New Directions for Toxicology at the US National Toxicology Program

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National Institutes of Health

Fourth AIMBE/NIH Workshop on Validation and Qualification of New In Vitro Tools and Models for The Pre-clinical Drug Discovery Process

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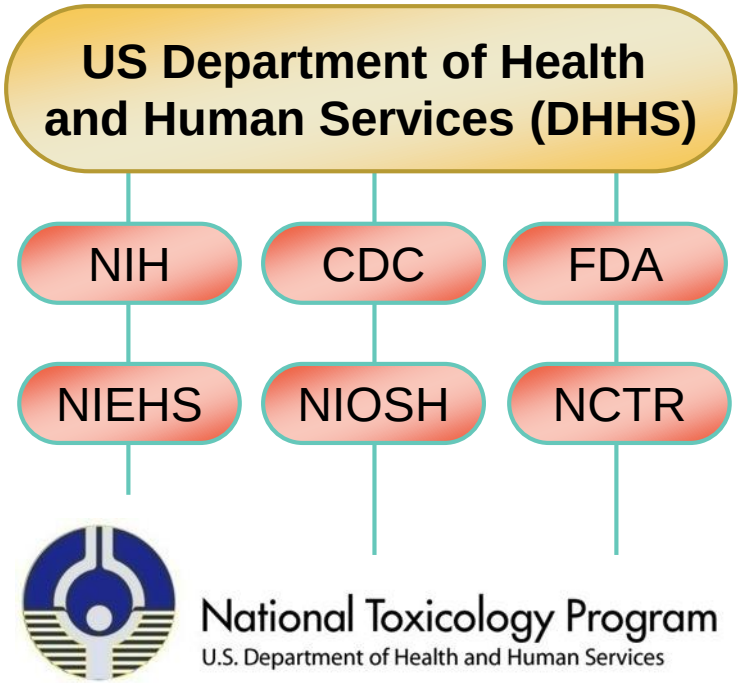
What is the US National Toxicology Program (NTP)?

- **Interagency program**
  - Established in 1978
  - Headquartered at NIEHS
- **Research on "nominations"**
  - Thousands of agents evaluated in comprehensive toxicology studies
  - Results communicated through technical reports, scientific publications, and the web
- **Analysis activities**
  - Report on Carcinogens
  - Office of Health Assessment & Translation
  - NTP Interagency Center for the Evaluation of Alternative Toxicological Methods



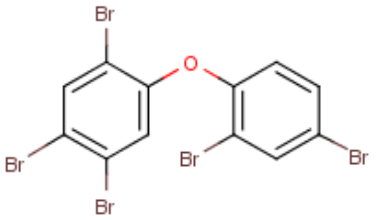
http://ntp.niehs.nih.gov

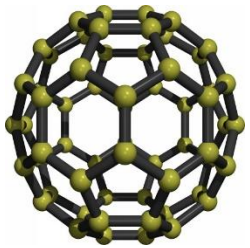
What are the mission and goals of the NTP?

- *Mission*:
  - Evaluate agents of public health concern by developing and applying tools of modern toxicology and molecular biology
- *Goals*:
  - Coordinate toxicological testing programs within the Department of Health and Human Services.
  - Develop and validate improved testing methods that reduce, refine, or replace the use of animals.
  - Develop approaches and generate data to strengthen scientific knowledge about potentially hazardous substances.
  - Communicate information about potentially hazardous substances to health regulatory and research agencies, scientific and medical communities and the public.

What are current areas of emphasis?

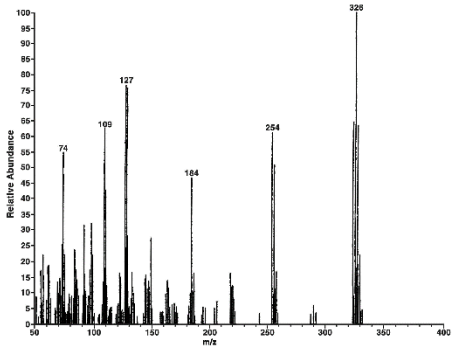
- Combination AIDS therapeutics
- Complex occupational exposures
- Dietary supplements
- Green chemistry
- Endocrine active compounds
- Flame retardants
- Food and drinking water contaminants
- Industrial chemicals
- Nanoscale materials
- Persistent environmental contaminants
- Personal care products
- Radiofrequency radiation





How does the NTP perform its toxicology studies?

- Utilizes NTP contracts, Interagency agreements, and in house capabilities
- Multiple capability-based contracts
  - Not one contract per study
- High quality physicochemical characterization and stability of materials
- Primarily GLP-compliant rodent *in vivo* studies
  - *In vitro*, mechanistic studie, ADME studies, toxicogenomics, genetically-modified models
- High quality pathology review

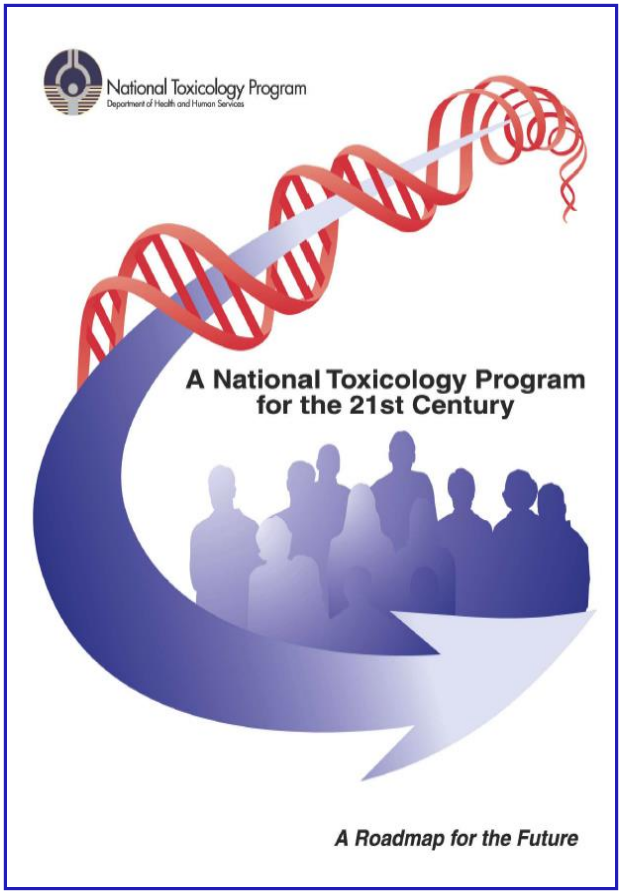


What are the standard NTP assays?

- Prechronic (14 and 90-day toxicology screens) Harlan SD rats, B6C3F1 mice, both sexes
- Two-year rodent cancer studies
- Genetic toxicology (Salmonella mutation assay, blood and bone marrow micronucleus, pig-A assay, comet assay)
- Reproductive assessment by continuous breeding in rats (RACB)
- Developmental assessments (follows FDA segment 2 guidelines)
- Immunotoxicity in mice (immune cell counts, functional responses, *in vivo* challenge assays, hypersensitivity assays)
- Absorption, Distribution, Metabolism, Excretion (ADME) studies
- Toxicokinetic studies
- Toxicogenomic studies

NTP Roadmap 2004

- Review and refine traditional toxicology assays
- Develop rapid, mechanism-based predictive screens for environmentally induced toxicity and disease
- Improve the utility of NTP products for public health decisions



Conceptual shift for environmental health science

OLD… chemicals act by overwhelming the body's defenses by brute force at very high doses

NEW… chemicals can act like hormones and drugs to disrupt the control of development and function at very low doses to which the average person is exposed

NEW… susceptibility to environmentally induced disease can vary widely, can persist long after exposure, and potentially across generations

Refinements to traditional toxicology assays

- Modified one generational study design largely replacing Reproductive Assessment by Continuous Breeding
- Perinatal dosing as a default approach in rat studies
- Bisphenol A Regulatory Agency/Academic Consortium
- Pathology enhancements- extended mammary and brain sectioning, digital conversions, atlas and diagnostic harmonization
- Diversity Outbred mouse model
- Mouse methylome project
- Tox21
- Systematic Review extension to *in vitro* data

