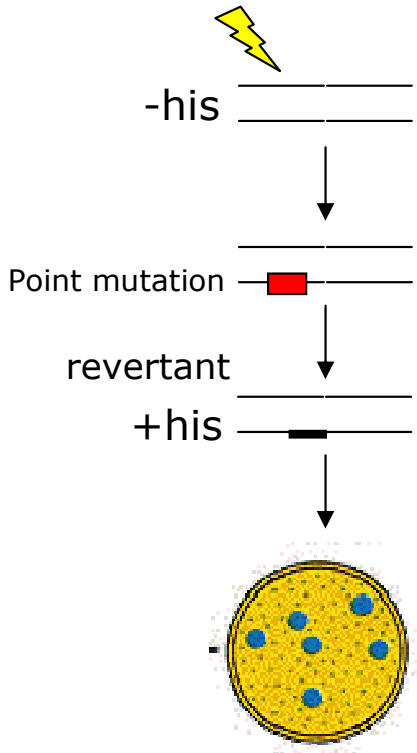
- **Transcriptomic analysis of stress response**
  - Insight into underlying mechanisms
  - Enables evaluating relevance to humans
  - Compound specific biomarkers

Address relevance

- **Address underlying processes in chemical carcinogenesis**
  - Genetic damage and underlying biological processes
- **Facilitate understanding relevance of findings**
  - Mechanistic information is central to risk assessment for drugs and chemicals!!!
- **High throughput and low cost paradigm, 3R principle**

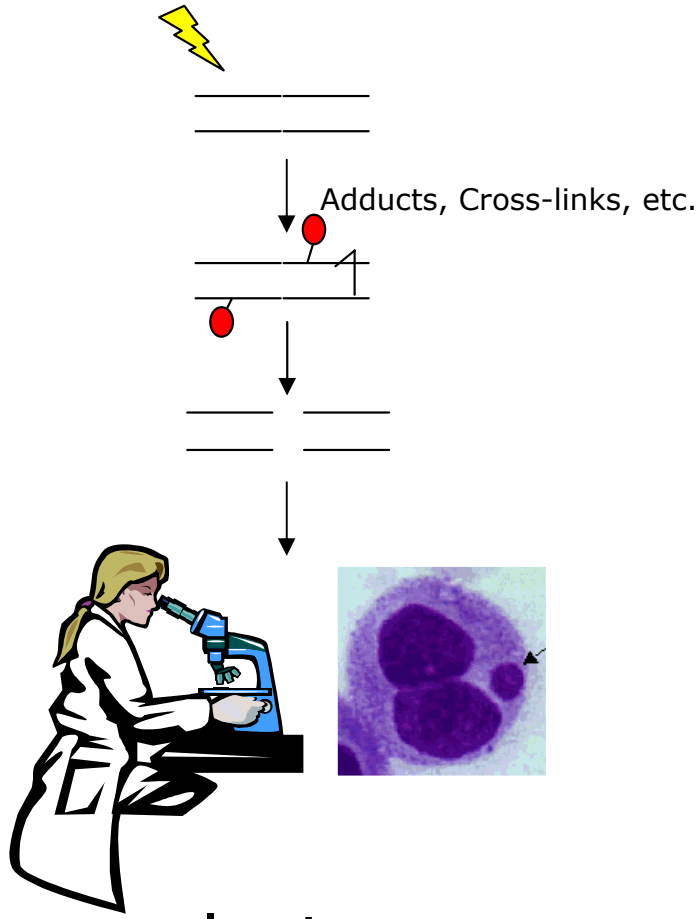
What does AMES IVMN and DEL Detect?

AMES



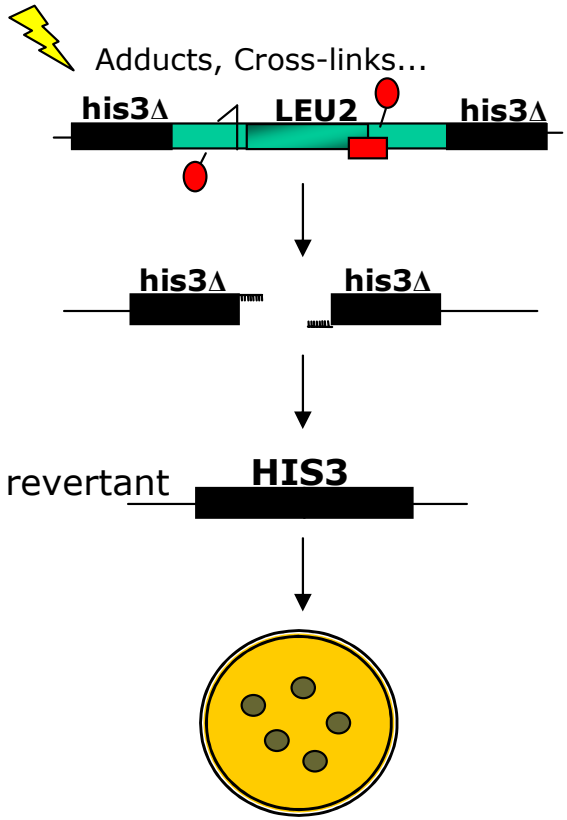
mutagens

IVMN



clastogens

DEL



mutagens & clastogens

Response of carcinogens and noncarcinogens in the yeast DEL recombination assay and the Ames assay

