



NEW METHODOLOGY FOR PHENOTYPING P450 POLYMORPHISMS AND SUSCEPTIBILITY TO ENVIRONMENTAL TOXICANTS USING HUMANIZED BUDDING YEAST

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Outline of talk

- Aflatoxins and hepatocarcinogens
- Activation of aflatoxins
- Biomarkers for aflatoxin exposure in yeast
- Characterization of P450 Polymorphisms
- High-throughput screening for AFB1 resistance
- Identity of “Candidate Genes”
- Conclusions and Future Directions



Aspergillus Contamination in Corn and Peanuts



<http://aes.missouri.edu/delta/croppest/aflacorn.stm>



<http://cgiarweb.s3.amazonaws.com/wp-content/uploads/2012/04/groundnuts.jpg>

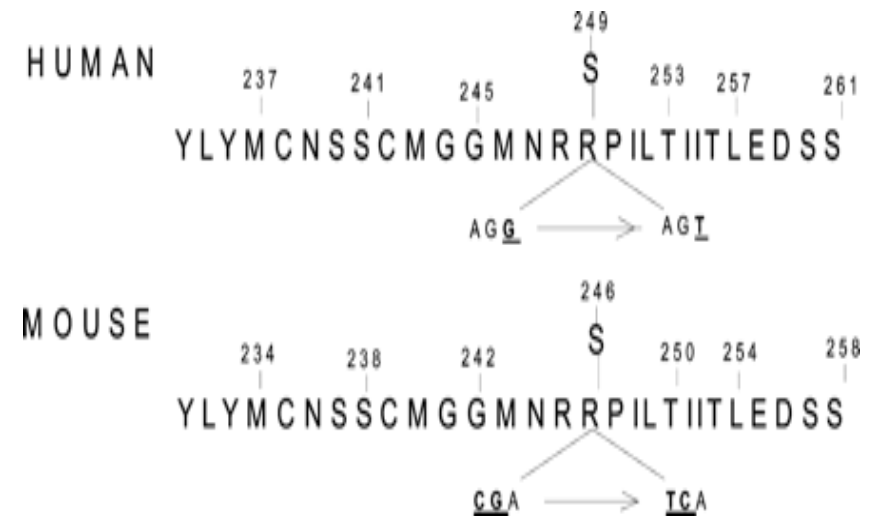
AFBI FDA limits = 20 ppb for humans, up to 500 ppb can be blended for animals



Hepatocellular Carcinoma is Concentrated in AFB1 Contaminated Areas in China



Hepatocellular carcinoma incidence correlates with aflatoxin B1 (AFB1) and HBV exposure

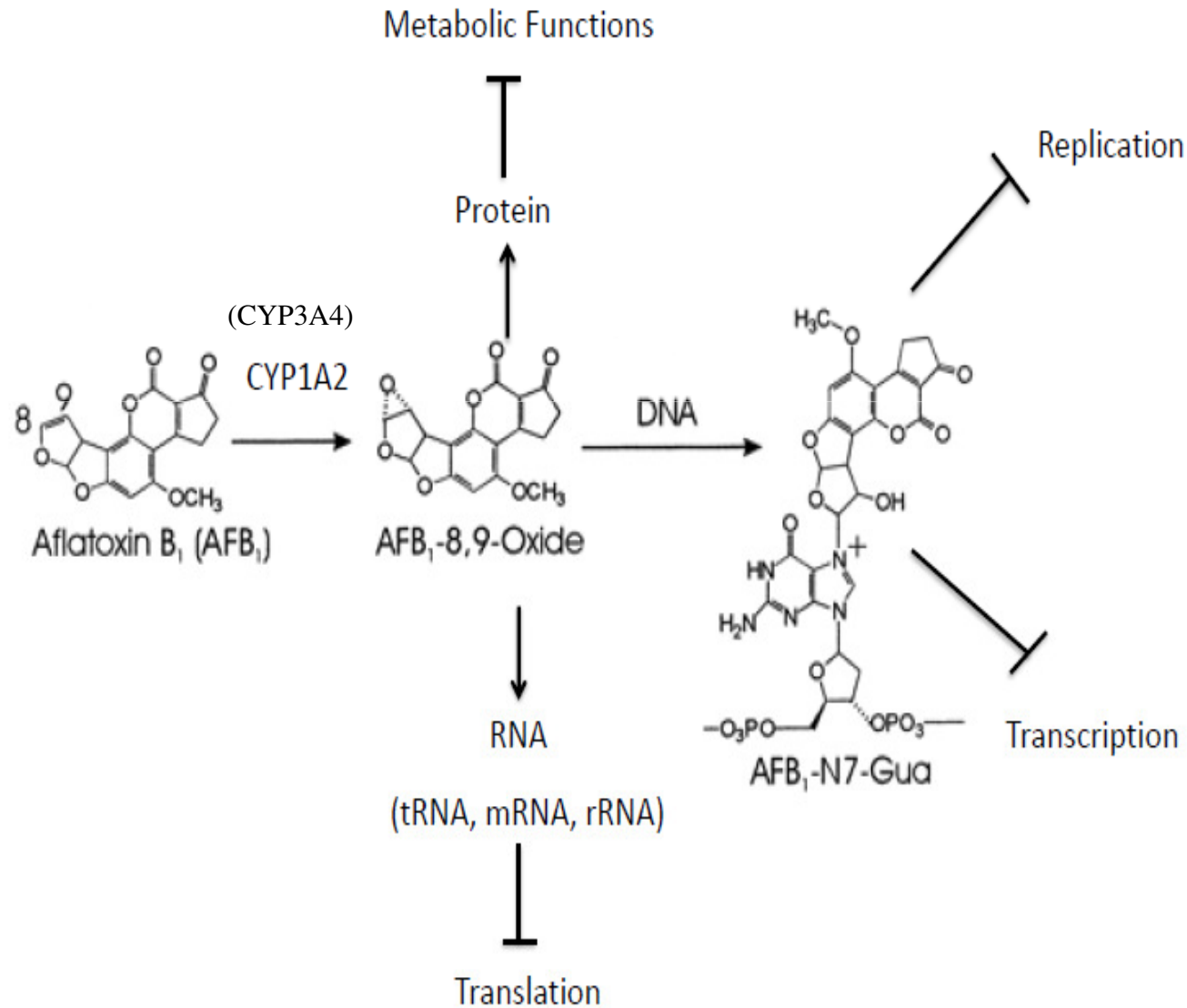


Specific P53 S(249) mutation is found in HCC corresponding to AFB1 exposure

Sell, Cancer Res. 63:7554, 2003



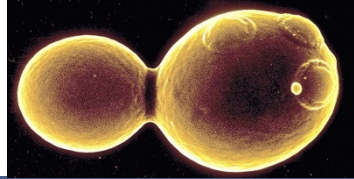
AFB1 Requires Bioactivation





Risk Factors Involved in Hepatocarcinogenesis:

- Aflatoxin exposure
- Hepatitis B exposure
- Detoxification by Phase II enzymes (GSTa3, mouse)
- *Polymorphisms in P450 genes*
- *Polymorphisms in DNA repair/cell metabolism/oxidative stress response*

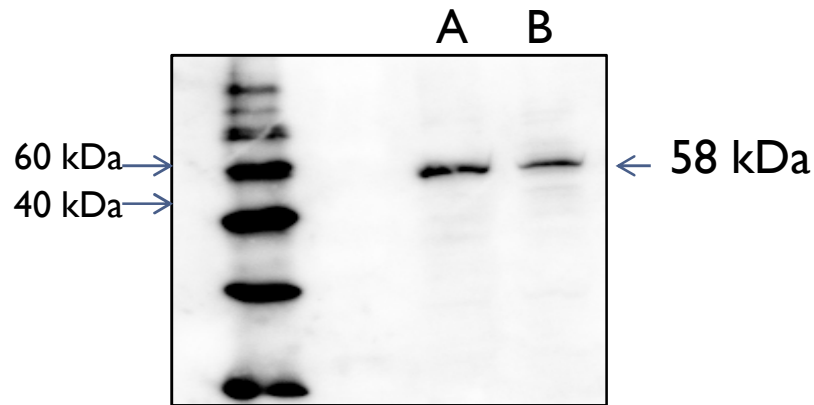
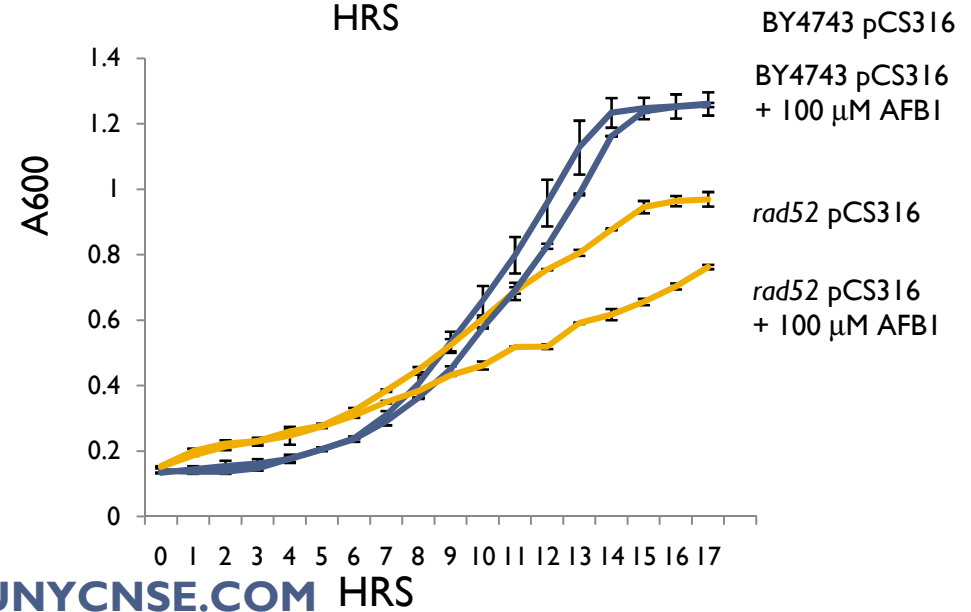
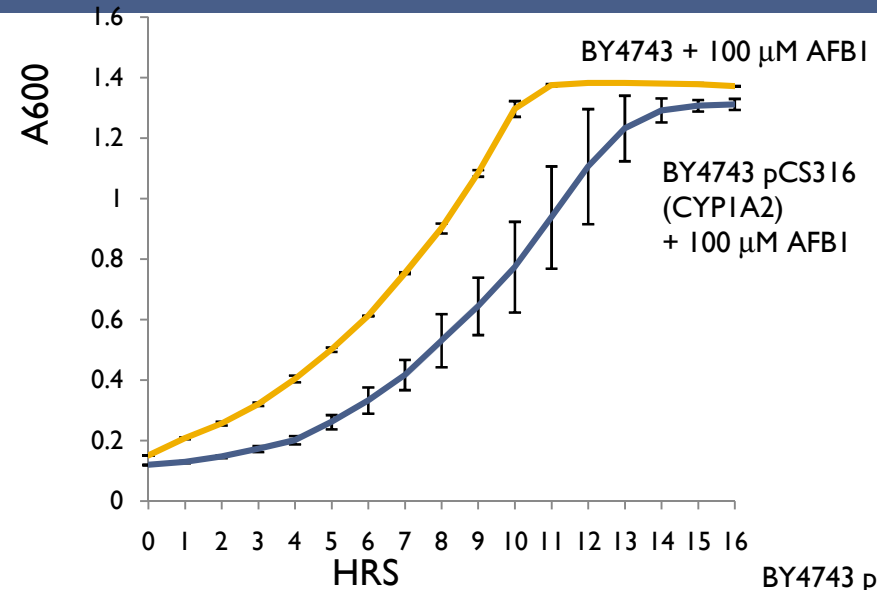
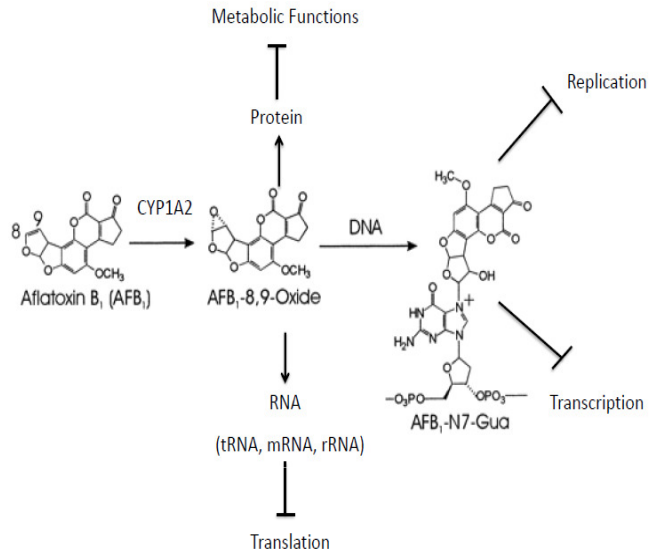


Why Yeast? It is a eukaryote!