Modified one-generation prechronic toxicity study

- Modification to the traditional utero-lactational and Seg III designs
- Can be used to set doses for perinatal exposure cancer bioassays
- Continuous exposure from implantation through sexual maturity
- The first cohort of animals provides target organ toxicity, but also can be used to evaluate immunological or behavioral end points
- The second cohort evaluates developmental toxicity
- The third cohort may be used to evaluate breeding and littering
- All F1 animals after PND 4 are taken to adulthood for pathology examination and there are two cohorts for fertility/ fecundity assessment
- Details of design available on NTP website
  - http://ntp.niehs.nih.gov/?objectid=72015D9F-BDB7-CEBA-F4EB4F9BF507820C

NTP Bisphenol A studies

- Comprehensive GLP perinatal, 2-year, 7 days per week, 5-dose level gavage study in SD rats
- 2.5 to 25,000 µg/kg bw/day
- Control for litter effects, BPA in caging, water, feed, etc.
- Concurrent “positive” control
- Core protocol for interim (1 year) and 2-year animals
  - Vaginal cytology starting at 4 months to evaluate onset of aberrant cycles
  - Clinical chemistry, sperm analysis, organ weights, and target organ histopathology on interim sacrifice animals
  - At 2 years, complete necropsy with selected target organ histopathology
- Subset of animals for behavior testing
- All other animals for NIEHS-funded grantee studies; tissues from the same animals shared when feasible

Consortium members and areas of study

Name	Disease Focus	Endpoint	Aims Funded
Gail Prins	Prostate cancer	Prostate gene expression and cancer development *(PND 21; 6, 12, and 24 months)*	• Prostate gene expression • Prostate methylation • Renewal of stem cells • Assess PIN and cancer
Heather Patisaul	Learning and behavior	Brain transcriptomics *(Birth)* Behavior *(PND 21 and 90)*	• Brain gene expression • Behavioral assessment (*PND 21 and 90)*
Norbert Kaminski	Immune function	Spleen assessed *(PND 90 and 12 months)*	• Spleen T and B cells subpopulations • Response to stimulation • Estrogen receptor (ER) characterization • Gene expression
Kim Boekelheide	Testis function/sperm counts *(Continuous dosing only)*	Testis and epididymis (*PND 90 and 12 months)*	• Histological and morphological assessment of testis • Caudal sperm transcriptome • Caudal sperm methylome

Consortium members and areas of study

Name	Disease Focus	Endpoint	Aims Funded
Ana Soto	Breast cancer	Breast development and cancer *(PND 21 and 90; 6 months (whole mounts))*	• Breast morphology as precursor of cancer (*PND 21)* • Gene expression and DNA methylation (*PND 21)* • Assess pre-neoplastic lesions and neoplastic lesions (*PND 90 and 6 months)*
Shuk Mei Ho	Uterine cancer *Continuous dosing only*	Uterus histology and gene expression *(6, 12, and 24 months)*	• Histological identification of uterine hyperplasia/adenocarcinoma • Laser capture to assess methylome and transcriptome to identify early cancer genes
Nira Ben Jonathan	Obesity/adipose tissue	Adipose tissue disposition and weight gain *(PND 90; 6 and 12 months)*	• Fat depots and selected adipokines, gene expression • Serum hormones • Adipose cell number and size • BPA in fat tissues

Consortium members and areas of study

Name	Disease Focus	Endpoint	Aims Funded
Fred vom Saal	Male urogenital abnormalities	Urogenital system analysis *(Birth; 12 and 24 months)*	• 3D reconstruction of urogenital system • Examine animals for voiding and laser capture to assess gene expression in epithelium and stroma
Jodi Flaws	Ovarian function	Ovary *(Birth, PND 21 and 90, and 12 months)*	• Follicle number • Steroidogenic enzymes
Tom Zoeller	Thyroid and brain anatomy	Thyroid and brain development *(PND 15 and 21)*	• Changes in brain gene expression and histology due to BPA impact on thyroid hormones
Nestor Gonzalez-Cadavid	Penile function	Penile erection mechanism *(12 months)*	• Erection capability, transcriptomic profile, and stem cell analysis
Andrew Greenberg	Diabetes, blood glucose, and pancreas	Blood glucose and pancreas assessment *(12 months)*	• Assess blood glucose over time, beta cell mass, and insulin content

2004 NIEHS/NTP-Perlegen mouse sequencing project

Frazer et al. Nature, 448:1050, 2007; Yang et al. Nature Genetics 39:1100, 2007

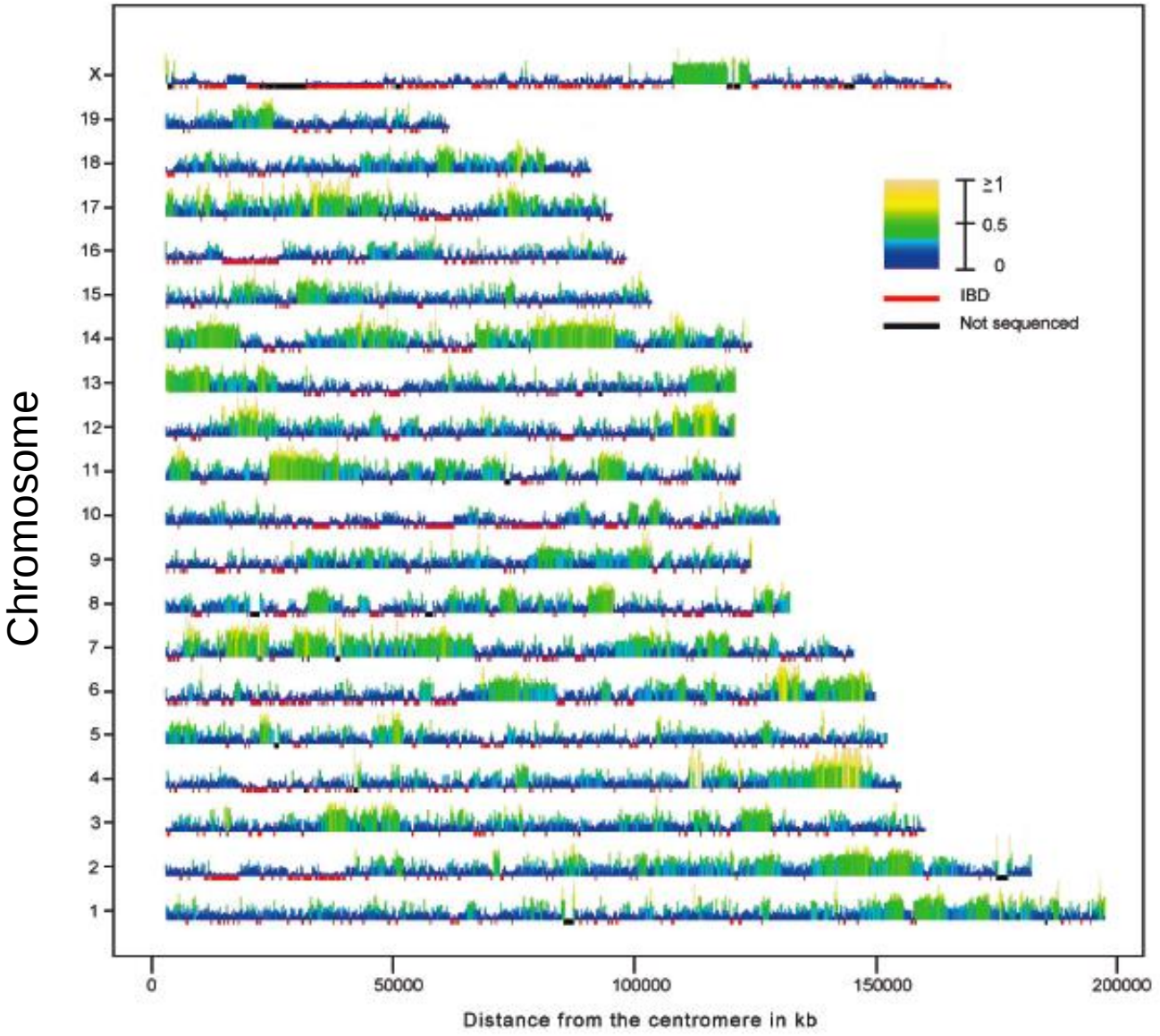
Lab derived inbred strains

Mus musculus
129S1/SvlmJ*
A/J*
AKR/J
BALB/cByJ*
C3H/HeJ
DBA2/J
FVB/NJ
NOD/LtJ*
BTBR T+tf/J
KK/HIJ
*CC (different experts)

