Insights from Animal Models for Cryptococcal Meningitis

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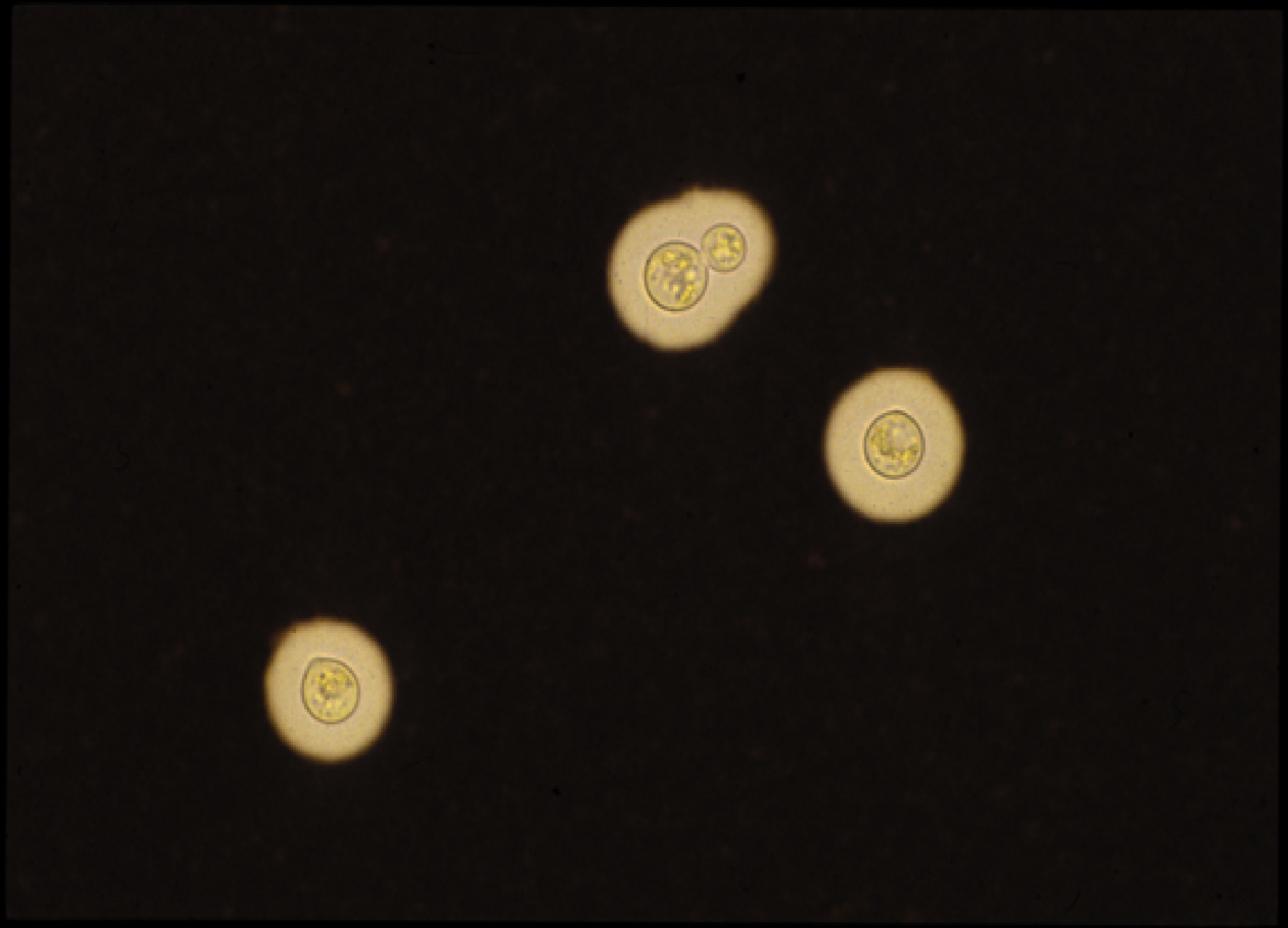
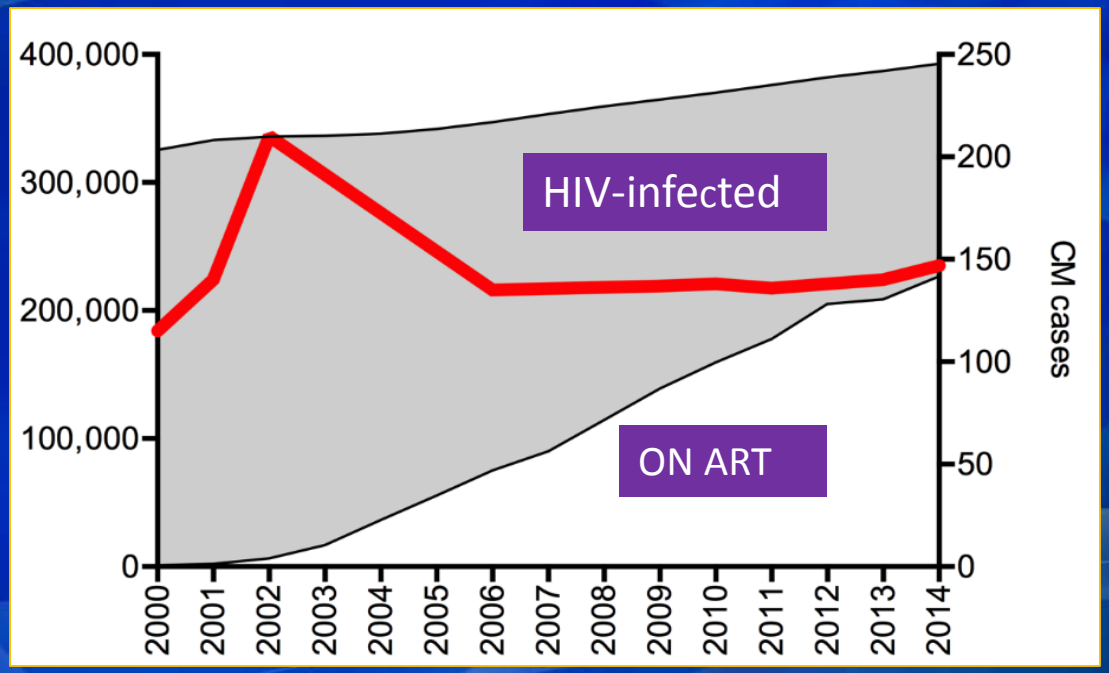


The incidence of cryptococcal meningitis remains high despite ART roll-out in Africa Botswana Data



2014 CM Incidence 15/100,000 (95% CI 13-16) overall
78/100,000 (95% CI 70-87) HIV+ve

SA 2002-4 15.6/100,000
95/100,000

McCarthy et al. AIDS 2006

Jarvis et al, 2016

Influence of initial therapy on outcome *

- 204 pts. (severe-non-severe) DUMC 1996-2009
- Impact on overall mortality, attributable mortality, persistence
- Attributable mortality 20% severe disease vs. 5% non-severe disease
- Flucytosine exposure was associated with lower overall mortality (HR 0.4 95% CI 0.2-0.9) and attributable mortality (HR 0.5 95% CI 0.2-1.2)
- Receiving a non-recommended 2012 IDSA Guideline Regimen had a higher relative of persistence (HR 1.9, 95% (CI 0.9-4.3) and higher rate of attributable mortality (HR 2.3; 95% CI 1.0-5.0)