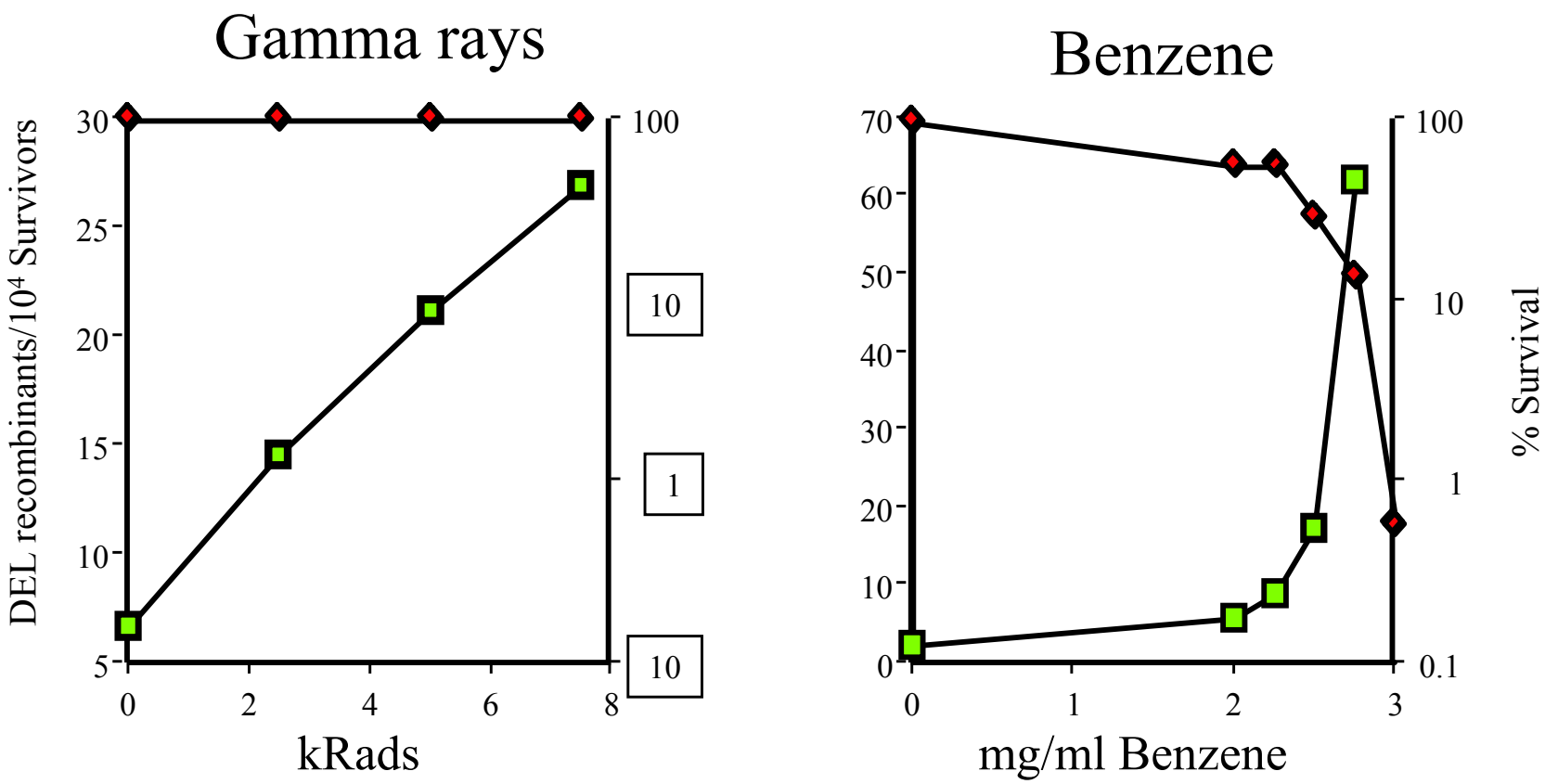
Compound	Carcinogen	Assay Response	
		DEL	Salmonella
Safrole	+	+	-
Ethionine	+	+	-
Urethane	+	+	-
Auramine O	+	+	-
Methylene chloride	+	+	-
Carbon tetrachloride	+	+	-
Cadmium chloride	+	+	-
Cadmium sulfate	+	+	-
3-Amino-1,2,4-triazole	+	+	-
Acetamide	+	+	-
Thioacetamide	+	+	-
Thiourea	+	+	-
DDE	+	+	-
Ethylenethiourea	+	+	-
Aniline	+	+	-
o-Toluidine	+	+	-
o-Anisidine	+	+	-
Hexamethyl phosphoramide	+	+	-
Acrylonitrile	+	+	-
Benzene	+	+	-
Arsenate	+	+	-
Catechol	+	+	-
Aroclor 1221 (PCB)	+	+	-
UV irradiation	+	+	+
γ-ray exposure	+	+	+
4-NQO	+	+	+
MMS	+	+	+
2-amino anthracene	+	+	+
2-nitrofluorene	+	+	+
2-acetoamidofluorene	+	+	+

Compound	Carcinogen	Assay Response	
		DEL	Salmonella
EMS	+	+	+
Nitrogen mustard	+	+	+
Epichlorohydrin	+	+	+
Aflatoxin B_1	+	+	+
Ethylene dibromide	+	+	+
Dimethylhydrazine	+	+	+
Cyclophosphamide	+	+	+
Formaldehyde	+	+	+
Ethylene oxide	+	+	+
Propylene oxide	+	+	+
2,4-Diaminotoluene	+	+	+
TPA	+	-	-
Diethylstilbestrol	+	-	-
Peroxysome Proliferators	+	-	-
Diethylhexylphthalate	+	-	-
Phenobarbital	+	-	-
2,6-Diaminotoluene	-	-	+
Hydroxylamine HCl	-	-	+
Sodium azide	-	-	+
5-Bromouracil	-	-	+
2-Aminopurine	-	+	+
Ethidium bromide	-	+	+
Benzoin	-	-	-
Methionine	-	-	-
Ethanol	-	-	-
Acetone	-	-	-
Caprolactam	-	+	+

Validation of the yeast DEL assay

- 60 chemicals tested (most of them false negatives or false positives with the Salmonella assay) DEL assay accuracy: 92%, sensitivity: 94%, specificity: 80%
- Accuracy of the Salmonella assay with these chemicals: 62%
- The yeast DEL assay reduces the number of false negatives and false positives of the Salmonella assay
- Carcinogen/Noncarcinogen pairs correctly identified: o-Toluidine/2,4-Dimethoxy aniline 2,4-Diamino toluene/2,6-Diamino toluene Ethionine/Methionine
- Very High correlation with clastogenicity of chemicals

The DEL assay detects Salmonella positive and negative carcinogens with different dose responses

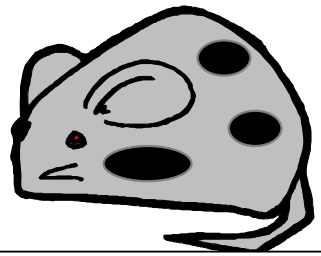


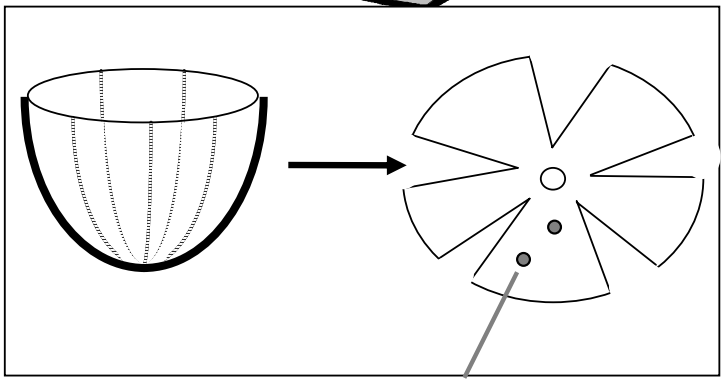
Galli and Schiestl (1995) *Carcinogenesis 16,* 659-663.

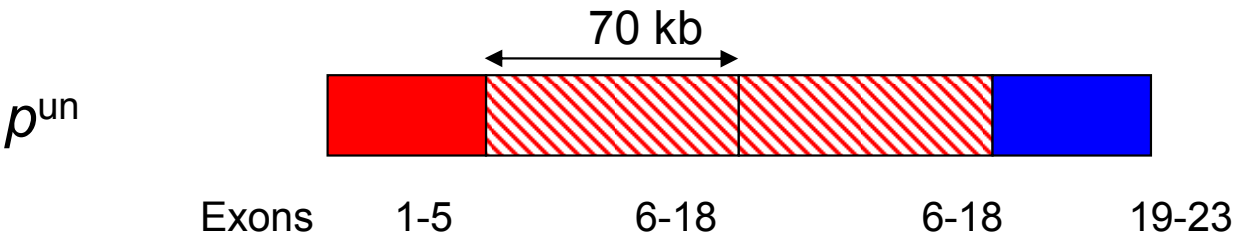
How DNA deletions are scored in vivo?