Wild derived inbred strains

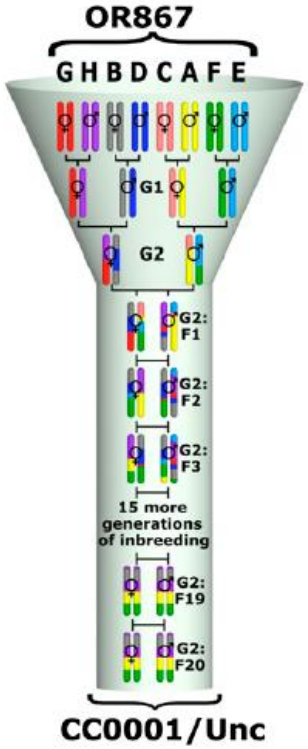
CAST/EiJ* *M.m.castaneous*
MOLF/EiJ *M.m.molossinus*
PWD/*PhJ** *M.m.musculus*
WSB/EiJ* *M.m.domesticus*

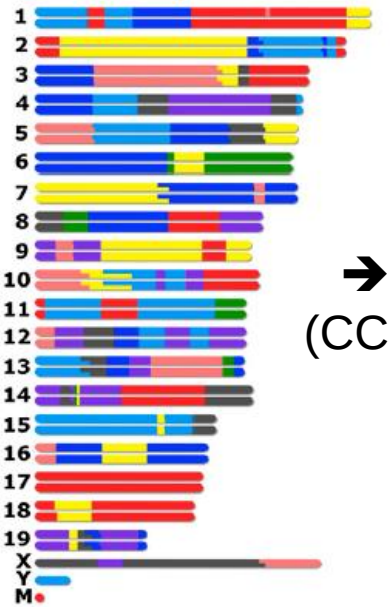
CC – NZO/LtJ*



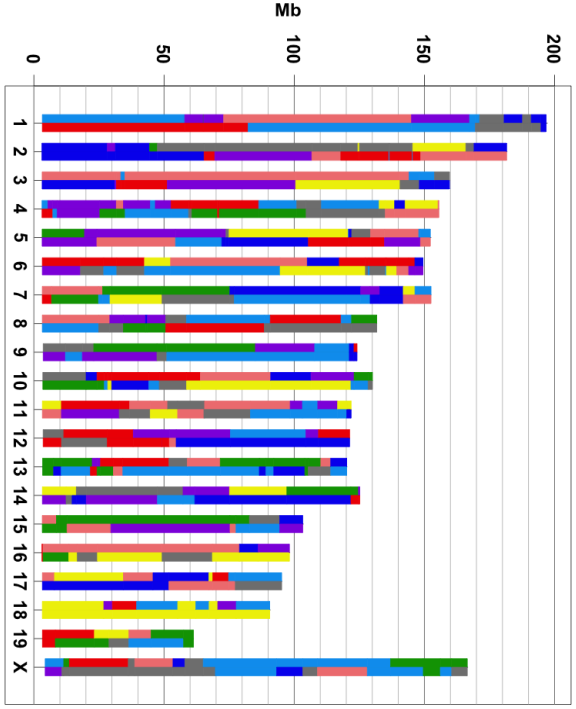
Collaborative Cross and Diversity Outbred models

≈45 million segregating SNPs

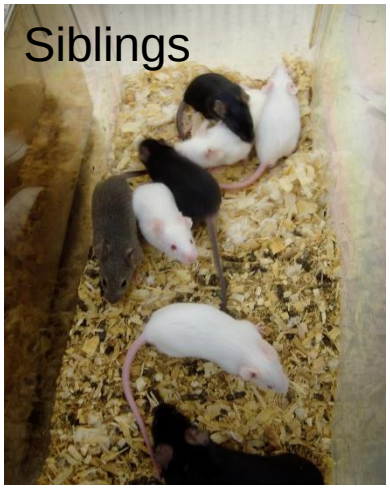




➔J:DO mice
(CC G2:F4-F12)



≥10% minor allele frequency



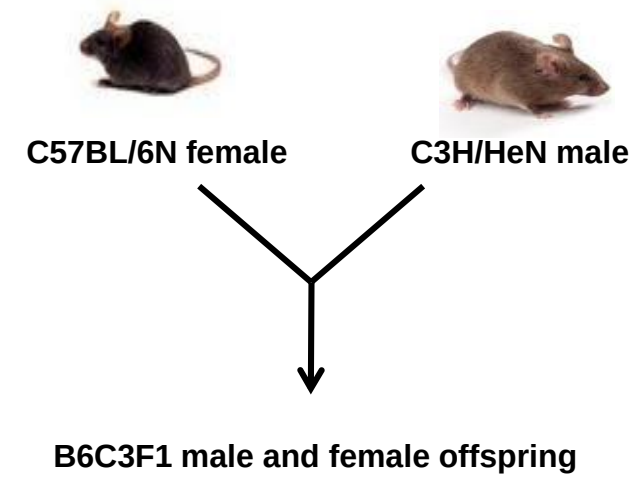
Benzene inhalation study with the DO mouse

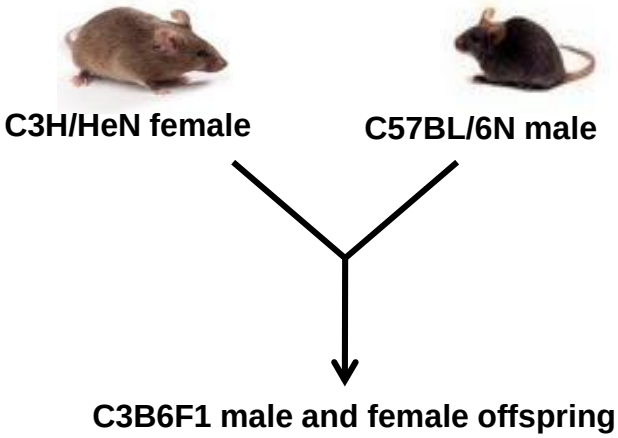
- Diversity outbred (J:DO) male mice selected from 175 breeding pairs
- Dose levels: 0, 1, 10, 100 ppm benzene, 28 days, 6 hr/day
- 600 mice total: 2 separate cohorts to assess reproducibility
- Endpoints for hematotoxicity and genetic damage
  - % reticulocytes and micronucleated reticulocytes in bone marrow and blood
  - Mouse Universal Genotyping Array (9K SNPs; MUGA)
  - Mapping & Linkage analysis (QTLRel)

- Mice showed a 205-fold difference in susceptibility
- Associated with variable expression of a sulfotransferace detoxification enzyme

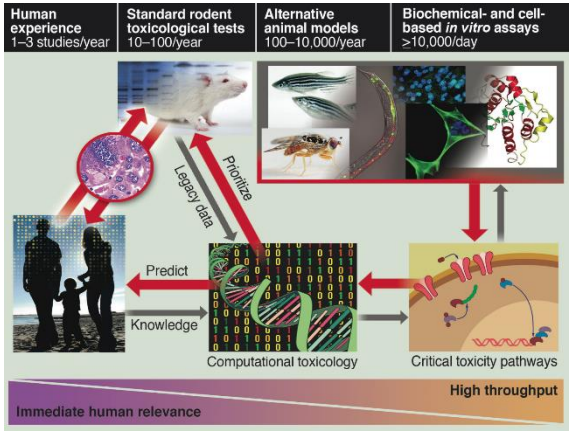
Mouse methylome project

- Goal:
  - Develop chip-based tools for rapid screening of segments of the B6C3F1 hybrid mouse genome susceptible to epigenetic modifications
- Currently:
  - Deep sequencing DNA, and assaying RNA expression
- Outcome:
  - Identify differentially methylated regions
  - Determine inheritance (parent of origin)
  - Integration of methylation with gene expression