- Budding yeast has no P450 enzyme that activates carcinogens; different genotoxic responses can be assayed after expression of different P450 enzymes
- 31% of yeast genes are orthologous are similar to human genes, including those that function in DNA repair, replication, and general housekeeping function
- DNA damage response can be easily profiled: checkpoint activation, cell-cycle arrest, DNA adducts
- Systems biology approach for genetic transcription, resistance profiling, and proteomics
- Damage to eukaryotic organelles, such as mitochondria, can be measured.

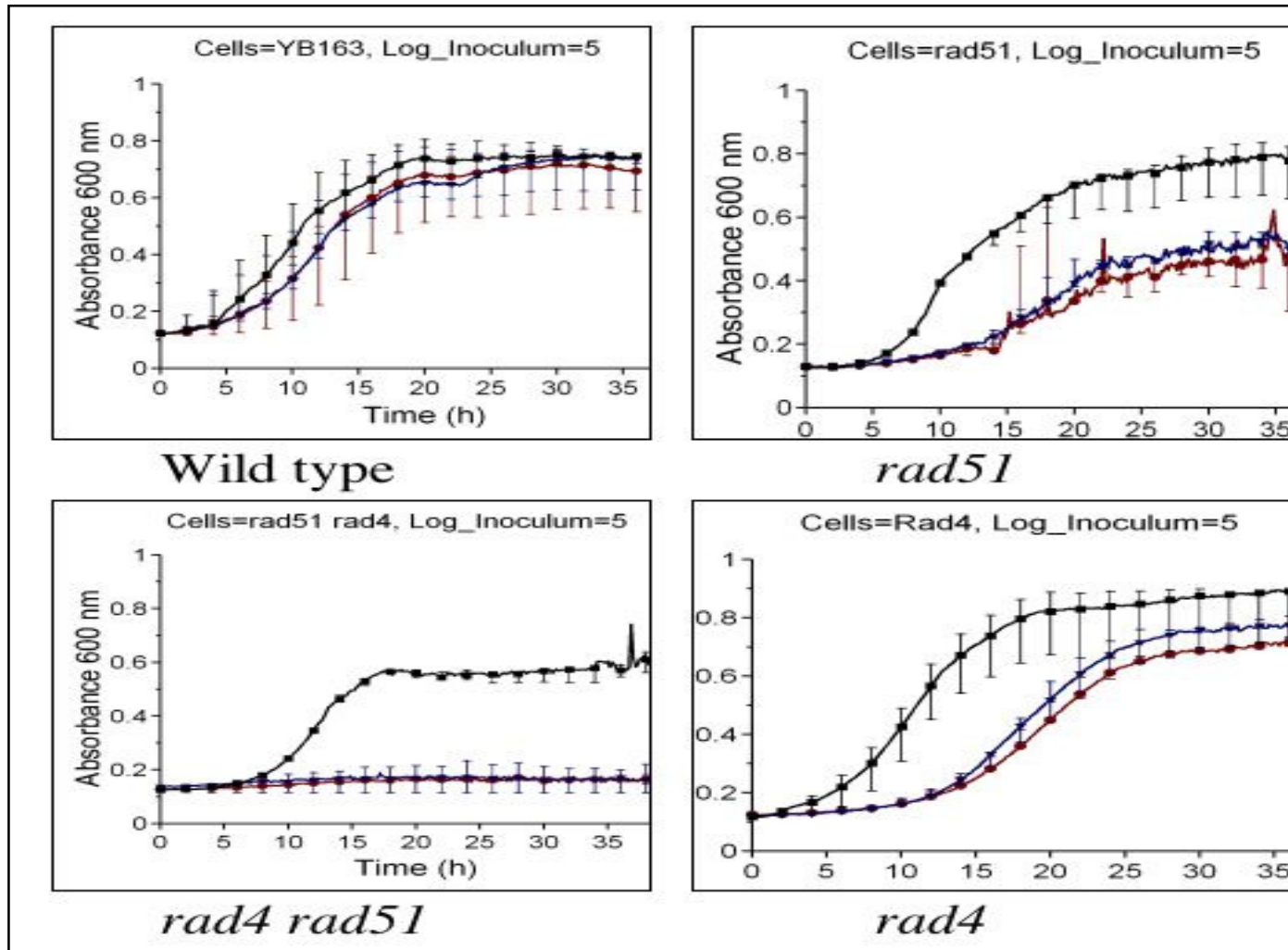


Yeast AFBI Sensitivity Requires P450 Expression





The *rad4 rad51* mutant exhibits extreme AFB1 Sensitivity



Black = 0 uM AFB1, blue = 25 uM AFB1, red = 50 uM AFB1



AFB1 Exposure Increases Expression of 14 DNA Repair Genes

Gene	Fold Stimulation	X-ray inducible	Mammalian Orthologue	Repair Pathway	Enzymatic Function
<i>RAD51</i>	7.7	+	RAD51	HR	DNA strand exchange
<i>POL3</i>	5.0	+	Pol δ	HR, NER, BER	Lagging strand synthesis
<i>RAD3</i>			XPD	NER	TFIIH component, helicase
<i>RFA1</i>	4.6	+	RPA	HR, NER, BER	Single-strand binding protein
<i>DST1</i>	4.6			NER	TFIIS
<i>RAD16</i>	4.5			NER	
<i>MLH1</i>	4.1		MLH1	MMR	Mismatch repair chaperone
<i>MMS21</i>	2.9			MMR	
<i>MET18</i>	2.5			NER	Transcription factor
<i>SRS2</i>	2.4	+		HR	Helicase
<i>RFA2</i>	2.2		RPA	HR, BER, NER	Subunit of RPA
<i>MSH6</i>	2.2		MSH6	MMR	
<i>RAD1</i>	2.1		XPF	NER	Excision of branched molecules
<i>MLH3</i>	2.0			MMR	Mismatch repair chaperone

Other genes induced:
> 2x

TOR1
TOR2
PTK1
BIM1
MAD1

HR, homologous recombination
NER, nucleotide excision repair
BER, base excision repair
MMR, mismatch repair

Keller-Seitz, M., Certa, Ulrich, Sengstag, C., Wurgler, F., Sun, M., and Fasullo, M. Transcriptional response of the Yeast to the carcinogen Aflatoxin B₁: Recombinational repair involving *RAD51* and *RAD1*. *Molecular Biology of the Cell*. 15:4321-4336, 2004



Expression of P450 Polymorphisms in Yeast

Rationale:

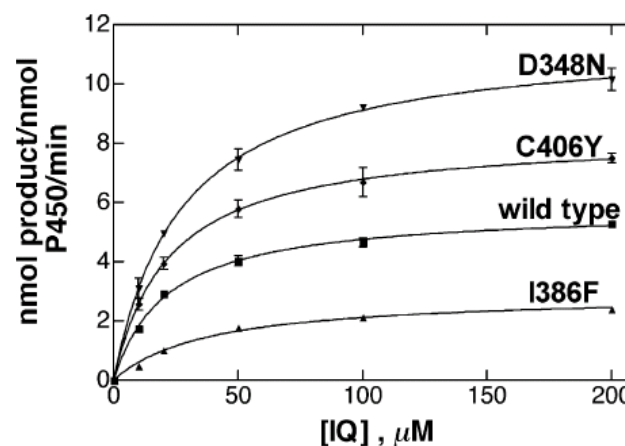
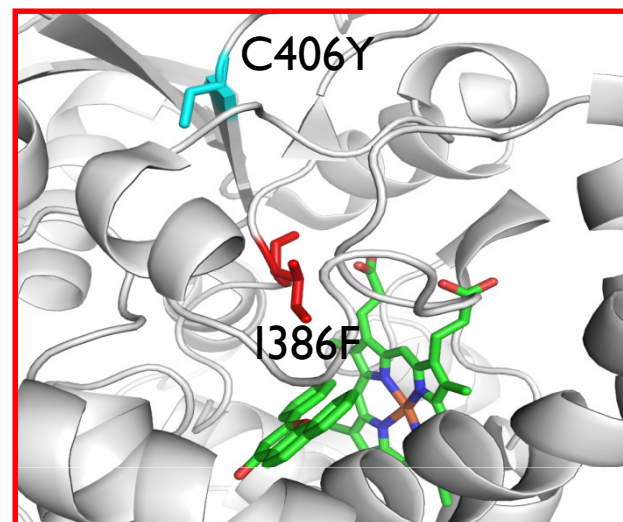
- Phenotype of rare polymorphisms in amino acid coding sequences are difficult ascertain by epidemiological data
- Full-length cDNA sequences can be expressed in yeast
- Biomarkers can be quantified
 - DNA adducts
 - Toxicity in DNA repair mutants
 - Rad51 foci



Cytochrome P450 1A2 alleles

Polymorphism	Population	Allele freq	Comments*
*2 (F21L)	Chinese	.003	
*3 (D348N)	French	.005	
*4 (I386F)	French	.005	
*5 (C406Y)	French	.005	
*6 (R431W)	French	.005	
*7 (Abnormal splicing)			
*8 (R456F)	Japanese	.004	EROD <.01
*11 (F186L)	Japanese		EROD <.3
*15 (P42R)	Japanese	.004	EROD <.01
*16 (R377Q)	Japanese	.004	EROD <.01
-3860G/-3113G/5347C	Chinese		
K254G	Laboratory	NA	EROD <1
S126K	Laboratory	NA	EROD <1

*7-ethoxyresorufin deethylation (EROD) activity is presented in fraction activity compared to wild type



Chevalier et al. Hum Mutat 17:355-356.

<http://jpet.aspetjournals.org/content/308/1/300.short>

Zhou et al. Mutat Res. (2004) 422

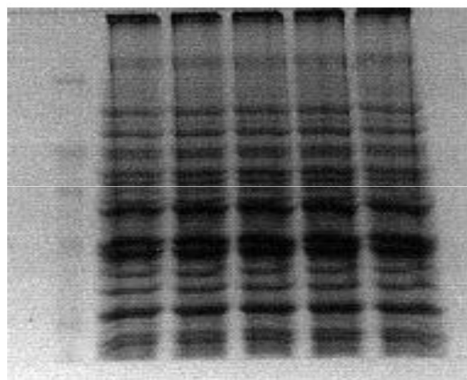


Concentrations of AFB₁ ⁷N-Gua Adducts in DNA.

Western



Coumassie Stain



CYP450 ^a	AFB ₁ ⁷ N-Guanine /mg DNA ^b	Ratio ^c
CYP1A2	5.0 ± 3.9 pmole	1
C406Y	2.2 ± 0.05 pmole	0.4
I386F	0.3 ± 0.16 pmole	<0.1
D348N	8.3 ± 6.9 pmole	1.7
None	≤0.05 pmole	<0.01

^aIndicates CYP1A2 allele expressed in yeast

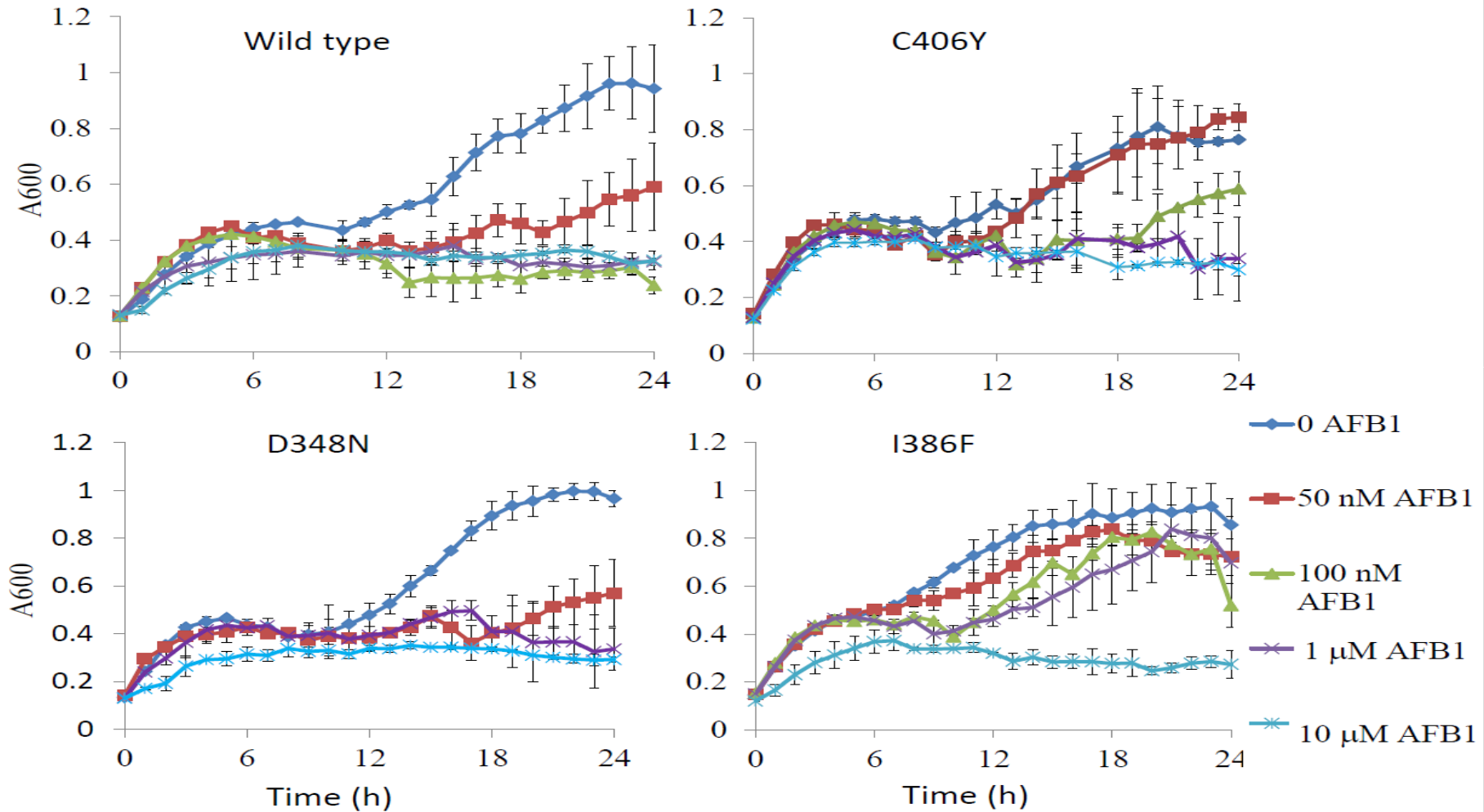
^bN > 2, generated after exposure to 50 μM AFB1