Mouse strain: *p*^{un} mouse (C57BL/6J-p^{un}/p^{un})

- dilute gray fur color
- pink eyes
- *p*^{un} mutation

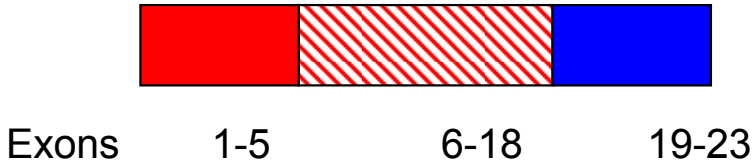






homologous ⇩ recombination (in embryonic life)

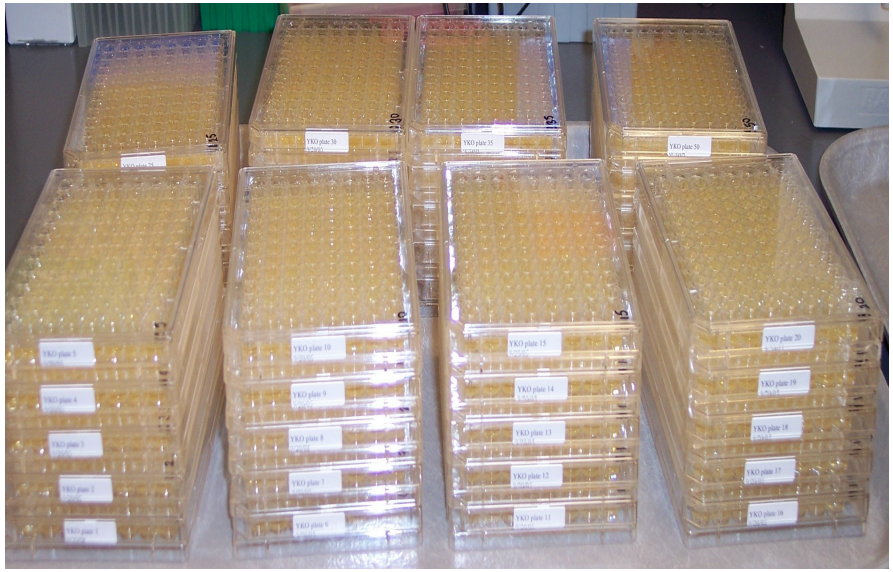
wild type *p*

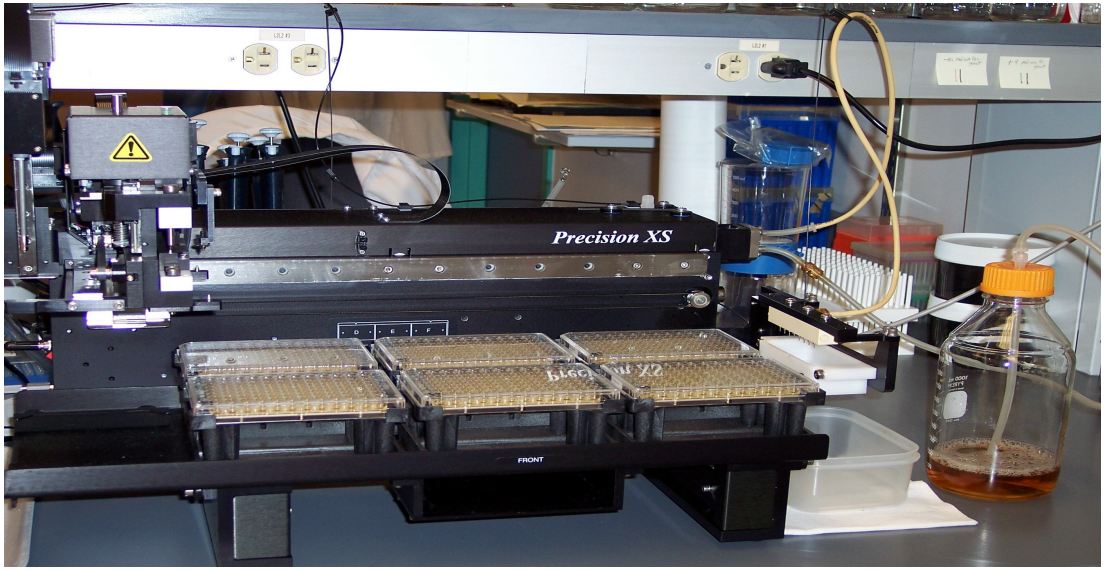


HR leads to a deletion of exons 6-18

High-throughput yeast format

- **Quantitative colorimetric assay for yeast growth**
  - MTS- a compound that is reduced by cells to form a colored product.
  - Cell proliferation is proportional to the quantity of product.
  - Absorbance is recorded at 490 nm.





Validated the well-based assay by using chemicals known to induce DEL events

Hontzeas N., Hafer K., and Schiestl R., Mutat. Res. **634 (2007) 228–234**

Compound	DEL (fold increase)[1]	DEL significance[2]
EMS (10μg/ml)	2.65	***
4NQO[4] (0.08μg/ml)	3.39	***
MMS (0.11μg/ml)	3.09	***
Camptothecin (13.93μg/ml)	1.77	***
ActD (25.11μg/ml)	1.26	**
Cr (3) (221.7μg/ml)	1.44	***
Cr (6) (69.99μg/ml)	3.78	***
Benzene (400μg/ml)	2.31	***
Cyclophosphamide (55.82μg/ml)	0.95	ns
Mitomycin C (13.37μg/ml)	1.29	**
Chlorambucil (9.13μg/ml)	3.58	***
Carmustine (10.70μg/ml)	7.62	***
Cisplatin (300μg/ml)	4.57	***
DMSO 1%[4]	0.96	ns
Acetone 0.4%[4]	0.96	ns
HCL-methanol (1:50) 0.5%[4]	0.95	ns

[1]Fold DEL increase was calculated by dividing the DEL induction measured for the respective compound concentration by that of the controls performed on the same plate. Each experiment was repeated at least 3 times on separate plates and similar results were attained in each measurement.
[2]Significance * ($p<0.05$), **($p<0.01$), ***($p<0.005$). ns-not significant ($p>0.05$).

Summary of the development of the DEL assay

- Proof of concept, the DEL assay detects all DNA lesions such as direct acting as well as indirect acting carcinogens
- Different dose response can differentiate between direct and indirect acting carcinogens
- The same chemicals induce DNA deletions in mammalian cells as well as in vivo in mice
- Isolated five mutants sensitive to hydrophobic toxins from a screen of the whole genomic mutant library and constructed a DEL tester strain with the best performing mutation
- Developed a High Throughput Format of the DEL assay and shown that it is as sensitive as the regular plating format
- The next step is to thoroughly validate the HTS assay

Toward agent-specific signatures:

Global analysis of molecular responses to stress with a functional genomics approaches