Develop mechanism-based predictive screens for environmentally induced diseases



- Workshops on:
  - HTS Assays
  - Chemical Genomics
  - HTS vendor meeting
- Collins *et al.* Transforming public health protection, *Science* 319:906-7, 2008
- Collaboration with NIEHS/NTP, EPA, National Human Genome Research Institute/NIH Chemical Genomics Center, FDA

Tox21

The Tox21 Community

FDA

NIEHS
National Institute of Environmental Health Sciences

National Toxicology Program
U.S. Department of Health and Human Services

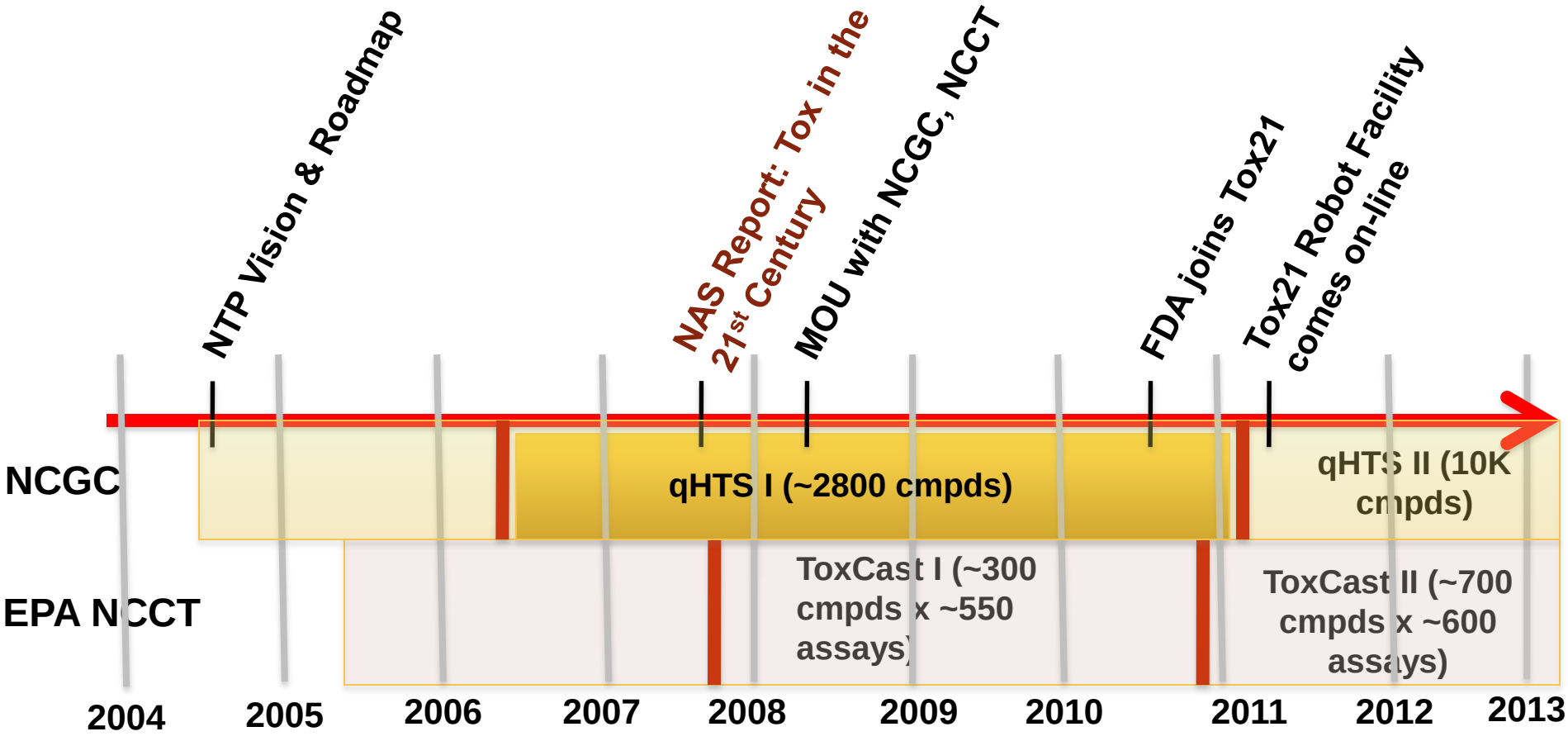
genome.gov
National Human Genome Research Institute
National Institutes of Health

NCGC
NIH Chemical Genomics Center

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
COMPUTATIONAL TOXICOLOGY

The Tox21 timeline



Tox21 - a "Community Resource" Project

Tox21 Phase II human nuclear receptor and related qHTS assays*

AhR full length receptor in HepG2 cells
AR full length receptor in MDA kb2 cells and partial receptor in HEK293 cells
ERα full length receptor in BG1 cells and partial receptor in HEK293 cells
FXR partial receptor in HEK293 cells
GR full length receptor in HeLa cells
PPARδ partial receptor in HEK293 cells
PPARγ partial receptor in HEK293 cells
PXR full length receptor in HepG2 cells
TRβ full length receptor in GH3 cells and partial receptor in HEK293 cells
VDR partial receptor in HEK293 cells
Inhibition of aromatase using MCF-7 cells

All NR assays conducted in agonist and antagonist modes

**Bolded text indicates completed assays*

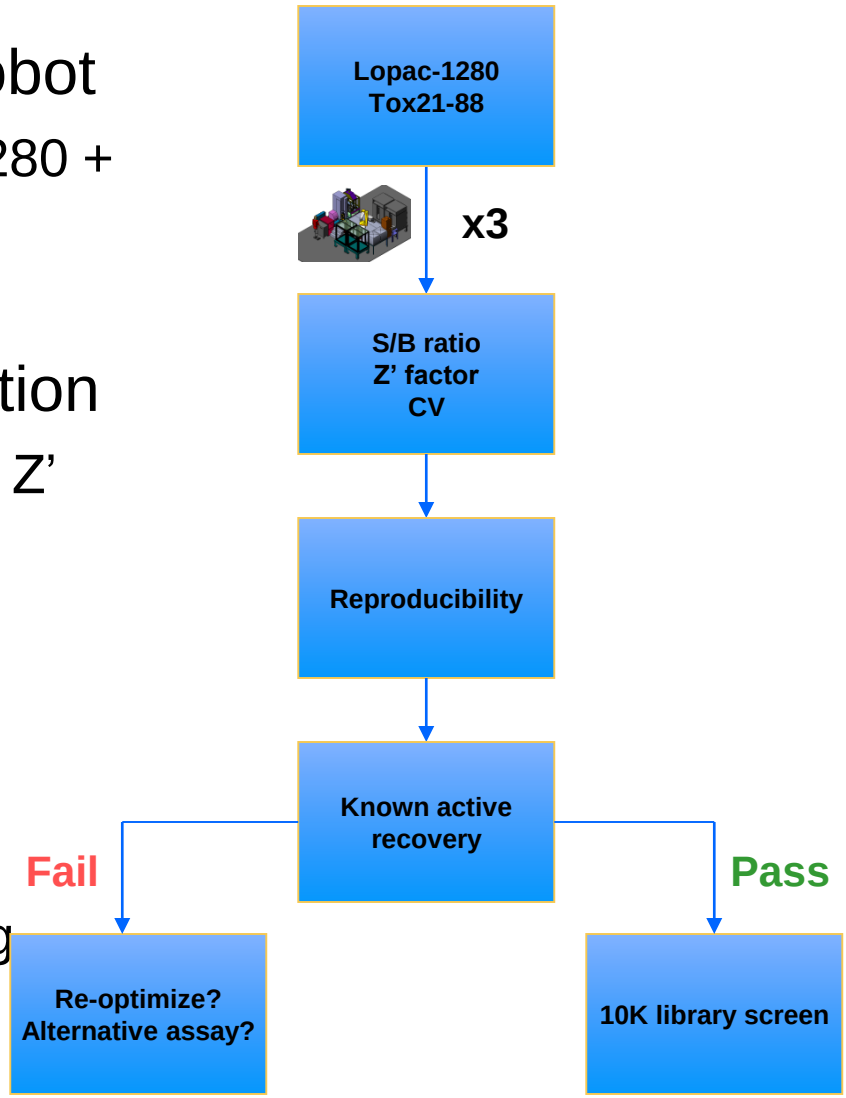
Tox21 Phase II stress response and other qHTS assays*