^cAdducts CYP1A2 allele/Adducts WT, numbers in bold indicate significant differences with wild type.

Fasullo, M. Smith, A., Egner, P and Cera, C. Activation of Aflatoxin B₁ by expression of CYP1A2 polymorphisms in *Saccharomyces cerevisiae*,. Mutation Research 761:18-26, 2014



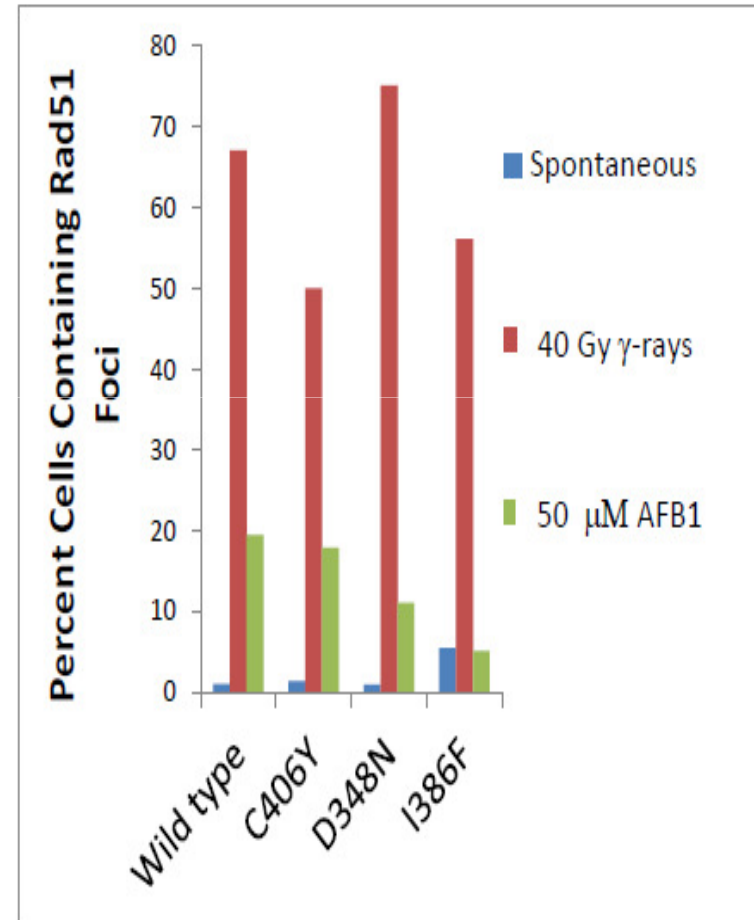
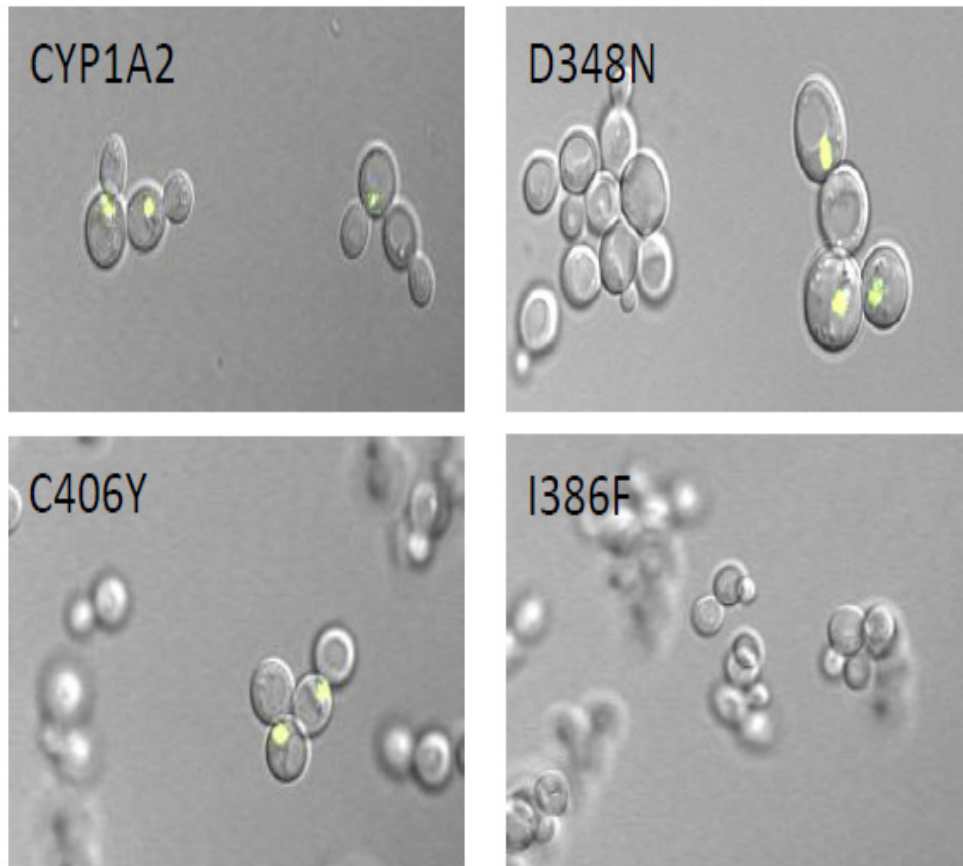
AFB1 Toxicity in *rad4 rad51* mutants expressing P450 Polymorphisms