Provide insight into mechanisms of action of genotoxicants and carcinogens

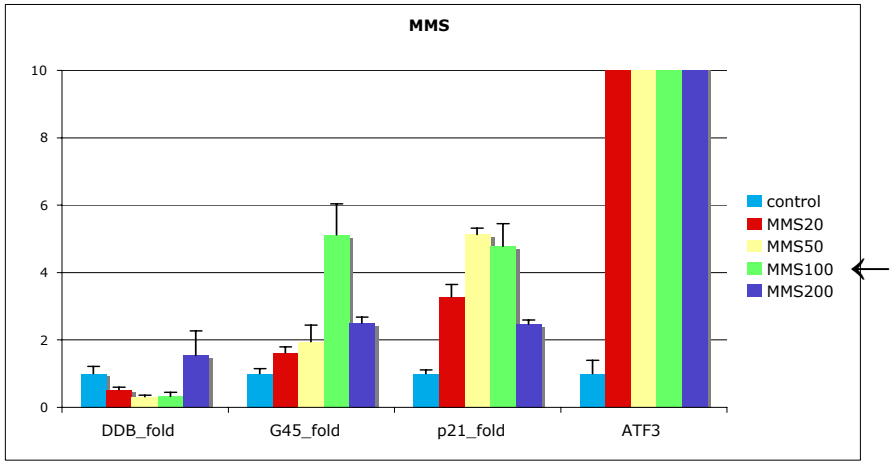
Development of Gene-Tox Signatures

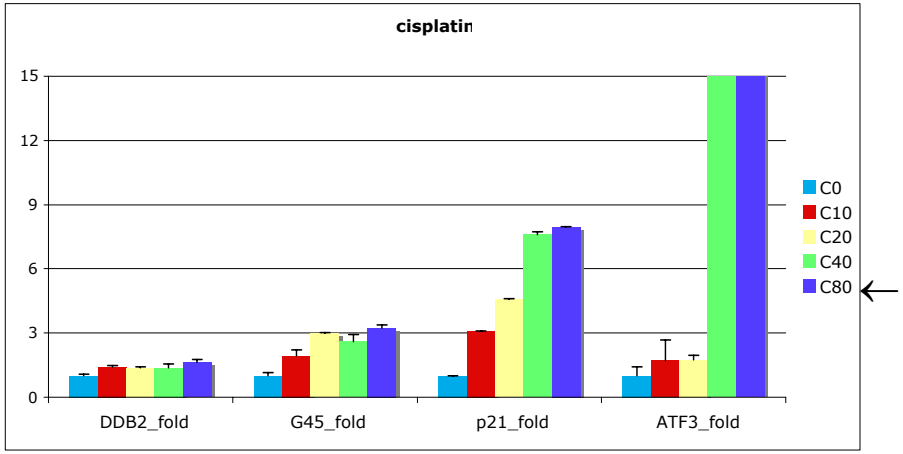
- TK6 cells
- acute response
  - little to no toxicity at this time, so secondary effects minimized
- optimize dose for signaling studies
  - typically robust responses at higher "physiologic" doses
  - very high doses can block transcription and general cell integrity
- agents from various classes/types of damaging agents

Genotoxic		Non-genotoxic
DNA damage	**antimetabolites**	
nitrogen mustard cisplatin camptothecin VP-16 bleomycin MMS x rays hydrogen peroxide cadmium chloride potassium chromate sodium arsenate	hydroxyurea Ara-C methotrexate PALA caffeine methotrexate 5-FU	heat shock NaCl thapsigargin (ER stress, intracellular calcium pump inhibitor) tunicamycin (ER stress, disrupt glycosylation of newly-synthesized proteins) 2-deoxyglucose ("glucose" poison) Antimycin A (mitochondria respiration inhibitor) AICAR (AMPK activator, mimic, mitochondrial disfunction and energy stress) p38 kinase activity inhibitor Microtubule inhibitors (stabilizers and destabilizers) HDAC inhibitors

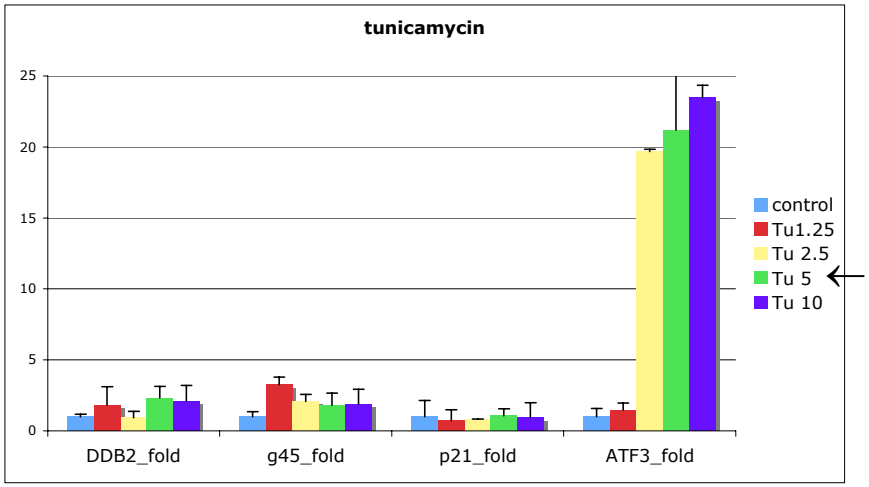
Pre-array RT-PCR for dose-optimization

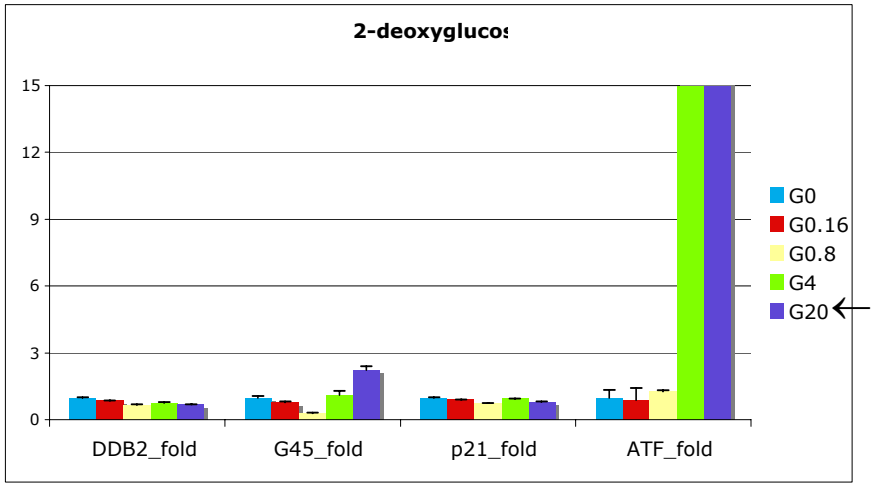
DNA damaging:





non-DNA damaging:

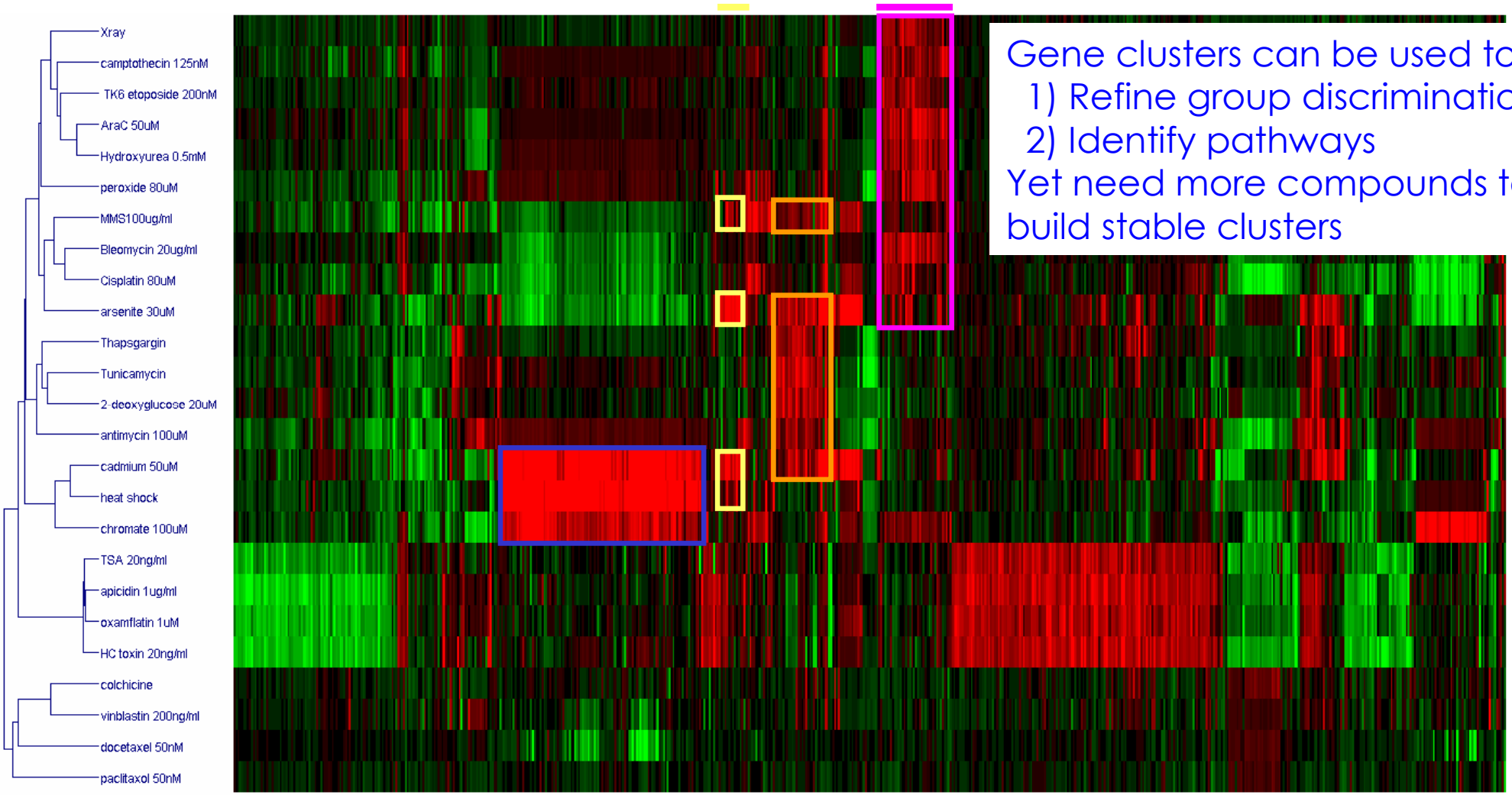




Pathway analysis of gene expression profiles

- Oxidative Stress Response
- p53 Signaling
- Acute Phase Response Signaling

- p53 Signaling
- Cell Cycle: G2/M DNA Damage Checkpoint Regulation



Gene clusters can be used to:
1) Refine group discrimination.
2) Identify pathways

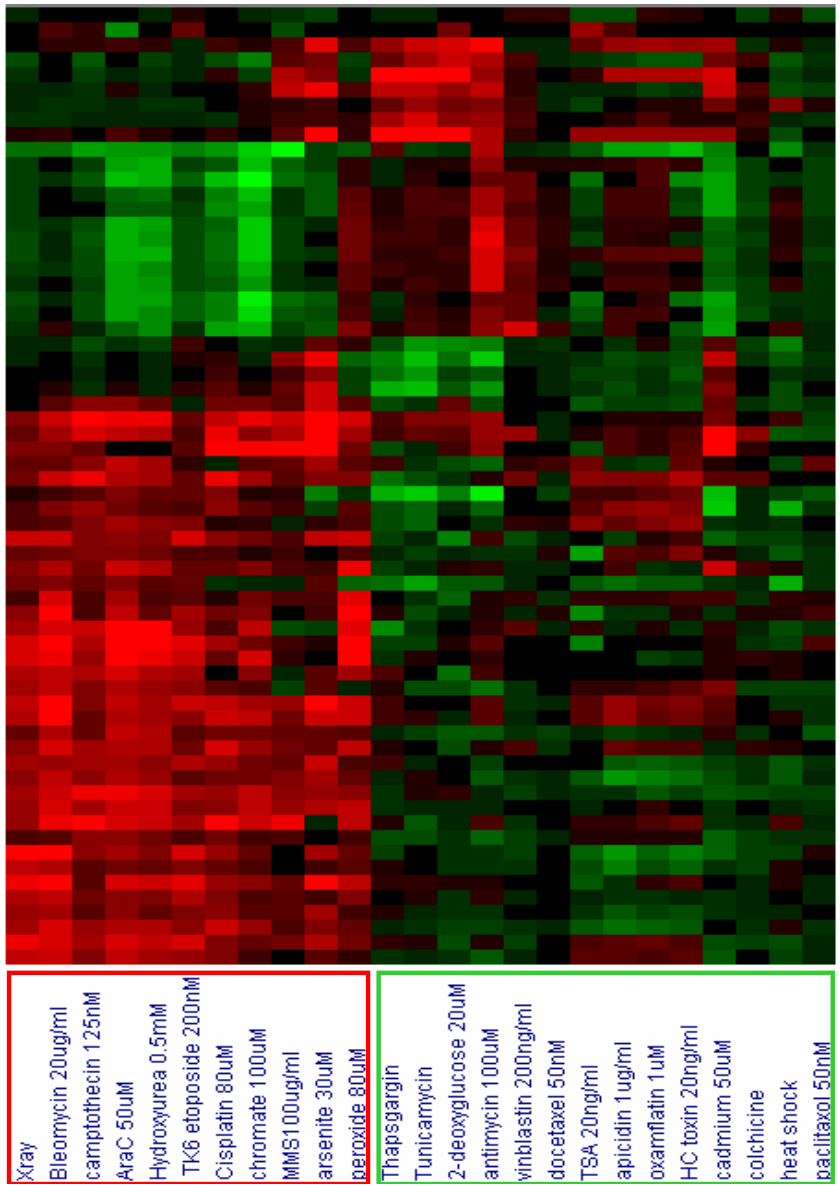
Yet need more compounds to build stable clusters

- Metabolism
- Protein perturbations
- NFKB signaling

- Endoplasmic Reticulum Stress Pathway
- p38 MAPK Signaling
- Glycine, Serine and Threonine Metabolism