Endpoint	Assay
Endoplasmic reticulum stress	Induction of lipid damage in HeLa cells
Genotoxic stress	**p53 activation in HCT-116 colon cancer cells**
	ATAD5 activation (DNA damage response element) in HEK293 cells
	Increased cytotoxicity in isogenic DNA-repair deficient chicken DT40 cell clones (Rev3 (-/-), rad54/ku70 (-/-) vs wild type
Heat shock	**Hsp70 induction in HeLa cells**
Hypoxia	Induction of hypoxia inducible factor 1α in ME-180 cervical carcinoma cells
Inflammation	Induction of NFκB in ME-180 cells
Oxidative stress	**Induction of antioxidant response element Nrf2 in HepG2 cells**
Other	Activator protein-1 activation in ME-180 cells
	Caspase 3/7 activation in multiple cell lines
	Cytotoxicity (LDH release, ATP levels) in multiple cell lines
	Mitochondrial membrane potential in HepG2 cells

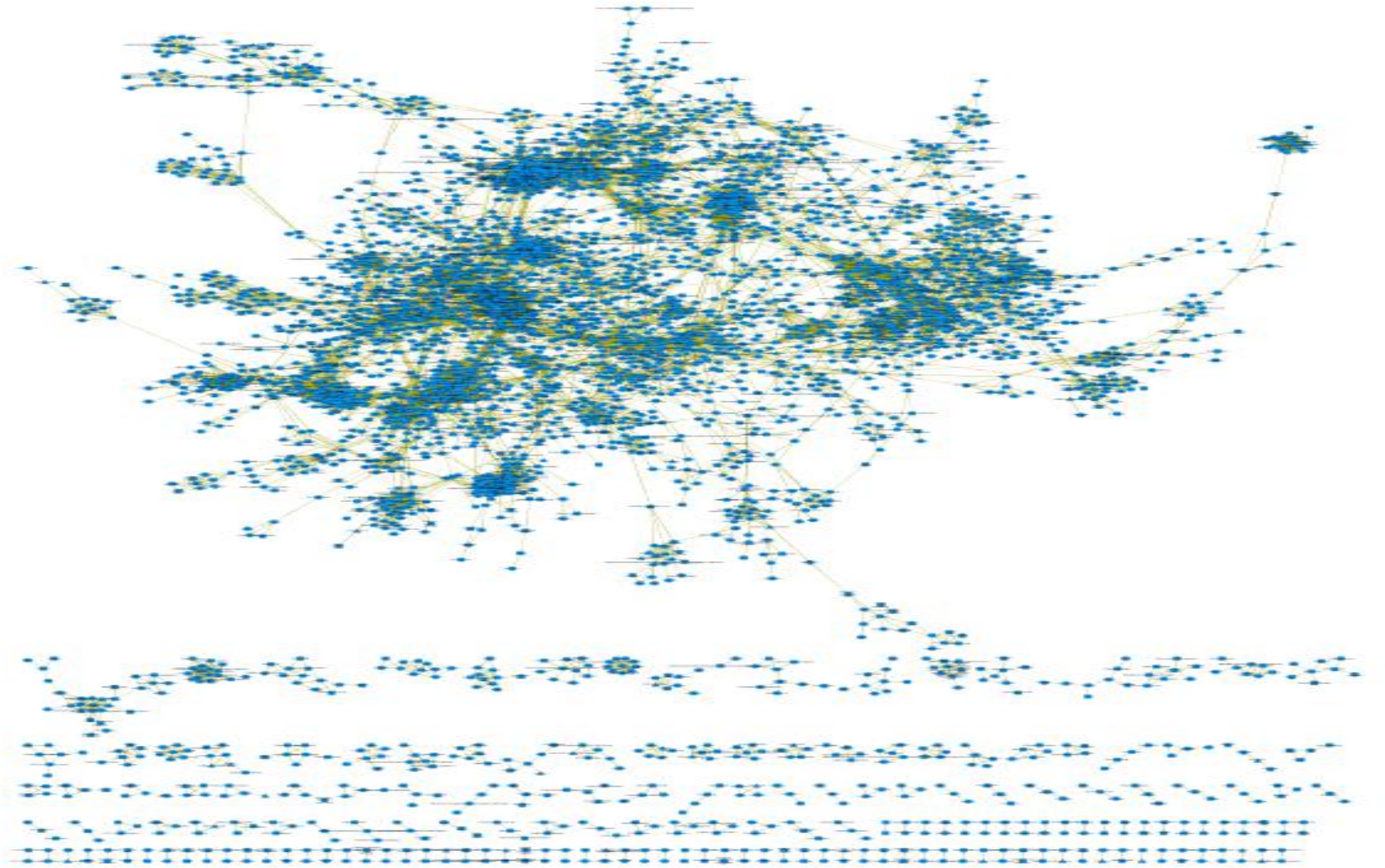
**Bolded text indicates completed assays*

NCGC qHTS Assay Evaluation Process

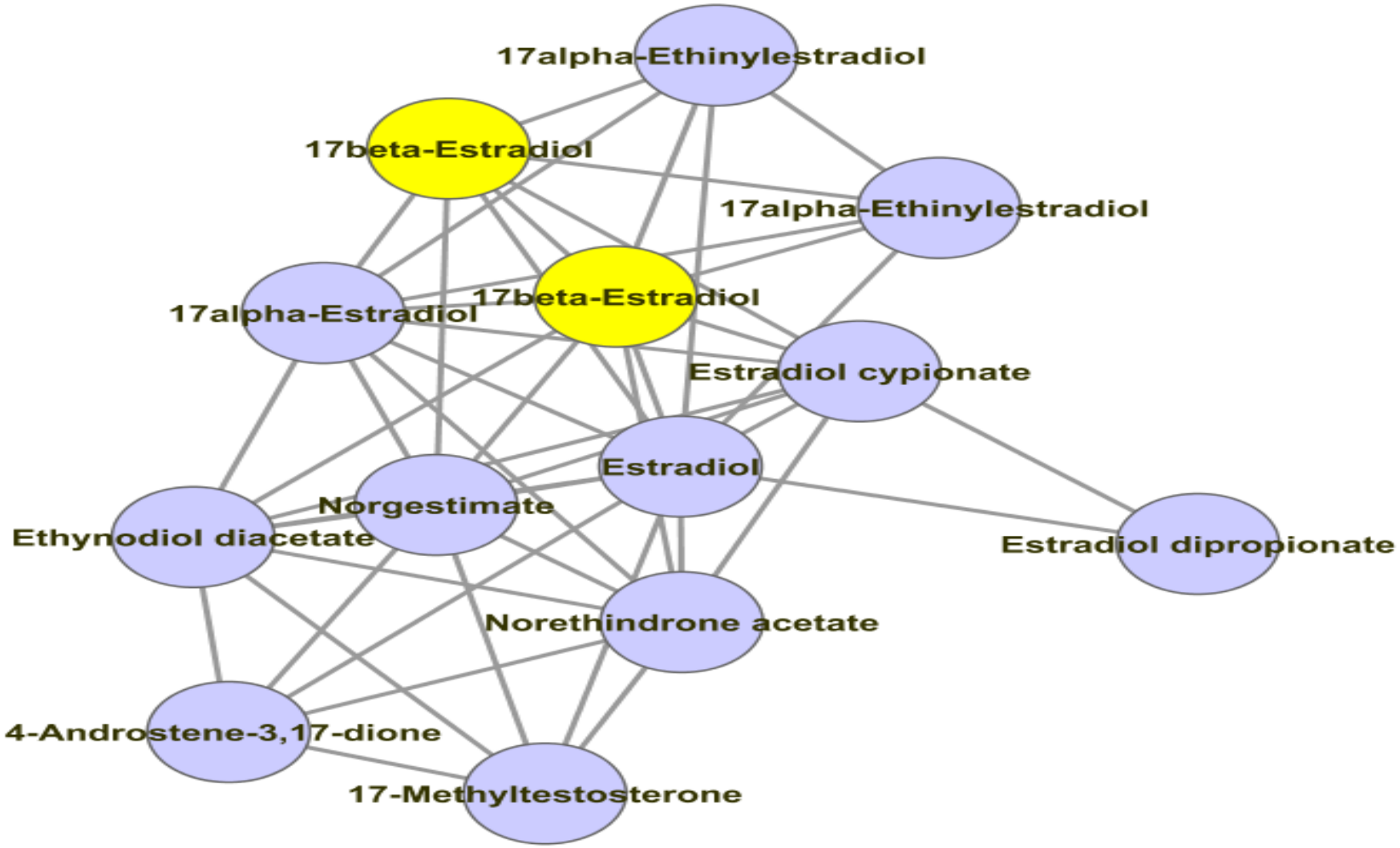
- Online validation on Tox21 Robot
  - Tox21 validation plate (Lopac-1280 + 88 Tox21 replicates)
  - Triplicate runs
- Acceptance criteria consideration
  - Performance metrics - S/B ratio, Z' factor, CV
  - Reproducibility
  - Ability to identify reference compounds/known actives
- Pass
  - Proceed to 10K library screening
- Fail
  - Go back to optimization?
  - Select alternative assay?