* Bratton-Wernik et al Antimicrob Agents Chemother 57: 2793-2800, 2013

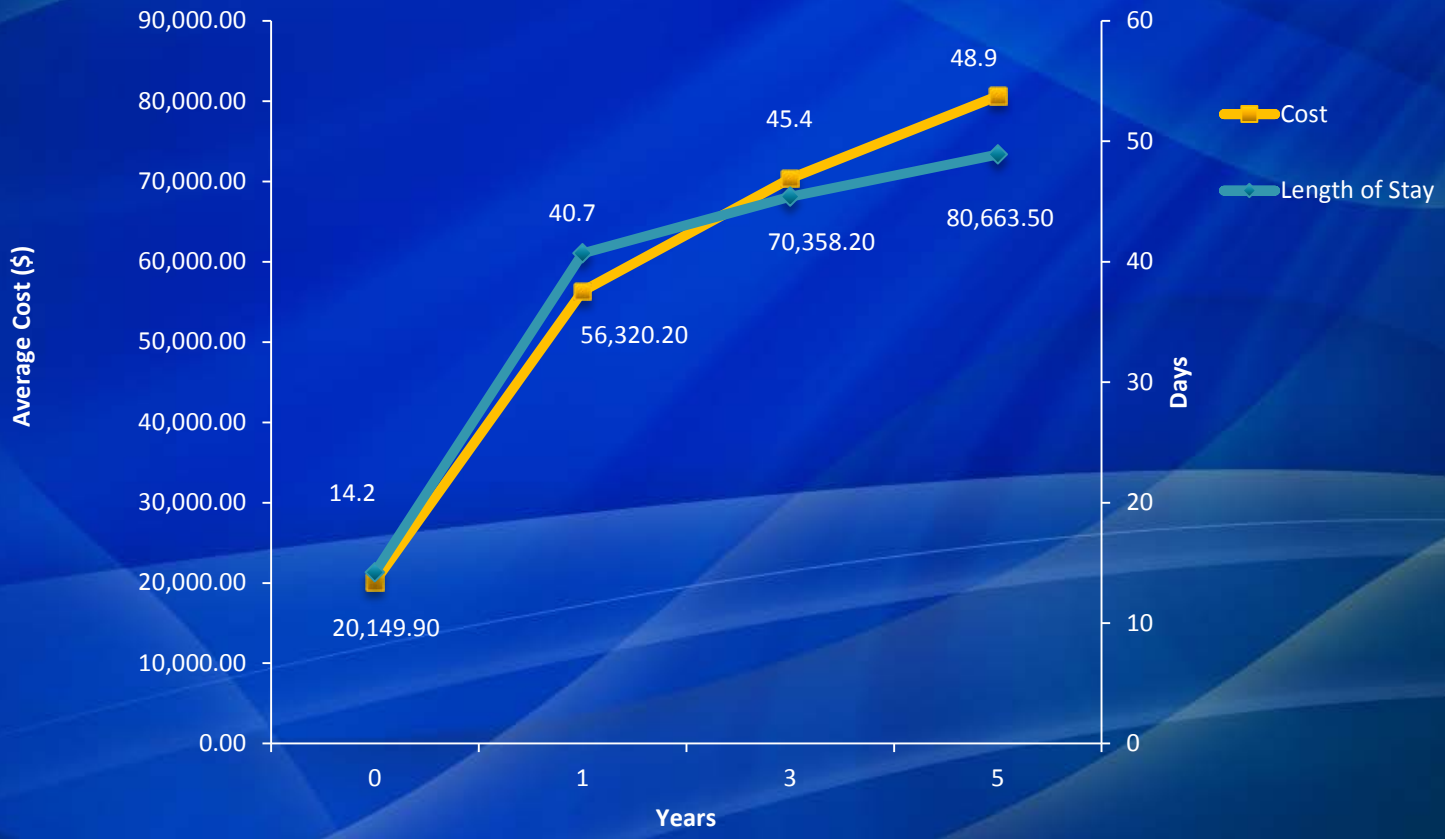
Patient mortality and IRIS through one year of follow-up at Duke

Severe (n=131)

	HIV+ (n=74)	Transplant (n=18)	HIV- /non-transplant (n=39)
	n (%)	n (%)	n(%)
Attributable mortality	12 (16)	3 (17)	12 (31)
All-cause mortality	15 (20)	5 (28)	16(41)
IRIS*	3 (4)	2 (11)	1(3)

* Immune Reconstitution Inflammatory Syndrome

Average cost of Cryptococcal meningitis Treatment from Initial Diagnosis to 5 years



Truven Health Analytics Market Scan® Database (Inpatient/Outpatient)

The Union of TB and Cryptococcal meningitis Dexamethasone Treatment

TB meningitis (Yes)*

- Acute survival benefit
- No reduction in proportion of severely disabled

Cryptococcal meningitis (No)[t]

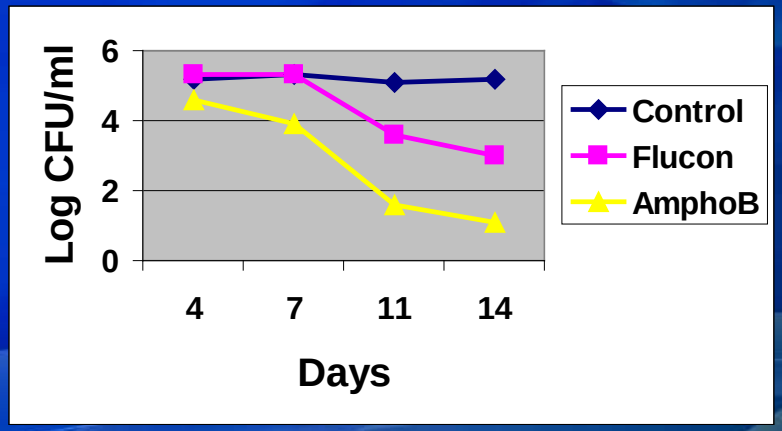
- More adverse events and disabilities
- No mortality benefit
- Fungal clearance worse in Dexa group

* Thwaites GE eta al. 351: 1741-1751,2004

[t]Beardsley MD et al. N. Engl J. Med. 374:542-54,2016

Rabbit Model

-Yeast inoculated directly into the CSF of rabbits immuno-compromised with corticosteroids.
-Infection followed over time by sampling CSF for culture.
-Used in both antifungal drug studies and pathogenesis.



-Model has predicted how antifungals will work in humans.
-Model allows for recovery of yeast from site of infection.
-However: body temp 39° C, cumbersome, only CSF

What the Rabbit Model of Cryptocccal meningitis showed that was confirmed in Humans?

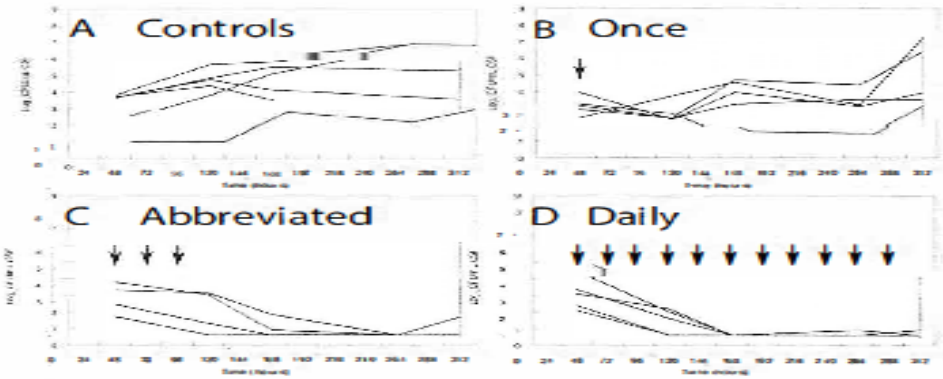
- Corticosteroids were highly immunosuppressive
- Antigen does not go across BBB
- Quantitative CSF yeast counts for antifungal treatment success
- Azoles had fungistatic activity (with both high and low CSF penetration) Amphotericin B fungicidal
- Amphotericin B potent antifungal activity despite very low (Below MIC) drug levels in CSF
- Combination of Amphotericin B and azole showed additive effects. (Flucytosine toxic to Rabbits)
- Good correlation with rabbit and human outcome: (1) Amphotericin B best but with lipid products higher doses improved killing