Visualization of Genotoxic Classifier

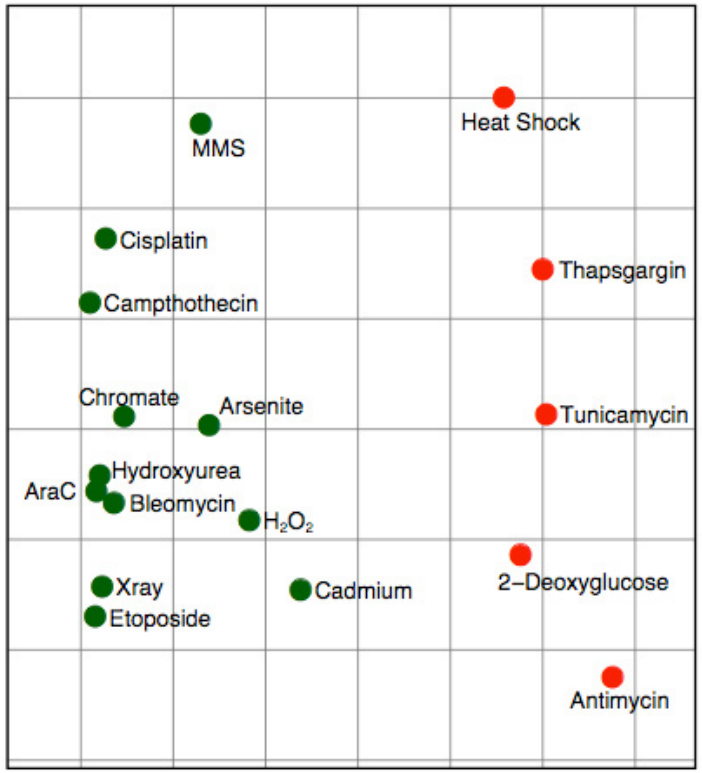
Heatmap of 58 Gene Genotoxic Classifier



Genotox Repressed

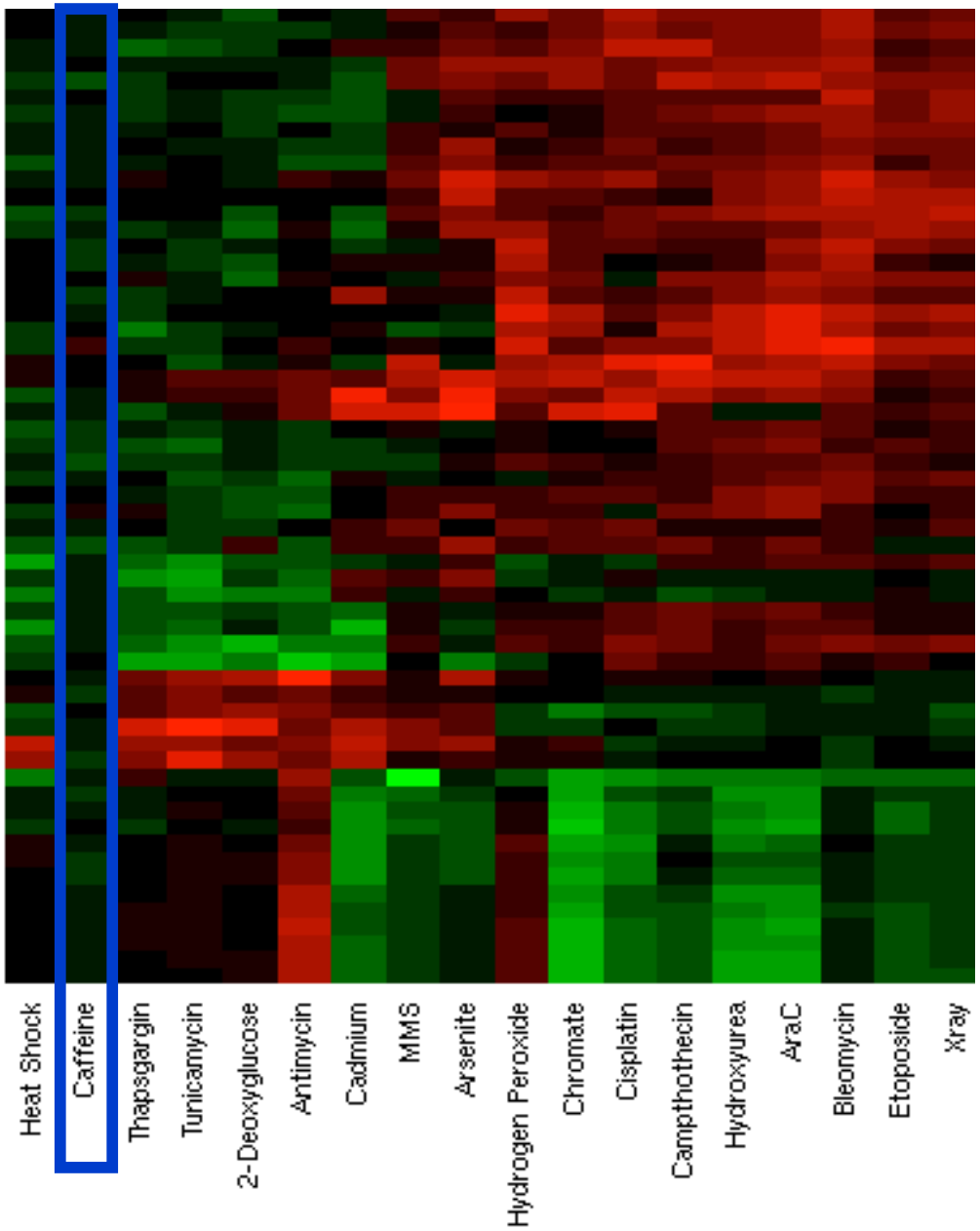
Genotox Induced

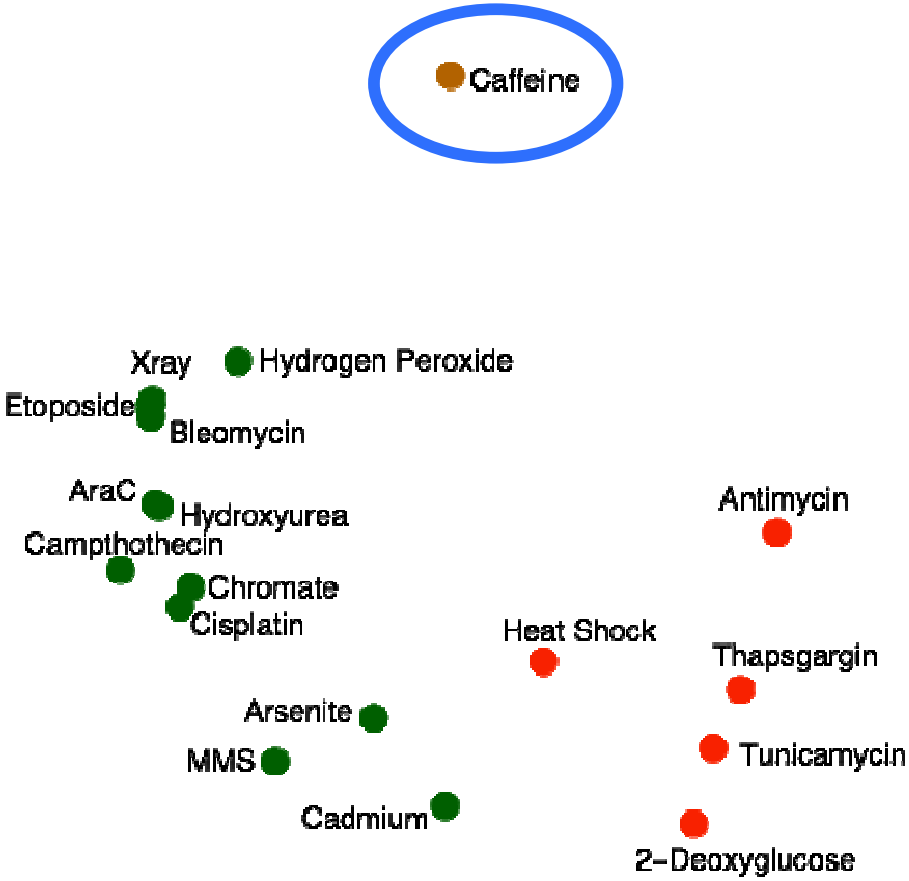
MDS Using Genotoxic Classifier



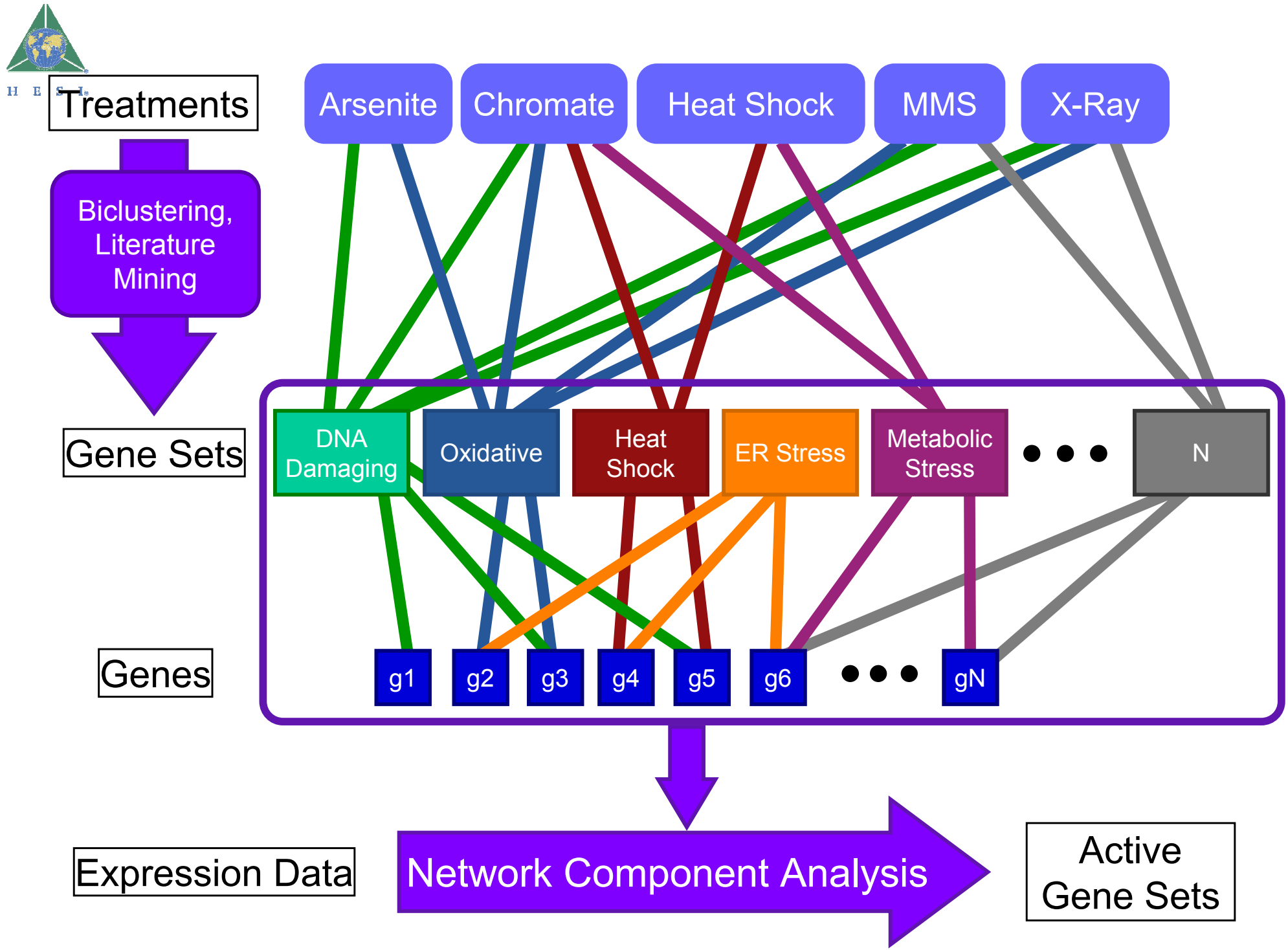
Genotoxic Nongenotoxic

Caffeine Is Non-genotoxic

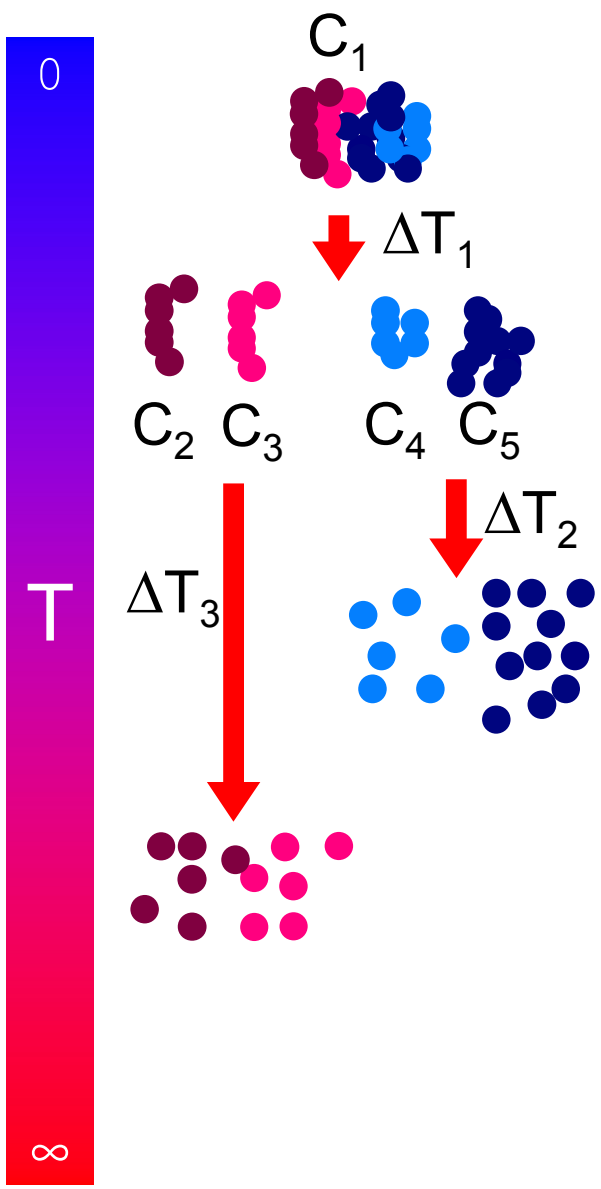




Genotoxic Nongenotoxic


Treatments
Arsenite
Chromate