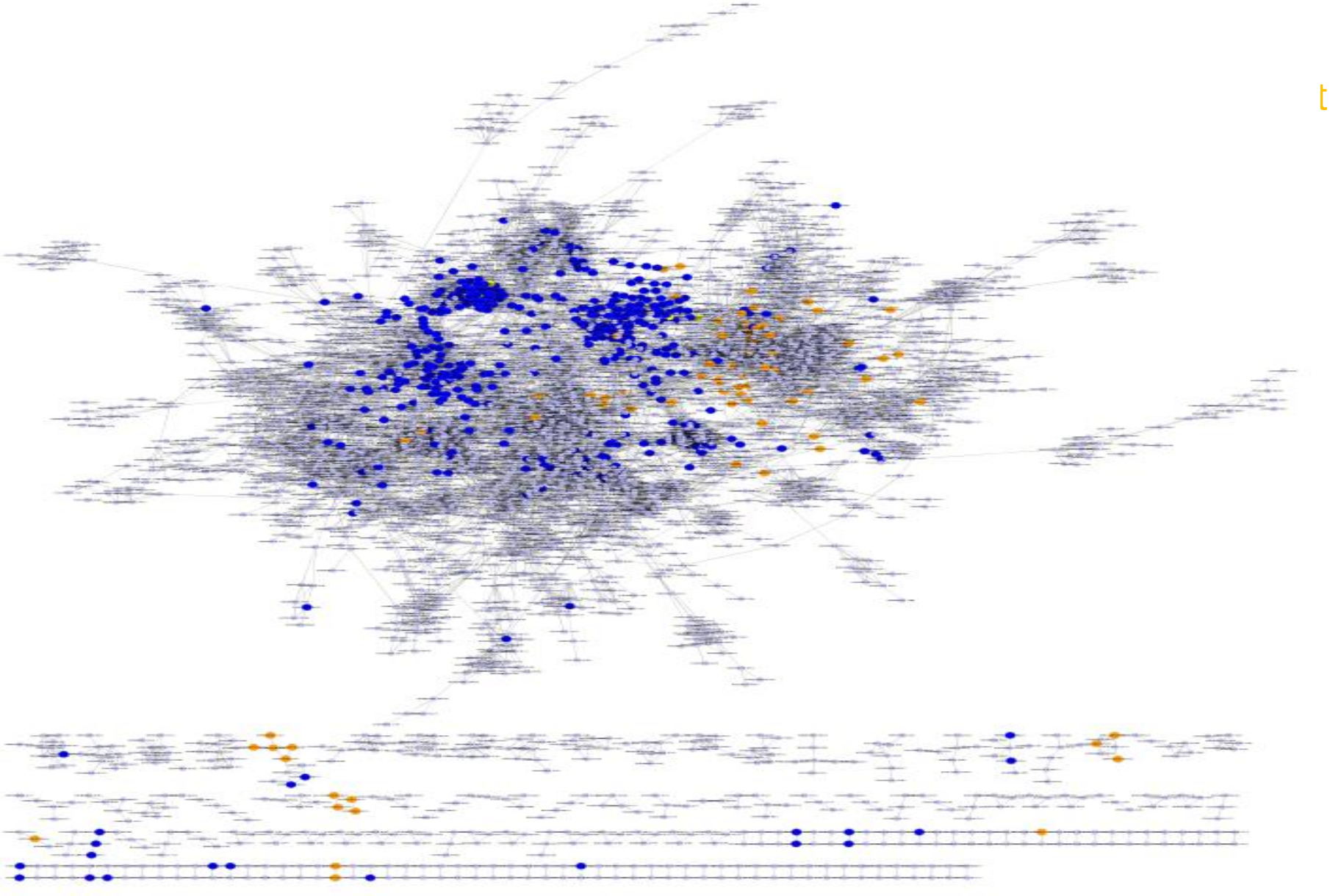
pAC50 with Pearson correlation >0.7
Connectivity network for all assays to date