(2) Most azoles are equal despite differences in CSF penetration

(3) Failure in rabbit =failure in human (The lipid nystatin story)

- Yeast gene expression in rabbits vs humans (similarities/differences)

Intermittent AmBisome Dosing in Rabbits [t]



[t]Lester et al Antimicrob Agents Chemother, 2017
AmBition Study (Human-Pilot) 1,2,3 doses of AmBisome= 14 does with a fluconazole backbone

What's New in Anticryptococcal Therapy?? Short course Liposomal AmB therapy (single dose)
Is the animal model right? Maybe
The Ambition Study
(Joseph Jarvis and group Botswana; CROI, 2017 presentation)

Results from short course polyene*

- Principle: In animal model (rabbits) 3 days of AmB deoxycholate treatment equivalent to 14 days. Livermore et al mBIO 5:e00725-13,2014

- Principle: In animals, single dose of AmBisome equivalent to multiple doses. Lesner et al Antimicrob. Agents Chemother., in press
- Principle: 80 randomized patients (Ambition study)

(1) On a 1200md/d fluconazole base

(2) 4 groups AmBisome 10md/kg day

1. Ambisome 3mg/kg/d for 14 days;
2. AmBisome 10 mg/kg on day 1,
3. AmBisome 10mg/kg on day 1, 3;
4. Ambisome 10 mg/kg on days 1, 3,7.

(3) Outcome: (1) No difference in 2wk or 10wk mortality (2) All 3 short course treatment arms were not inferior to control by primary endpoint EFA; improved toxicity profile compare to

- daily AmB . Jarvis CR01, 2017

History around quantitation of microbe burden and clinical management decisions

- Bacterial counts in urinet
- HIV viral loads
  (HCV and CMV)
- CSF yeast counts (cryptococcal meningitis)

[t] (MacDonald et al N. Engl. J. Med. 256: 915-22, 1957)

Quantitative Yeast counts in CSF Early Fungicidal Activity (EFA)

Studies	References
Rabbit cryptococcal meningitis (Amphotericin B the best and combination better)	**Perfect et al Am.J. Path. 1980 and JID, 1982**
Human Studies:	**References:**
(a) AmB+ flucytosine	(Brouwer Lancet et al , 2004)
(b) Antigen titers and yeast counts	(Brouwer et al, JID 2005)
(c) Gamma interferon and yeast counts	(Siddigui J. immunol. et al, 2005)
(d) Fluconazole doses	(Longley et al, CID 2008)
(e) Outcome and yeast counts	(Bicanic et al, CID 2009)
(f) Combination Flucytosine/Fluconazole; Combination high dose AmB/Flucytosine	(Nussbaum JC et al, 2010)(Bicanic, CID 2008) (Day et al NEJM, 2013) (Jarvis et al CID 58:736-45, 2014)
(g) Stop Steroid Study	(Beardsley et al NEJM 374: 541-24,2016)
(h) EFA as Surrogate for all cause mortality	(Montezuma-Rusca et al PLos one 11:*e*0159727,2016)

Quantitative CSF yeast counts Why not just do it? (Reasons against using the test)

- Microbiology Labs don't like to do it
- Methodology (some individual level clearances variable between St. George's, ACTG, and calibrated loop methods)[+]
- Early Fungicidal Activity (EFA) is an insufficient endpoint for all cause mortality in multiple studies□ but validated in individual studies•

[t] Dyal J et al Med Mycol. 54: 361-9,2016 □Montezuma-Rusca JM et al PLoS One 11:e0159727, 2016
•Day J et al N. Eng. J Med, 2013

Combination Antifungal Therapy* Efficiently killing a sugar-coated yeast

- 3 arm randomized controlled induction trial; 299pts (Vietnam)

  (1) Amphotericin B lmg/kg/d for 4wks

  (2) Amphotericin B 1mg/kg/d plus flucytosine (5Fc)

  100mg/kg/d for 2wks

  (3) Amphotericin B 1mg/kg/d plus fluconazole 400mg for 2wks

- Am B + 5FC compared to AmB monotherapy

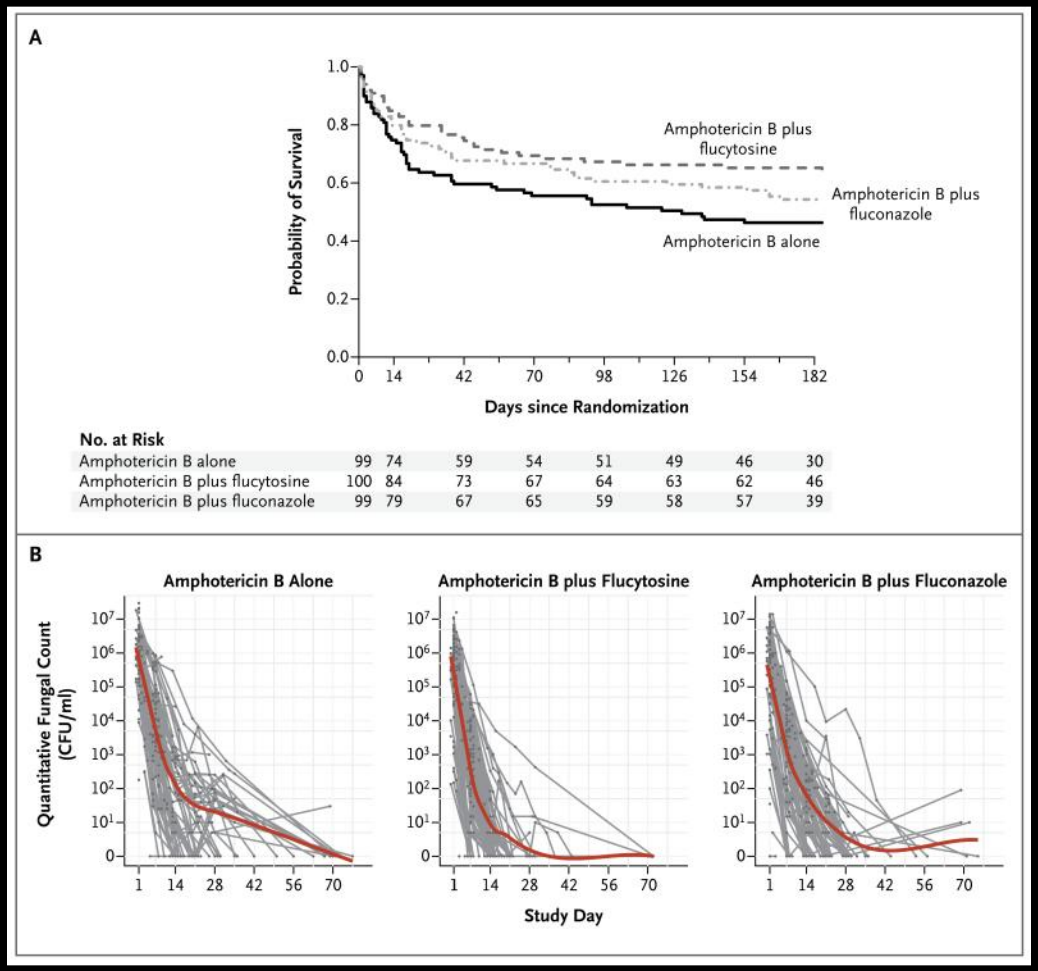
  (1) Fewer deaths (15 vs 25 p= 0.08)