Fasullo, M. Smith, A., Egner, P and Cera, C. Activation of Aflatoxin B₁ by expression of CYP1A2 polymorphisms in *Saccharomyces cerevisiae*,
Mutation Research 761:18-26, 2014



Rad51 Foci Appear in Yeast Expressing CYP1A2 alleles



Fasullo, M. Smith, A., Egner, P and Cera, C. Activation of Aflatoxin B₁ by expression of CYP1A2 polymorphisms in *Saccharomyces cerevisiae*., Mutation Research 761:18-26, 2014



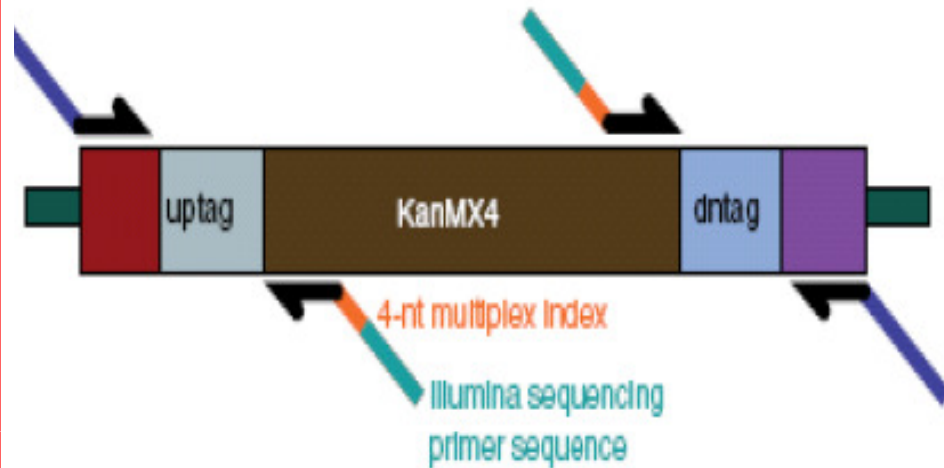
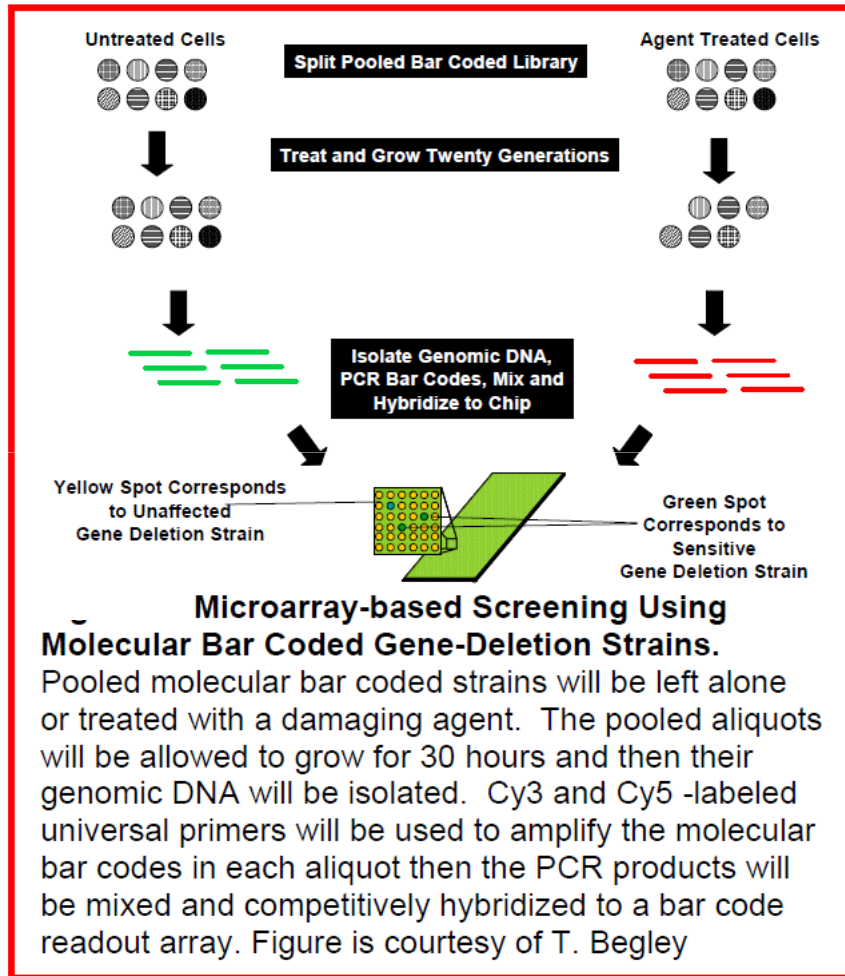
Summary of CYP1A2 allele expression in Yeast

- Full-length CYP1A2 proteins can be expressed in yeast
- DNA damage biomarkers can be used to differentiate AFB1 activation by polymorphic P450 enzymes
- Methodology could be expanded to other CYP1A2 alleles or genotoxins



“Humanized” Yeast Deletion Collection

- >5,000 strains, each strain contains a deletion for a single gene
- Each gene deletion is marked by an antibiotic cassette and two molecular bar codes (identifiers)
- The presence of the strain in the pooled collection can be quantified by detecting the molecular bar codes
- Human CYP1A2 has been introduced into 90% of the collection



- Centralized KanMX4 gene
- Flanked by uptag and dntag (~20-25 unique BP)
- Bound 4-nt multiplex index and the Illumina sequencing primer sequence



- Uptags counted for three different exposures
 - ▣ Normalize counts/Binomial Statistics

- Compare $\log_2 N$ ratios (treated/untreated)
 - ▣ For three independent experiments