17β-Estradiol (Pearson >0.7) nearest neighbors

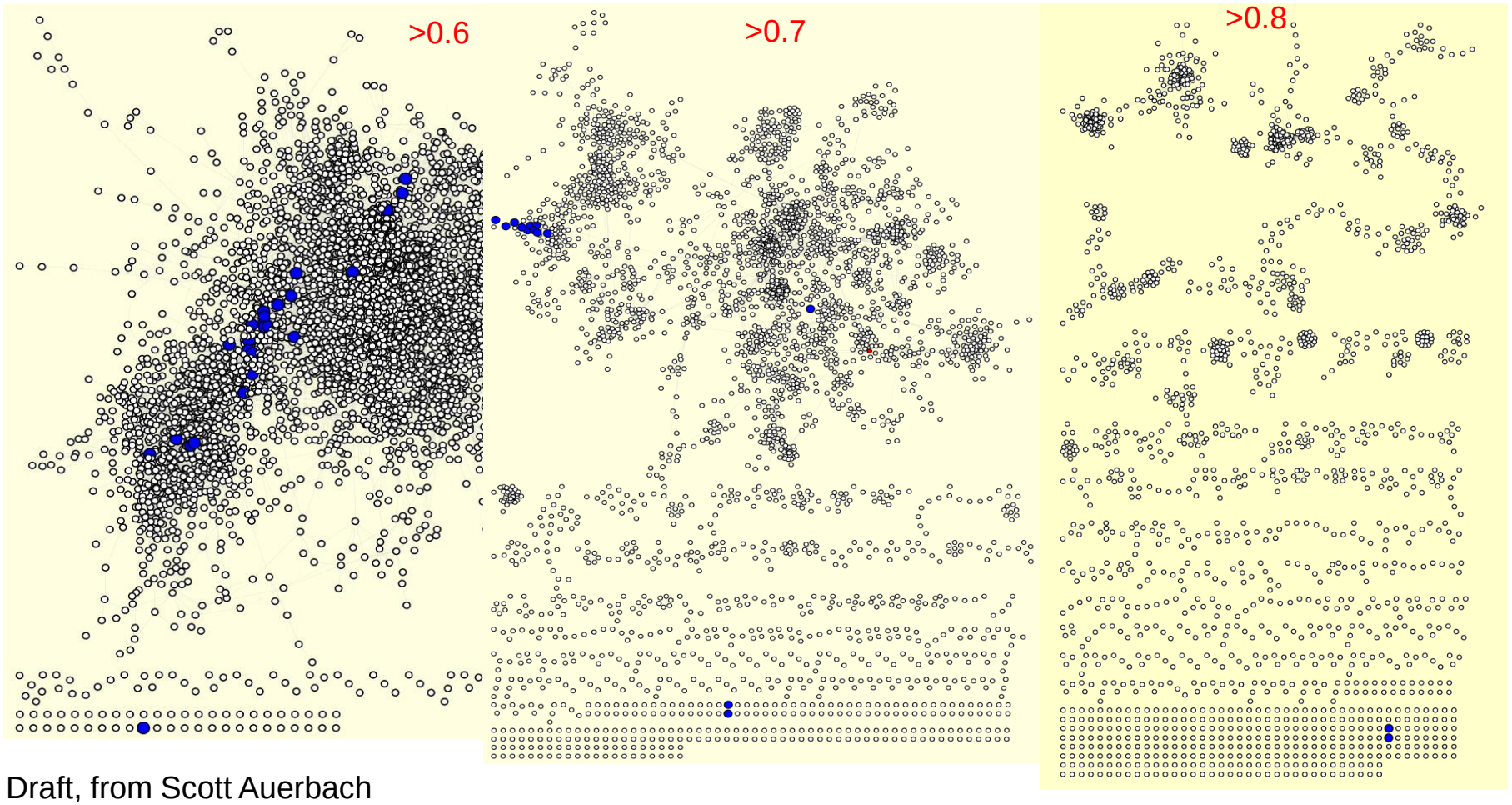


ER actives (pAC50 with Pearson correlation >0.7) Connectivity network for all assays with ER “painting”



Sensitivity Analysis for Similarity Profiling

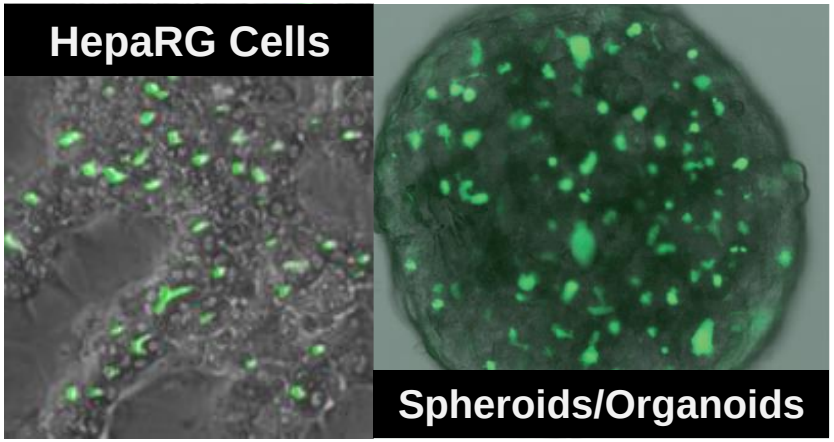
- Impact of changing stringency criteria
- Whole network or specific set of assays



Draft, from Scott Auerbach

Future Data Streams: Tox21 Phase III – Improving on Biological Coverage & Relevance (2013 - ?)

- Develop more physiologically-relevant in vitro and lower organism models and assays
- Incorporate xenobiotic metabolism & longer-term exposures
- Increase use of in silico models (e.g. xenobiotic metabolism, toxicity) and quantitative extrapolation models
- Integrate data-rich assay approaches capturing various molecular pathways & cellular phenotypes
- Utilize Adverse Outcome Pathways (AOPs)
- Expand collaborations and interactions



Near-Term Targeted Assays

•High Content screening assays
- Hoechst: Cell loss & nuclear size
- DHE: Oxidative stress/ROS
- p53: DNA damage
- pH2A.X: Genotoxicity
- JC-10: Mitochondrial damage (MMP)
- Caspase 3: Apoptosis
- Lipitox: Steatosis & Phospholipidosis
- Reactive metabolites/ROS: GSH depletion

•Gene expression assays
- ~1000 genes, multiple species

Tox21 challenges and questions

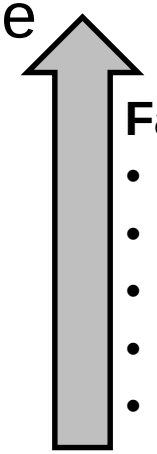
- Major challenges and areas under development:
  - Metabolism
  - Multiplexed endpoints
  - Higher order cell and tissue interactions
- Major questions:
  - How Tox21 results can inform traditional studies and vice versa
  - Whether identification of affected pathways can predict disease
  - How Tox21 data can be best used to protect public health

What is a Systematic Review?

- A scientific investigation that focuses on a specific question, and uses explicit, pre-specified methods to identify, select, summarize, and assess the findings of similar studies
- Provides greater transparency
- Used to:
  - Reach evidence-based conclusions
  - Clarify need for additional research
  - May or may not result in quantitative meta-analysis
- Existing methodologies are primarily used for assessment of healthcare interventions
  - e.g., Cochrane, AHRQ, GRADE

Develop Conclusions on Confidence in Body of Evidence

- Consider factors that can increase or decrease confidence for human and animal data
- Similar factors apply to non-traditional toxicology data
  - risk of bias (internal validity)
  - consistency
  - directness/applicability ≈ relevance of concentration and biological activity or process
  - magnitude of effect ≈ potency
  - dose-response
  - publication bias



Factors Considered for Human and Animal Evidence

Factors Increasing Confidence

- magnitude of effect
- dose response
- residual confounding
- consistency
- other

Factors Decreasing Confidence

- risk of bias (internal validity)
- unexplained inconsistency
- indirectness/applicability
- imprecision
- publication bias

Factors Considered When Evaluating Non-Traditional Toxicology Data

Weak Support	Strong Support
Relevance of biological process or pathway to human health	
limited relevance or uncharacterized	generally accepted as relevant
Consistency	
no studies or unexplained inconsistency	consistency across multiple studies (preferably more than 2 in different model systems)=
Relevance of concentration	
"high" concentration effects	ects
Potency	
weak response relative to positive con	
Dose response	
no dose response gradient or single concentration tested	displays expected dose response gradient
Publication bias	
strongly suspected	undetected

Also need to address consideration of similarity of structure or biological activity to more characterized analogue, metabolites, physical chemistry properties