  (2) Better 70 day survival p=0.04

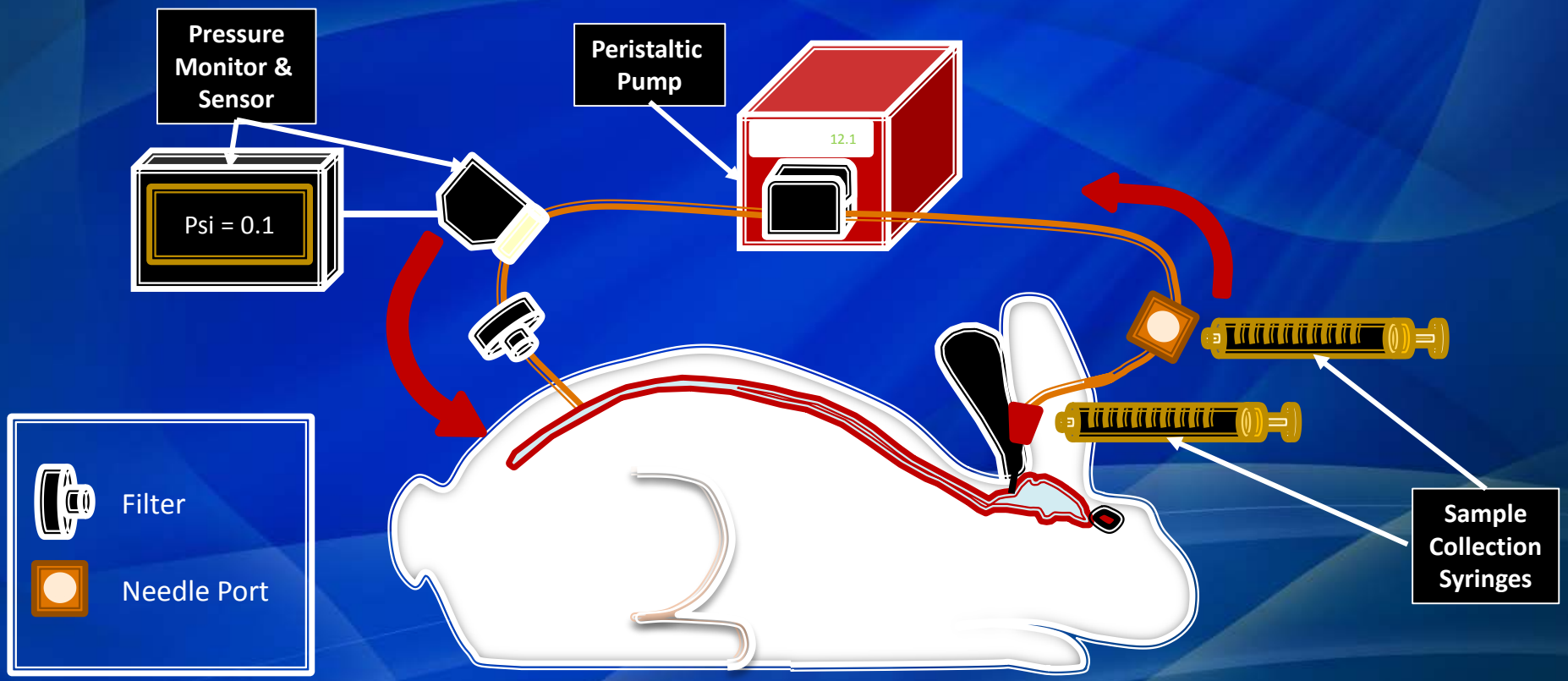
- AmB + 5FC cleared yeasts at 0.45 log10 CFU/ml/day significantly faster than AmB + FLU or AMB.

- **This the early fungicidal activity (EFA)**

* Day et al, N. Engl. J. Med 368:1291-1302


A
1.0
0.8
0.6
0.4
0.2
0.0
Probability of Survival
Amphotericin B plus flucytosine
Amphotericin B plus fluconazole
Amphotericin B alone
0
14
42
70
98
126
154
182
Days since Randomization
No. at Risk
Amphotericin B alone 99 74 59 54 51 49 46 30
Amphotericin B plus flucytosine 100 84 73 67 64 63 62 46
Amphotericin B plus fluconazole 99 79 67 65 59 58 57 39
B
Amphotericin B Alone
Amphotericin B plus Flucytosine
Amphotericin B plus Fluconazole
Quantitative Fungal Count (CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
1
14
28
42
56
70
Study Day

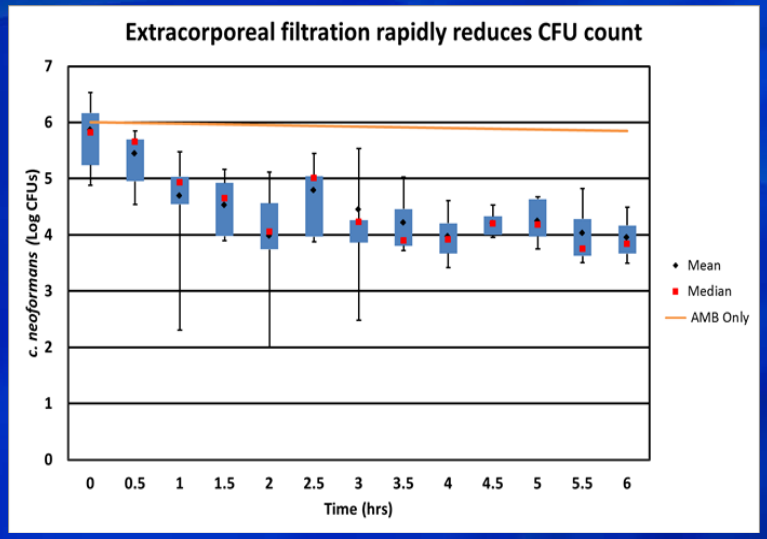
Neurapheresis: Rabbit Model



(A game changer- maybe/maybe not)

Premji and Cutshaw et al, IDSA week, abstr. 643, 2016

In vivo CFU Reduction



CSF CFU reduction due to 6 hours of neurapheresis. Filtration typically induced a 1-log reduction in CFU count after 1.5 hours and a 2 log reduction after 6 hours. AMB Only reduces CFUs by 1.25 log in 2 days

Cryptococcus can change and change rapidly

- Karyotypic Instability*
- Hetroresistance[t]
- Clinical Isolates with variation in chromosome copies within colonies [Δ]
- DNA mutation under stress[t]
- H99 (unique translocation) TGR1/YHPI[o]
- >500 C. neoformans genomes sequenced VNI, VNII,VNBl and VNBII
- Cryptococus gattii- molecular types (species) VGI, II, III,IV