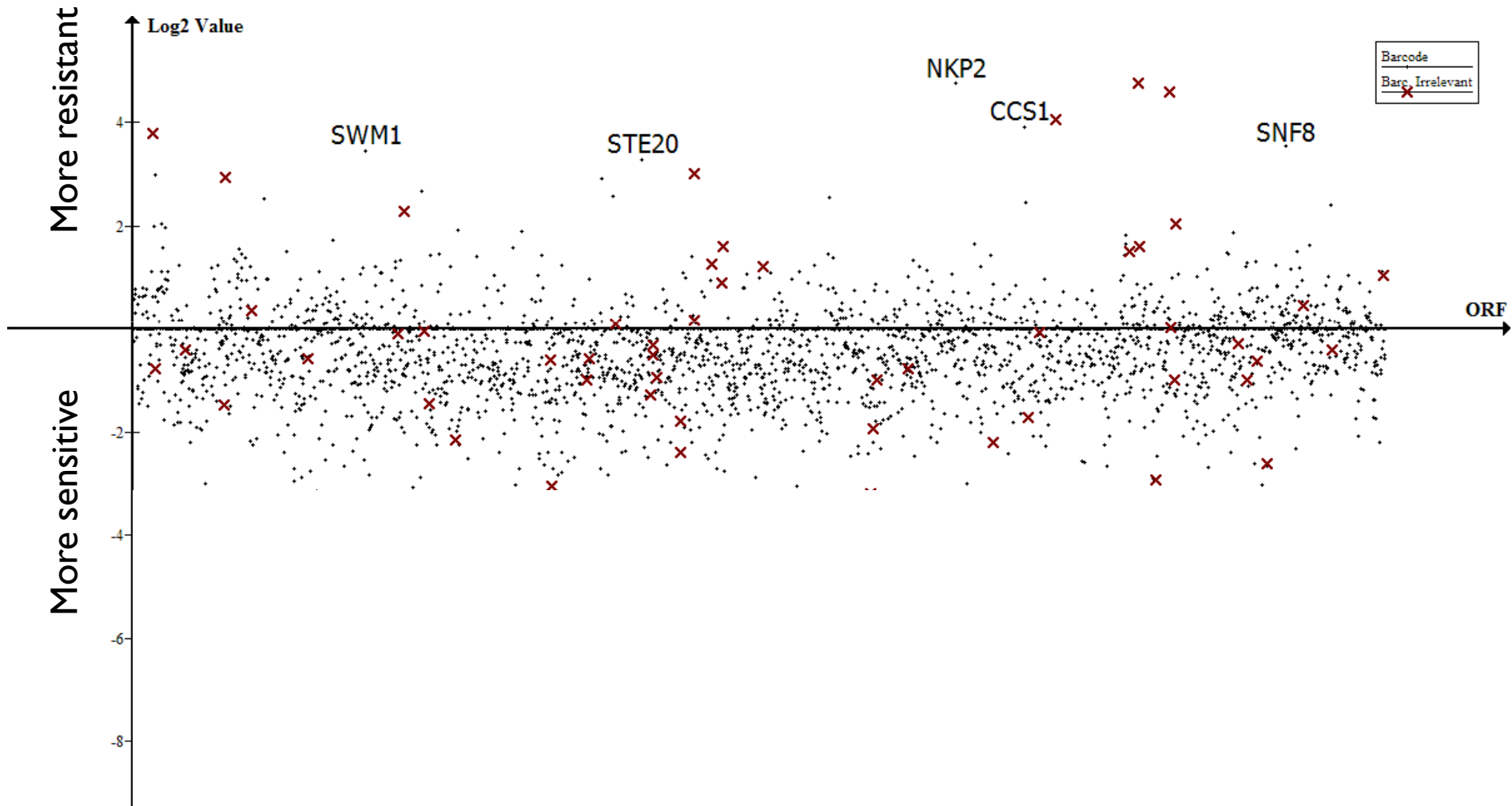
- Collect sensitive and resistant strains

- Group into Gene Ontology groups

- Validate groups by survival curves

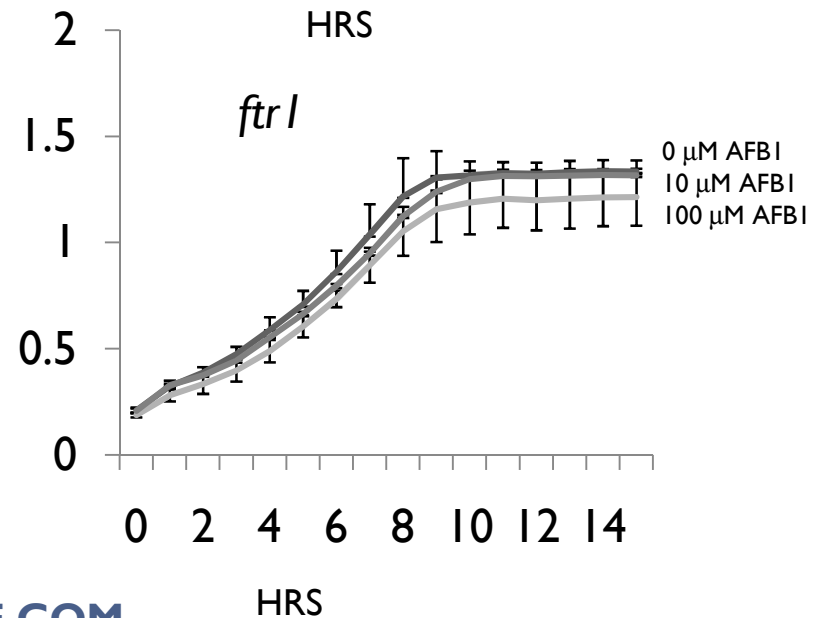
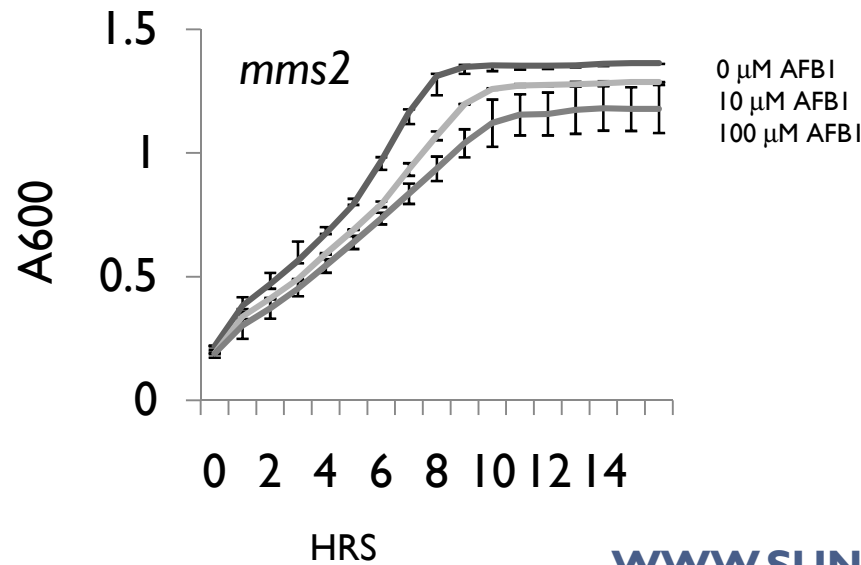
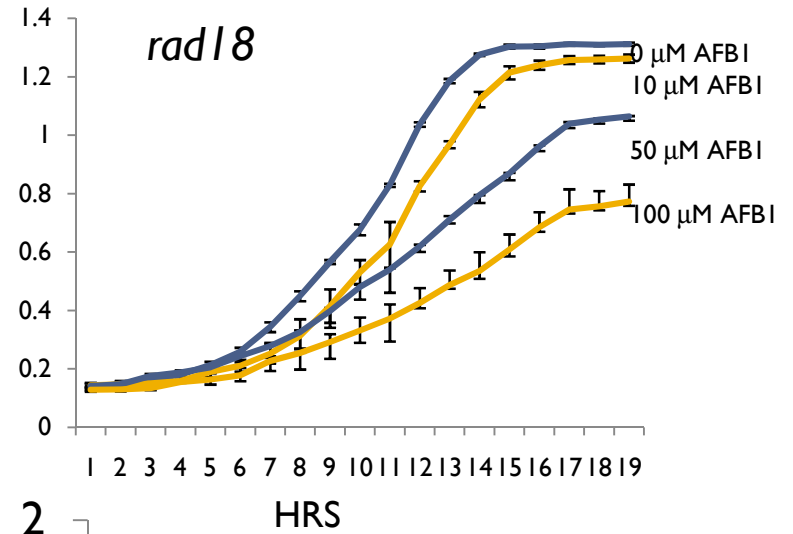
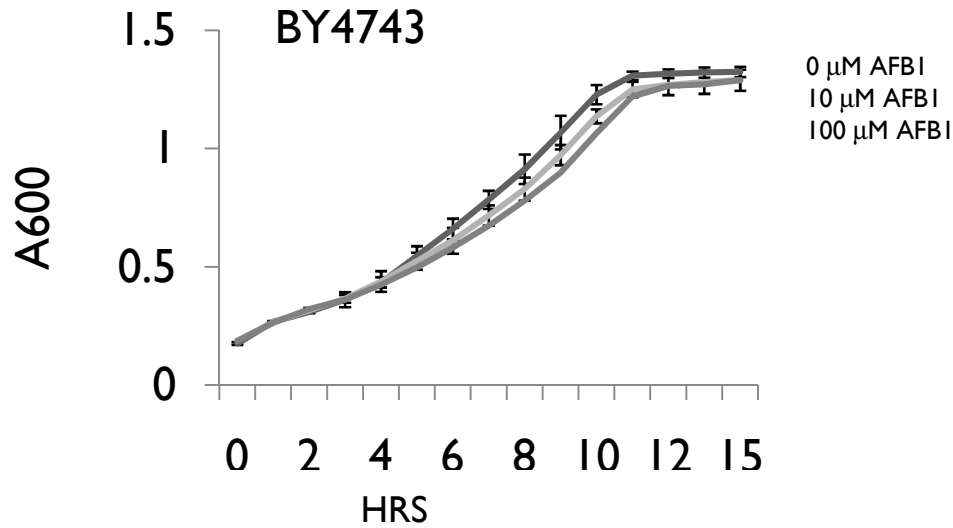