Heat Shock
MMS
X-Ray
Biclustering, Literature Mining
Gene Sets
DNA Damaging
Oxidative
Heat Shock
ER Stress
Metabolic Stress
N
Genes
g1
g2
g3
g4
g5
g6
gN
Expression Data
Network Component Analysis
Active Gene Sets

Superparamagnetic Clustering (SPC)



Based on statistical mechanics model of inhomogeneous ferromagnet.

Hierarchical and nonparametric.

Insensitive to initial conditions.

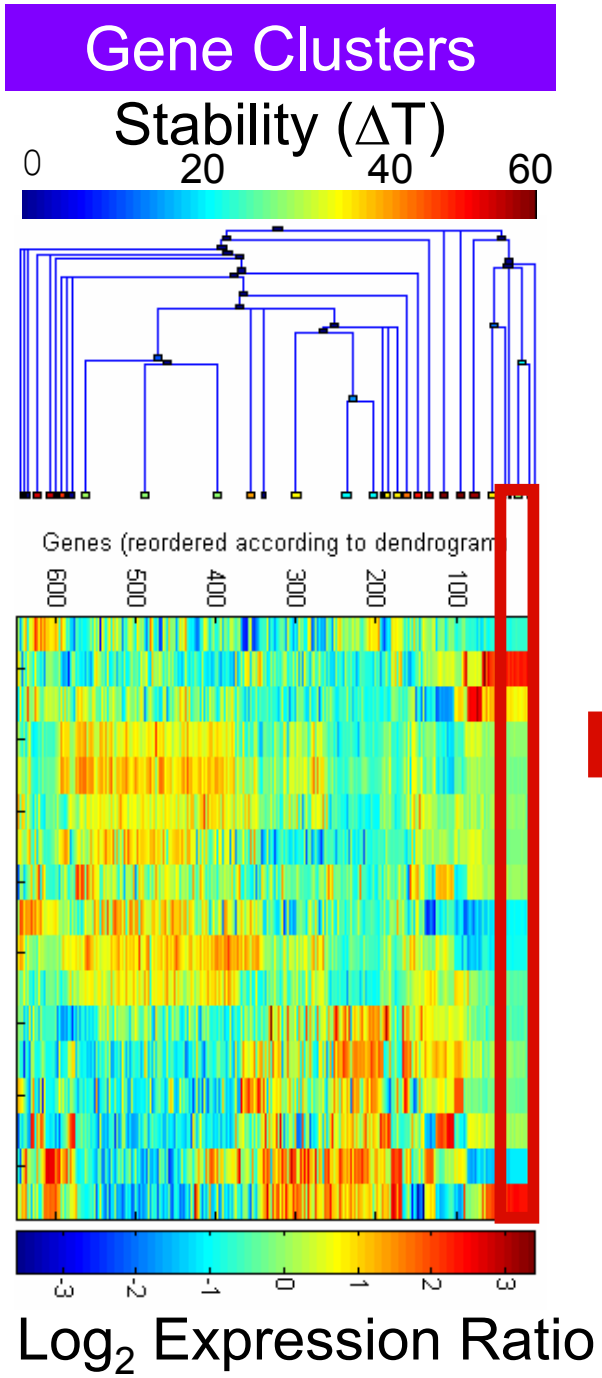
Does not spuriously identify clusters.