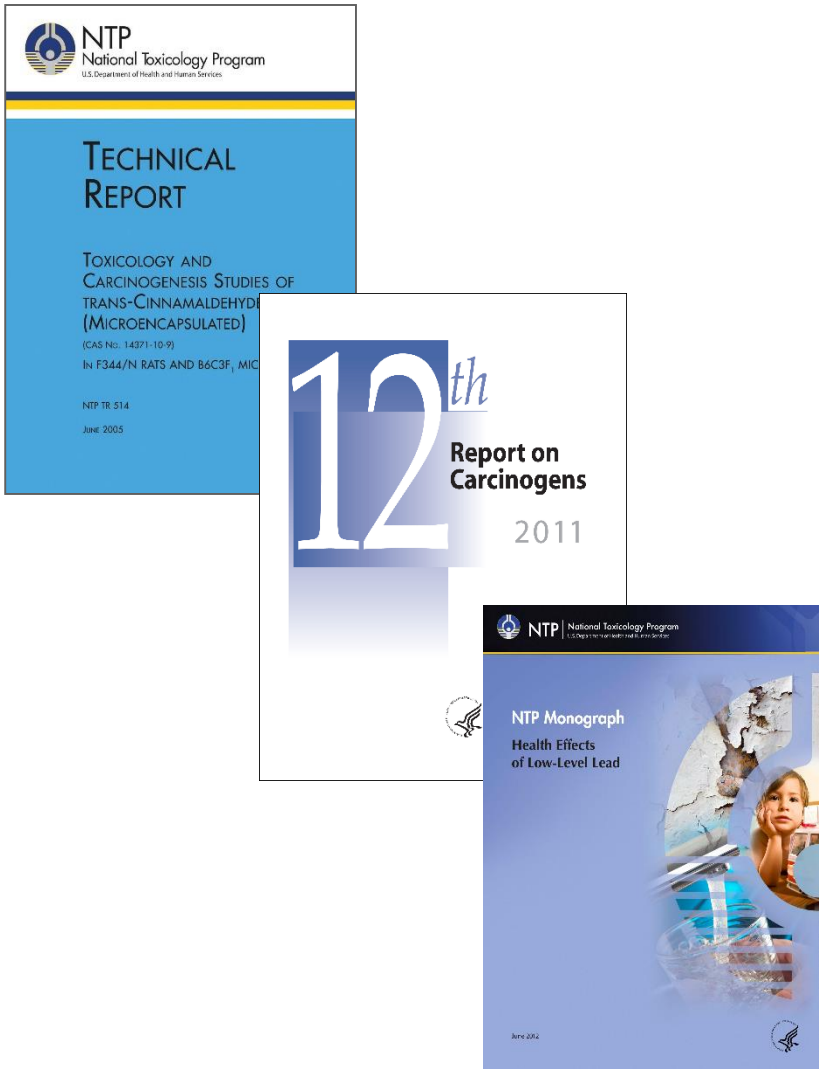
Recap: New areas of research emphasis

- Early life exposures
- Efficient use of animals
- Regulatory guideline vs. academic studies
- Epigenetic changes
- Differential susceptibility
- Predictive toxicity and disease- the Tox21 approach
- Systematic review for mechanistic studies

Questions?

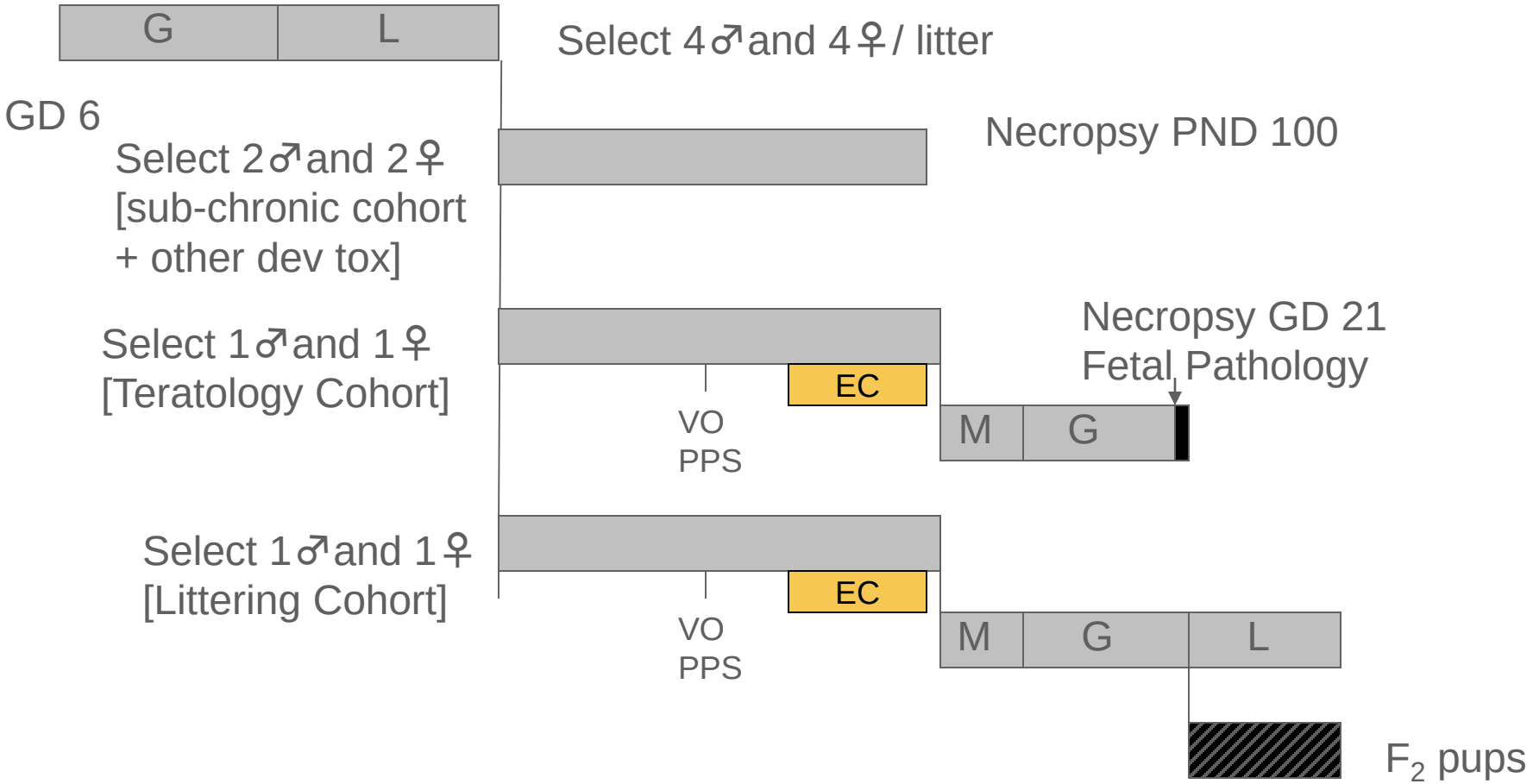
How does the US NTP report its findings?

- Technical Reports
  - ~600 two-year cancer assays
- Toxicity Reports
  - ~100 shorter term toxicity studies
  - Immunotoxicity
  - Developmental toxicity
  - AIDS therapeutics toxicity reports
  - Genetically modified models
- OHAT Monographs
- Report on Carcinogens
- All peer reviewed and available for free download from the NTP website
  - http://ntp.niehs.nih.gov/go/reports
- Journal articles ~300/year



NTP modified one-generation study

Timed – pregnant female rats: minimum of 20 litters/group; 3 dose groups + control



Other NTP resources

- Archives
  - Samples from >1400 NTP studies
  - >110,000 frozen samples
  - ~5 million tissue blocks
  - >200,000 formalin preserved tissues
  - Study data

- Techniques
  - Recover usable RNA from formalin fixed-paraffin embedded tissues for gene expression studies
- Databases
  - Bioassay pathology data
  - Other non pathology data from NTP studies
  - Chemical Effects in Biological Systems (CEBS) database
  - ICONIX/Drug Matrix microarray database
  - Tox 21 data

Same set of questions applied to different study designs

Risk of Bias Domain	Criterion	Animal	Controlled Exposure	Cohort	Case-Control	Cross-sectional	Case Series
Selection	Was administered dose or exposure level adequately randomized?	X	X				
	Was allocation to study groups adequately concealed?	X	X				
	Were the comparison groups appropriate?			X	X	X	
Confounding	Did the study design or analysis account for important confounding and modifying variables?	X	X	X	X	X	X
	Did researchers adjust or control for other exposures that are anticipated to bias results?	X	X	X	X	X	X
Performance	Were experimental conditions identical across study groups?	X	X				
	Did deviations from the study protocol impact the results?	X	X	X	X	X	X
	Were the research personnel and human subjects blinded to the study group during the study?	X	X				
Attrition	Were outcome data incomplete due to attrition or exclusion from analysis?	X	X	X	X	X	
Detection	Were the outcome assessors blinded to study group or exposure level?	X	X	X	X	X	X
	Were confounding variables assessed consistently across groups using valid and reliable measures	X	X	X	X	X	X
	Can we be confident in the exposure characterization?	X	X	X	X	X	X
	Can we be confident in the outcome assessment?	X	X	X	X	X	X
Reporting	Were all measured outcomes reported?	X	X	X	X	X	X
Other	Were there any other potential threats to internal validity (e.g., inappropriate statistical methods)?	X	X	X	X	X	X