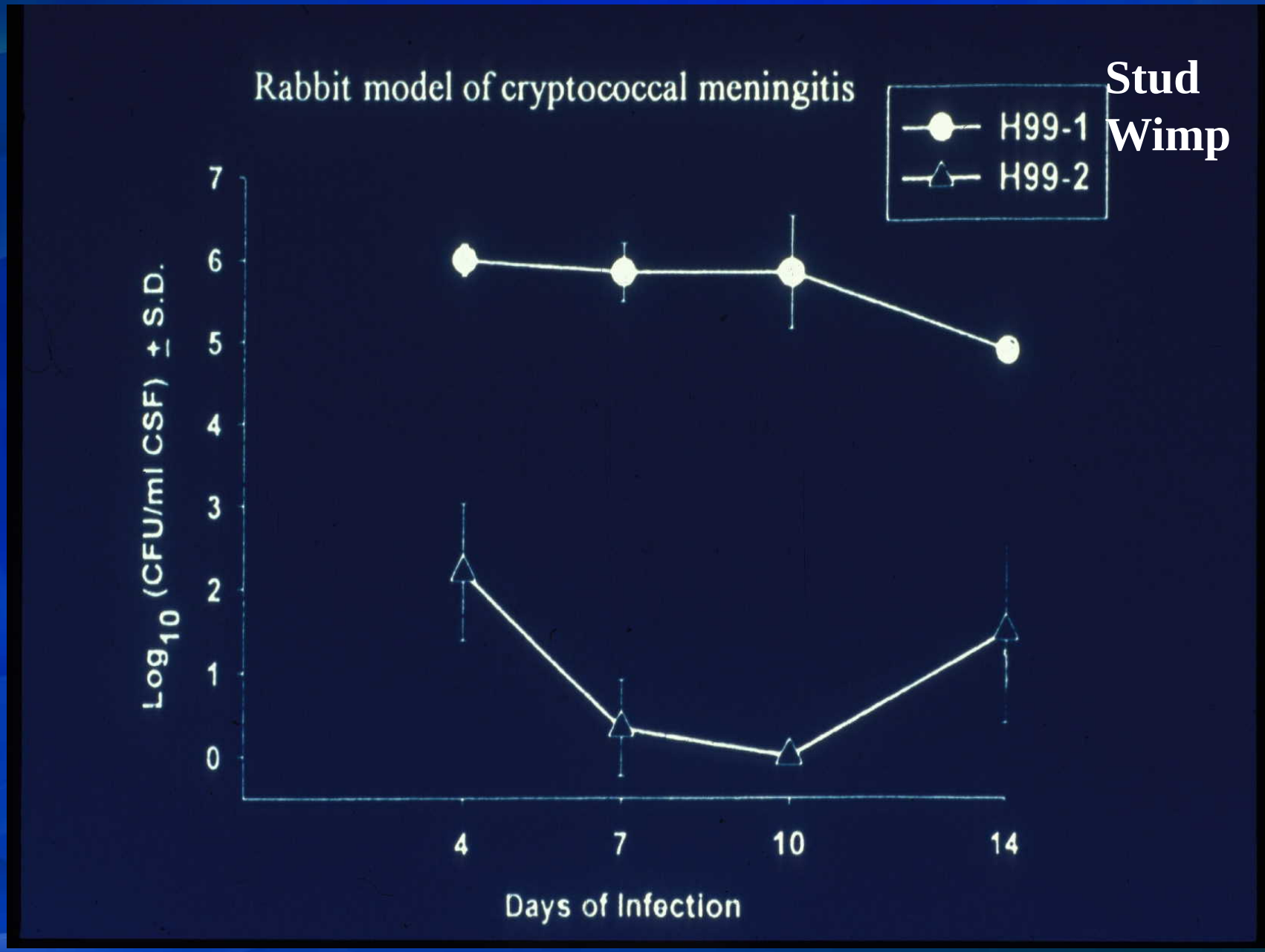
*Perfect et al J. Clin. Microbial. 31: 3305-3309,1993
Fries et al J. Clin. Microbiol. 34: 1531-4, 1996
Fries et al J. Infect. Dis. 178: 1761-1766,1998

[t] Sionov et al Plos Pathog 6:e 1000848, 2010
Ngamskulrungroj et al Plos One 7:e 33022, 2012

[Δ] Hu et al BMC genomics 12:256, 2011

[t] Magditch et al Plos Pathog. 8: *e*1002936

[o] Morrow et al mBIO 3*ie*00310-11


Rabbit model of cryptococcal meningitis
H99-1
H99-2
Log10 (CFU/ml CSF) ± S.D.
0
1
2
3
4
5
6
7
4
7
10
14
Days of Infection

Stud
Wimp

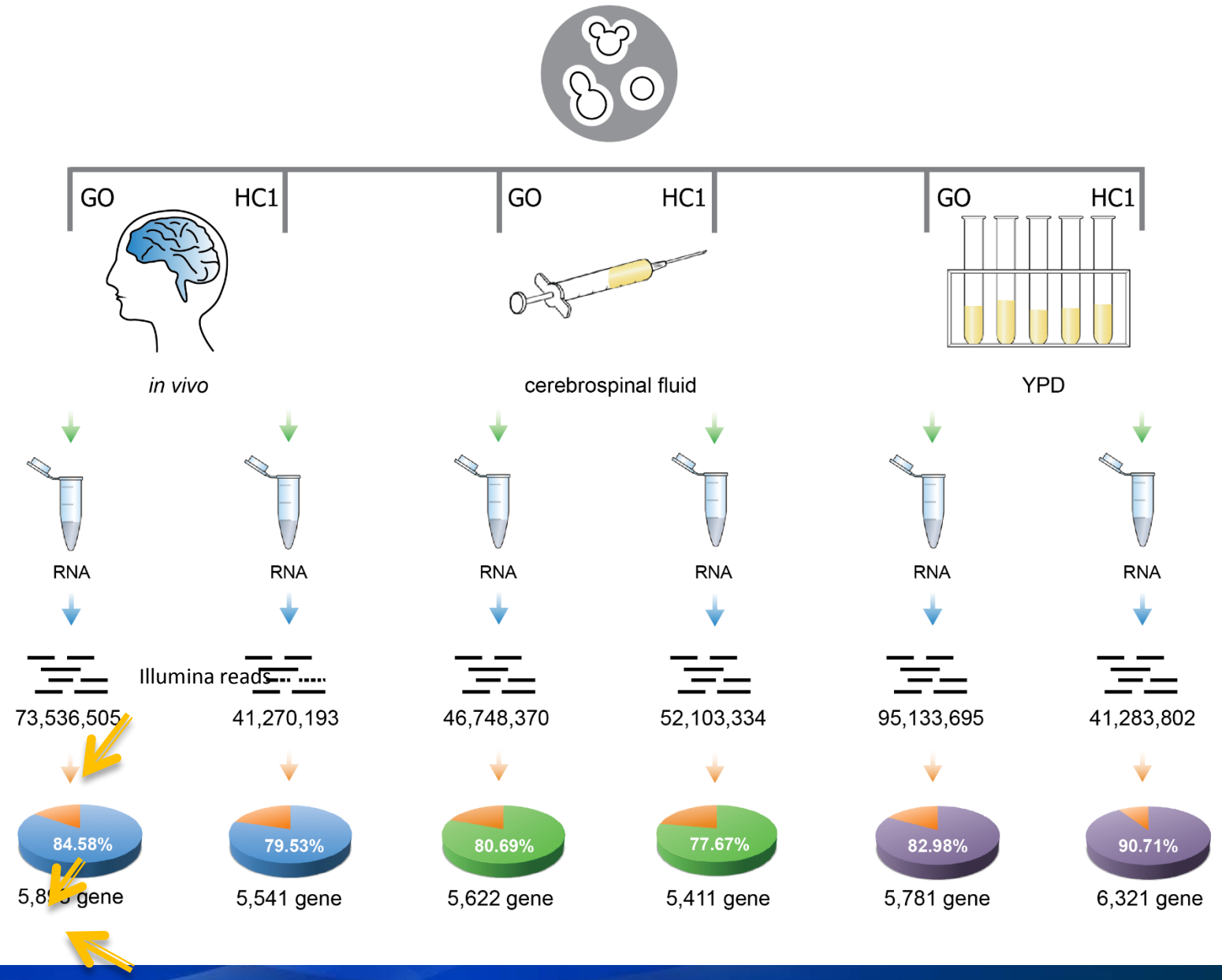
PLoS Genet. 2014 Apr 17;10(4):e1004261. doi: 10.1371/journal.pgen.1004261. eCollection 2014.