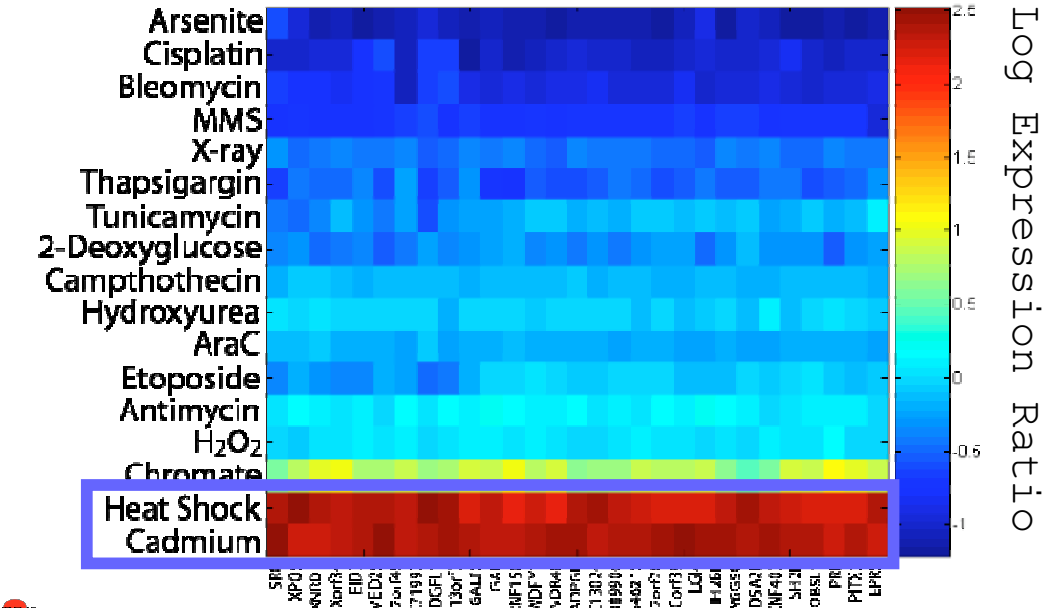
Control parameter (T) indicates the stability of the clusters.

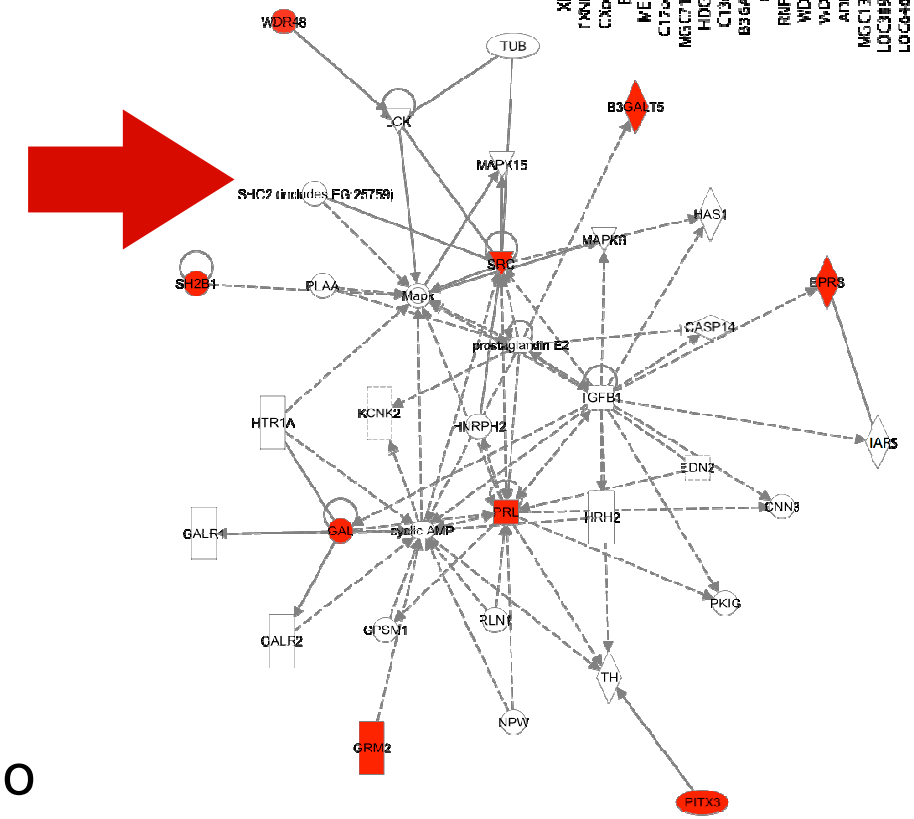
Example: $\Delta T_3 > \Delta T_2 > \Delta T_1$ therefore clusters C_2 and C_3 are more stable than clusters C_1, C_4, and C_5.

Domany 1999 Physica A 263:158-69.

Heat Shock / Cadmium Responsive Cluster







cAMP-Mediated Signaling

Summary

- Gene expression profiles provide insights into mechanisms
  - Pleiotropic effects
  - Pathway analysis provides substrate for human relevance and suggests links to carcinogenesis
- Phenotypic anchoring using stress genes enabled to compare gene expression signatures across compound classes
  - More agents will provide better resolution of toxic mechanisms
- Feasible approach for testing large set of chemicals
  - Single dose with a stress gene-based phenotypic anchor

Proposed testing paradigm

Ku et al.: Why not start with a single test. Tox Sci, 2007

Genetic damage
Genome instability

DEL recombination assay in yeast

- Addresses all spectrum of genetic lesions
- High throughput
- Initial mechanistic insights

HT screen

Relevance and Risk
Malfunctioning of checkpoints

System biology approach

- **Transcriptomic analysis of stress response**
  - Insight into underlying mechanisms
  - Enables evaluating relevance to humans
  - Compound specific biomarkers

Address relevance

- **Achieved proof of principle for DEL and toxicogenomic**
- **Understanding modes/mechanisms of action is common theme in risk assessment for drugs and chemicals**
- **Validation needed**

Research Proposal

- **Validate DEL assay as a high throughput alternative to genetox battery**
  - Assess concordance with genetox battery and rodent carcinogenicity testing
- **Validate transcriptomic systems toxicology approach for assessing genotoxic mechanisms**
  - Evaluate gene expression signatures across wide range of mechanisms and compounds
  - Mechanism-based gene signatures
  - Biomarkers
- **Research managed by collaborators**
  - Compound set 300-1500

Research implementation

- **Establish "consortium" of partners**
  - Government, academia and industry
  - Compound selection for testing
  - Data analysis and interpretation
  - Provide data and concepts for use in risk assessment
    - Drugs
    - Environmental chemicals
- **Laboratory work performed in academic labs or others**
  - DEL - Schiestl (UCLA)
  - Toxicogenomics – Fornace (Georgetown)
  - NIEHS or EPA?

Expected outcome

- **Testing paradigm suitable for validation by regulatory agencies eg. ICVAM/ECVAM validation, FDA etc**
  - High throughput
  - 3R compliant
- **Improved testing methods for chemical carcinogenicity**
  - Environmental chemicals and drugs
- **Decision on suitability of emerging technologies (DEL and toxicogenomics) in toxicological research**

Acknowledgments

Georgetown Univ

Henghong Li

Daniel Hyduke

UCLA

Zhanna Sobol

Alvaro Galli

Nicos Hontzeas

Pfizer

Ebru Caba

Warren Ku

Bob Mauthe

Denise Robinson-Gravat