Broad Spectrum of AFB1 Resistance and Sensitivity (Avg.) of 4300 ORFs Detected





Category	p-value	In Category from Cluster	k	f
negative regulation of DNA replication [GO:0008156]	0.0002065	RAD9 SML1 CSM3 TOF1	4	6
response to DNA damage stimulus [GO:0006974]	0.0002218	TEL1 MEC1 DUN1 RAD55 RAD9 RAD51 RAD4 MMS2 CKA1 REV7 MPH1 RAD52 SML1 OGG1 CSM3 MLH1 RAD14 PSY2 TOF1 NTG2 REV1 RAD17 RAD1 MEI5 RAD53 REV3	26	197
DNA damage checkpoint [GO:0000077]	0.0004004	MEC1 DUN1 RAD9 DOT1 RAD17 RAD53	6	17
DNA repair [GO:0006281]	0.0004235	TEL1 MEC1 RAD55 RAD9 RAD51 RAD4 RPH1 REV7 RRD1 MPH1 RAD52 OGG1 CSM3 MLH1 RAD14 TOF1 NTG2 REV1 RAD17 RAD1 MEI5 RAD53 REV3 CTF4	24	183
high-affinity iron ion transport [GO:0006827]	0.0009335	FTR1 AFT1 FET3	3	4
ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway [GO:0043162]	0.00168	STP22 VPS25 SNF7 SNF8 VPS28	5	15
meiotic DNA recombinase assembly [GO:0000707]	0.002225	RAD55 RAD52 MEI5	3	5
sulfate assimilation [GO:0000103]	0.003529	MET8 MET5 TRX1 MET16	4	11
histone phosphorylation [GO:0016572]	0.003922	TEL1 MEC1	2	2
DNA damage induced protein phosphorylation [GO:0006975]	0.003922	TEL1 MEC1	2	2
urea transport [GO:0015840]	0.003922	NPR2 DUR3	2	2
iron assimilation by reduction and transport	0.003922	FTR1 FET3	2	2





RECOMBINATIONAL REPAIR	NUCLEOTIDE EXCISION REPAIR	DNA DAMAGE TOLERANCE/ REPLICATION BYPASS	MISMATCH REPAIR	CHECKPOINT RESPONSE	MODULATION OF THE CHECKPOINT RESPONSE	DNA REPLICATION/ FORK INTEGRITY
<i>RAD51</i>	<i>RAD2</i>	<i>RAD5</i>	<i>MLH1</i>	<i>MEC1</i>	<i>PPH2</i>	<i>MUS81</i>
<i>RAD52</i>	<i>RAD14</i>	<i>RAD18</i>	<i>MSH3</i>	<i>TEL1</i>	<i>PSY2</i>	<i>SGS1</i>
<i>RAD55</i>	<i>RAD10</i>	<i>REV1</i>	<i>MSH4</i>	<i>RAD9</i>		<i>RTT109</i>
<i>RAD54</i>	<i>RAD1</i>	<i>REV3</i>	<i>MSH6</i>	<i>RAD53</i>		<i>RAD27/FEN1</i>
<i>MRE11</i>		<i>RAD30</i>		<i>DUN1</i>		<i>ESC2</i>
		<i>UBC13</i>		<i>RAD17</i>		<i>MPH1</i>
		<i>RAD6</i>				<i>POL32</i>
		<i>MMS2</i>				

Red color indicates meiosis specific



- AFB1 is a strong genotoxin in yeast, biomarkers include DNA adducts and Rad51 foci.
- Resistance genes includes DNA damage tolerance and downregulation of the Checkpoint Response
- Libraries may be useful for screening other CYP-activated xenobiotics and drugs
- Future Directions: Determine whether orthologous genes that confer resistance to AFB1 also do so in mice



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