Analysis of the Genome and Transcriptome of Cryptococcus neoformans var. grubii Reveals Complex RNA Expression and Microevolution Leading to Virulence Attenuation

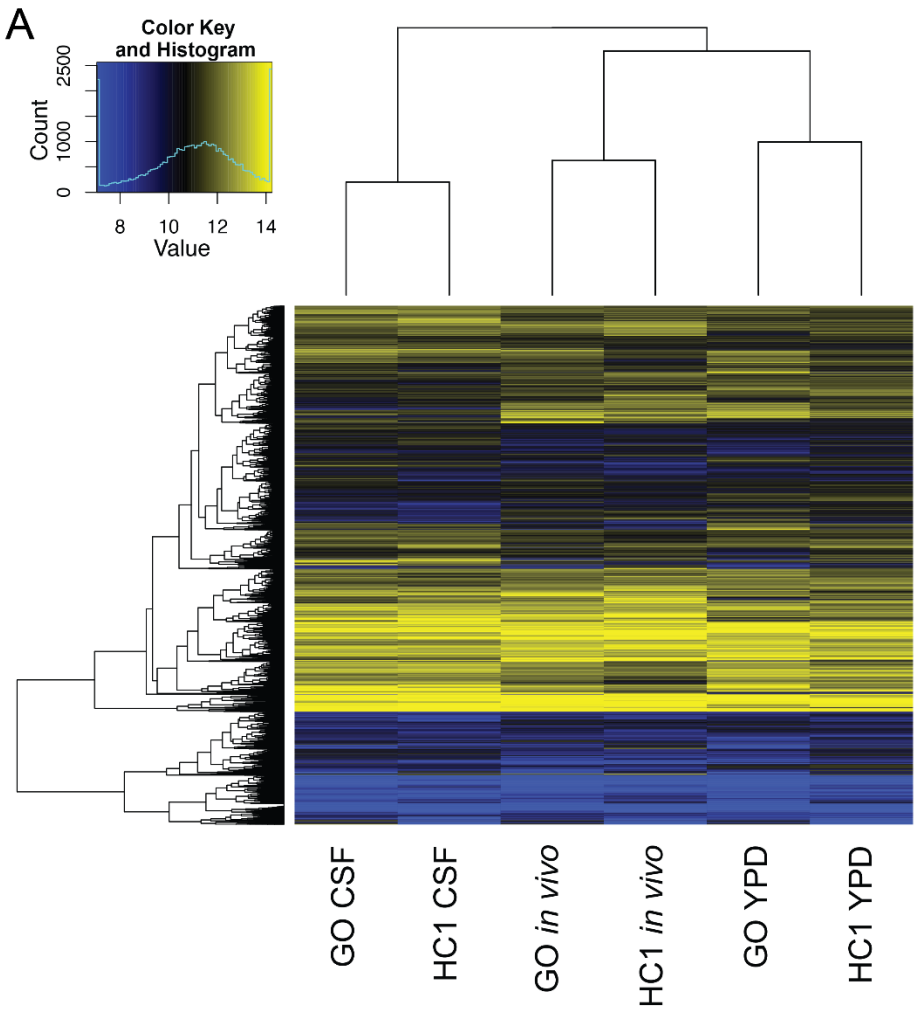
Janbon G, Ormerod KL, Paulet D, Byrnes EJ 3rd, Yadav V, Chatterjee G, Mullapudi N, Hon CC, Billmyre RB, Brunel F, Bahn YS, Chen W, Chen Y, Chow EW, Coppée JY, Floyd-Averette A, Gaillardin C, Gerik KJ, Goldberg J, Gonzalez-Hilarion S, Gujja S, Hamlin JL, Hsueh YP, Ianiri G, Jones S, Kodira CD, Kozubowski L, Lam W, Marra M, Mesner LD, Mieczkowski PA, Moyrand F, Nielsen K, Proux C, Rossignol T, Schein JE, Sun S, Wollschlaeger C, Wood IA, Zeng Q, Neuvéglise C, Newlon CS, Perfect JR, Lodge JK, Idnurm A, Stajich JE, Kronstad JW, Sanyal K, Heitman J, Fraser JA, Cuomo CA, Dietrich FS.

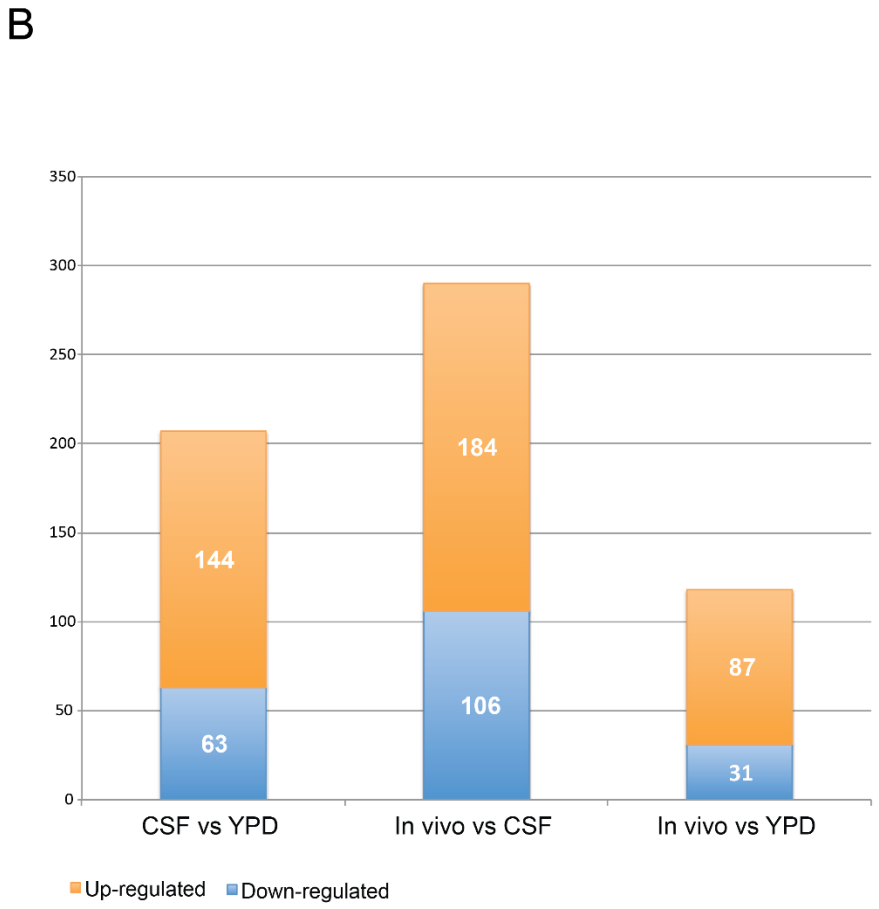
- Passaged strains of H99 through labs and animals

  H99 (Stud) vs H99 (Wimp) WGS
- Indel causing a frame shift in CNAG 06765 (hypothetical glutamine-rich protein with dimerization of Lis H domain)
- Deletion of CNAG 06765 in H99 stud caused reduction in mating, reduced melanin production and avirulence
- Named LMP1 (Low mating potential)
- **LMP1 has evolved in the clinical strains compared to environmental strains and highly expressed in the human CSF**



RPKM >= 10 Percentage of the gene number





A, Hierarchical clustering and heat-map analysis of the RNA-seq data. **B**, Up and down regulated gene numbers of the pairwise comparisons among the samples ($p < 0.01$).

Up-regulated Gene Group A

Gene ID	Gene Name
CNAG_05431*	**transcription factor PacC, *RIM101***
CNAG_00896	transcription factor
CNAG_00131	alcohol dehydrogenase
CNAG_00601	glycosyl hydrolase
CNAG_00490	acetyl-CoA C-acyltransferase
CNAG_02045	acetoacyl-CoA synthetase
CNAG_02562	acyl-Coenzyme A dehydrogenase
CNAG_05303	isocitrate lyase
CNAG_05264	alpha-amylase AmyA
CNAG_03019	long-chain-fatty-acid-CoA ligase
CNAG_00393	1,4-alpha-glucan-branching enzyme
CNAG_00531*	**ENA P-type ATPase 1,**
CNAG_00651	ATP-binding cassette transporter
CNAG_00864	myo-inositol transporter, *ITR2*
CNAG_01690	MFS transporter
CNAG_02288	succinate:fumarate antiporter
CNAG_03729	peroxisome targeting sequence binding protein
CNAG_05119	GabA permease
CNAG_05867	L-fucose transporter
CNAG_06241	acidic laccase, *LAC2*
CNAG_04392	sterol-binding protein
CNAG_06493	**conserved hypothetical protein**
CNAG_07512	**conserved hypothetical protein**
CNAG_07540	**conserved hypothetical protein**
CNAG_00456	**conserved hypothetical protein**
CNAG_00679	**conserved hypothetical protein**
CNAG_02044	**conserved hypothetical protein**
CNAG_05479	**conserved hypothetical protein**
CNAG_05870	**conserved hypothetical protein**
CNAG_05732	**conserved hypothetical protein**
CNAG_03142	**conserved hypothetical protein**
CNAG_03566	**conserved hypothetical protein**
CNAG_04837	**conserved hypothetical protein**
CNAG_02118	**conserved hypothetical protein**
CNAG_05159	**hypothetical protein**
CNAG_05632	**predicted protein**
CNAG_06000	**glycoprotein**
CNAG_02079	**cytoplasmic protein**
CNAG_01384	**bodown198**

Up-regulated Gene Group B

Gene ID	Gene Name
CNAG_03113	trehalose synthase
CNAG_00638	GTPase
CNAG_05095	putativepod-specific dehydrogenase SAC25
CNAG_00815	siderochrome-iron uptake transporter, *SIT1*
CNAG_01263	vacuolar transporter chaperone 4
CNAG_03143	**Heat shock protein 9 / 12**
CNAG_05147	**conserved hypothetical protein**
CNAG_05279	**conserved hypothetical protein**
CNAG_00654	**conserved hypothetical protein**
CNAG_01980	**conserved hypothetical protein**
CNAG_05652	**cytoplasmic protein**

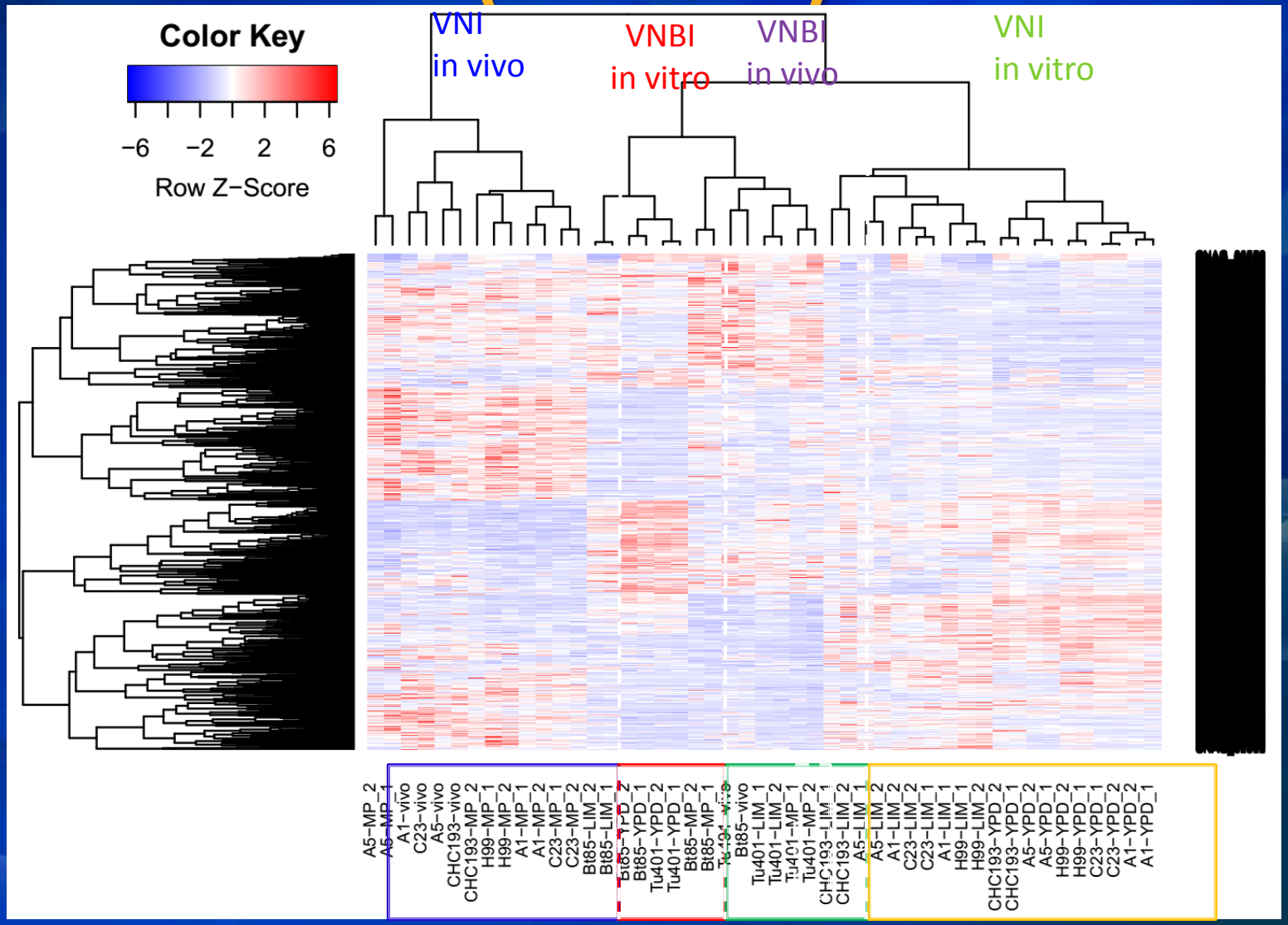
Transcription Factor

Metabolism

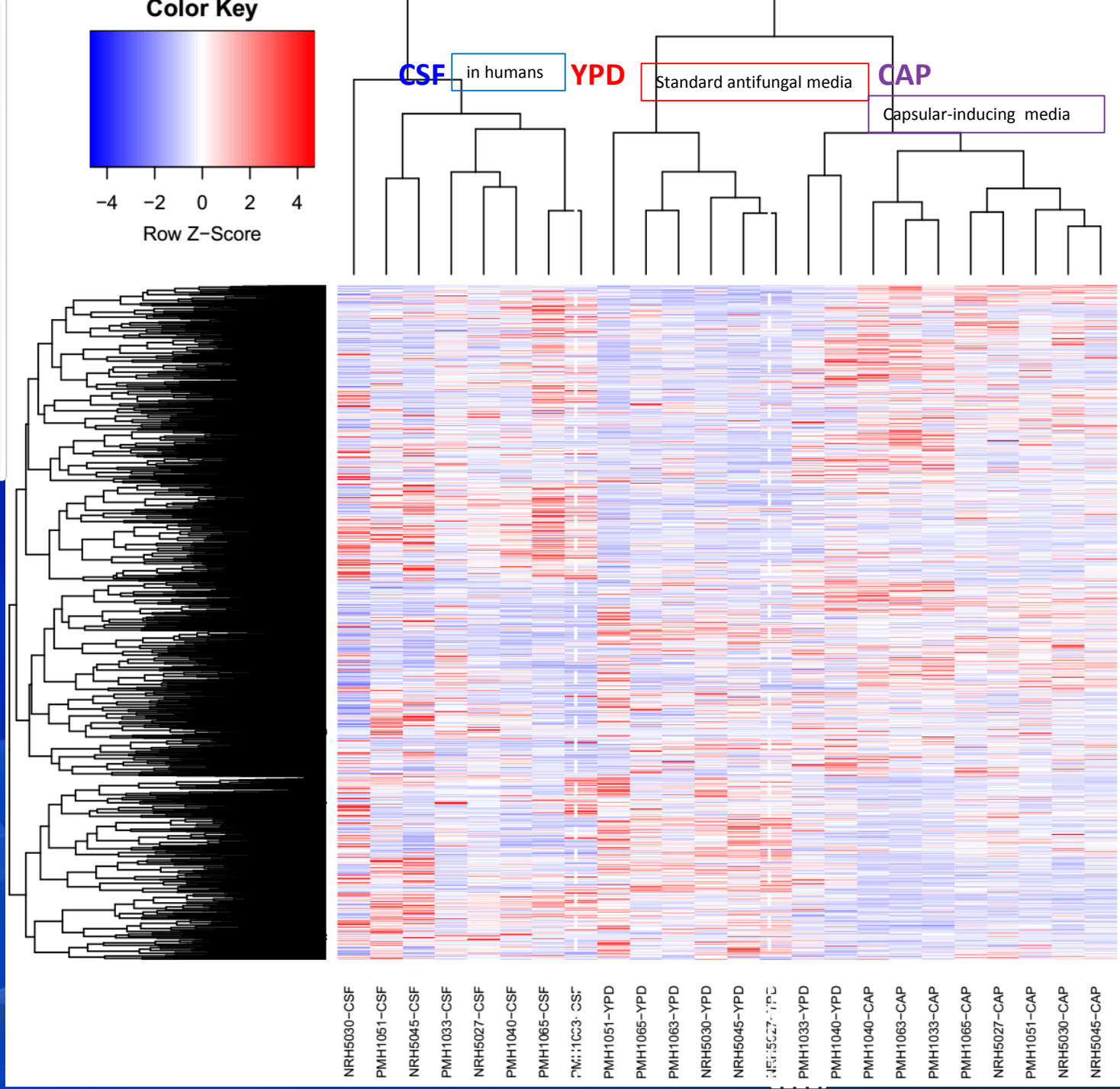
Transporter

We presently have 40 patients with Cryptococal in vivo RNA seq

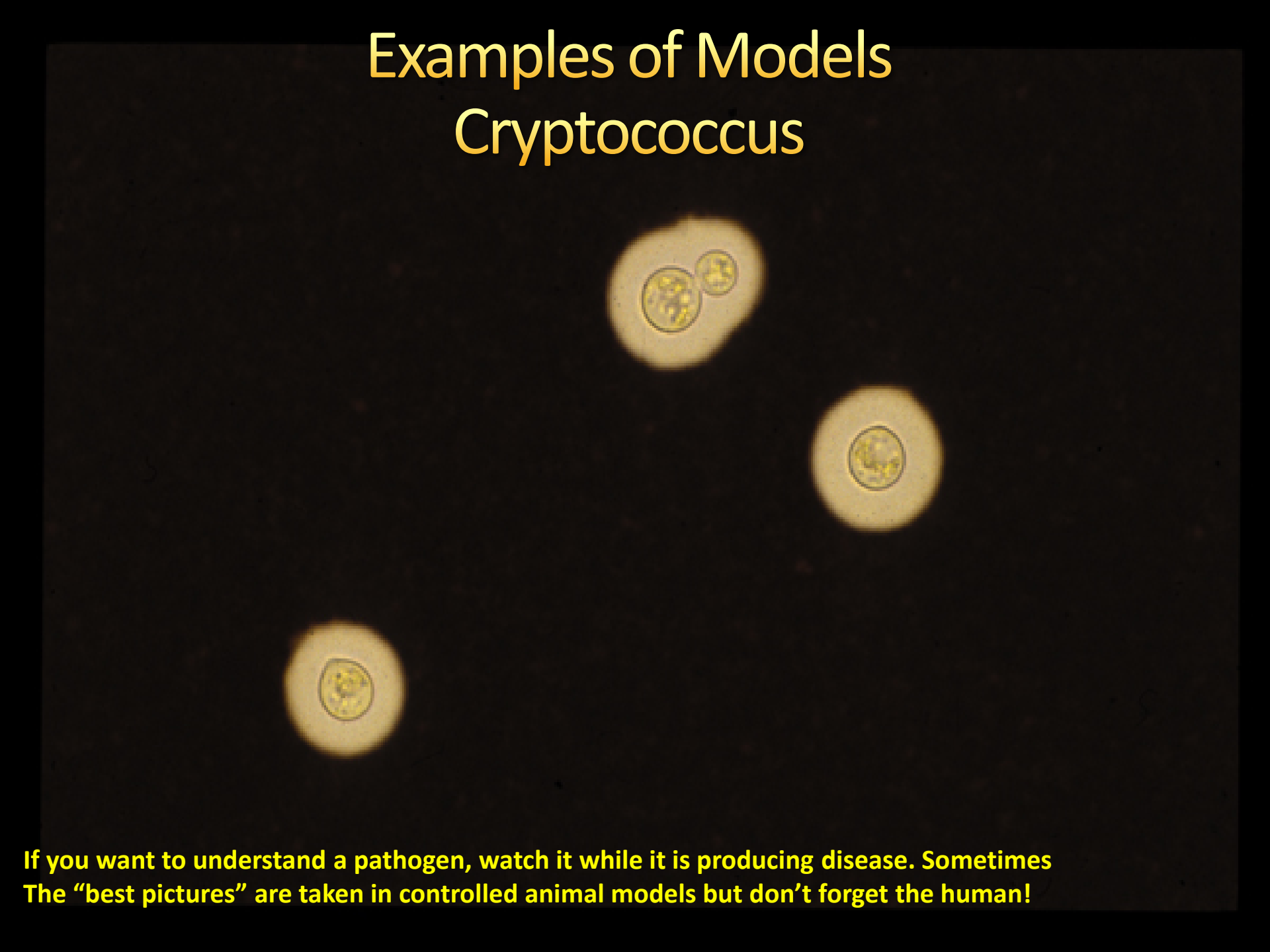
Heatmap & clustering *in vivo* (Rabbit CSF+MP) vs *in vitro* (YPD+LIM)



Expression profiles of 8 clinical isolates from Botswana
CSF= Cells directly from human Subarachnoid space
YPD= Yeast-Peptone-Dextrose media
CAP= Capsular-inducing media



Examples of Models
Cryptococcus



If you want to understand a pathogen, watch it while it is producing disease. Sometimes The “best pictures” are taken in controlled animal models but don’t forget the human!

Does in vitro phenotype predict in vivo phenotype?

(For example, a sick yeast for growth in vitro under nutritious conditions is a surrogate for outcome in animals?)

There are two prime examples in which this is not true in Cryptocccosis.

1. Can2 gene encoding beta-carbonic anhydrase is essential for growth under environmental ambient conditions but mutant grows well in host at high $C0_2$ levels*
2. PEX1 essential for optimal in vitro growth on glucose containing media but fully virulent.[t]

*Bahn YS et al Curr. Biol. 15:2013-20, 2005

[t] Idnurm A et al Eukaryot. Cell 6:60-72, 2007

Mouse Model

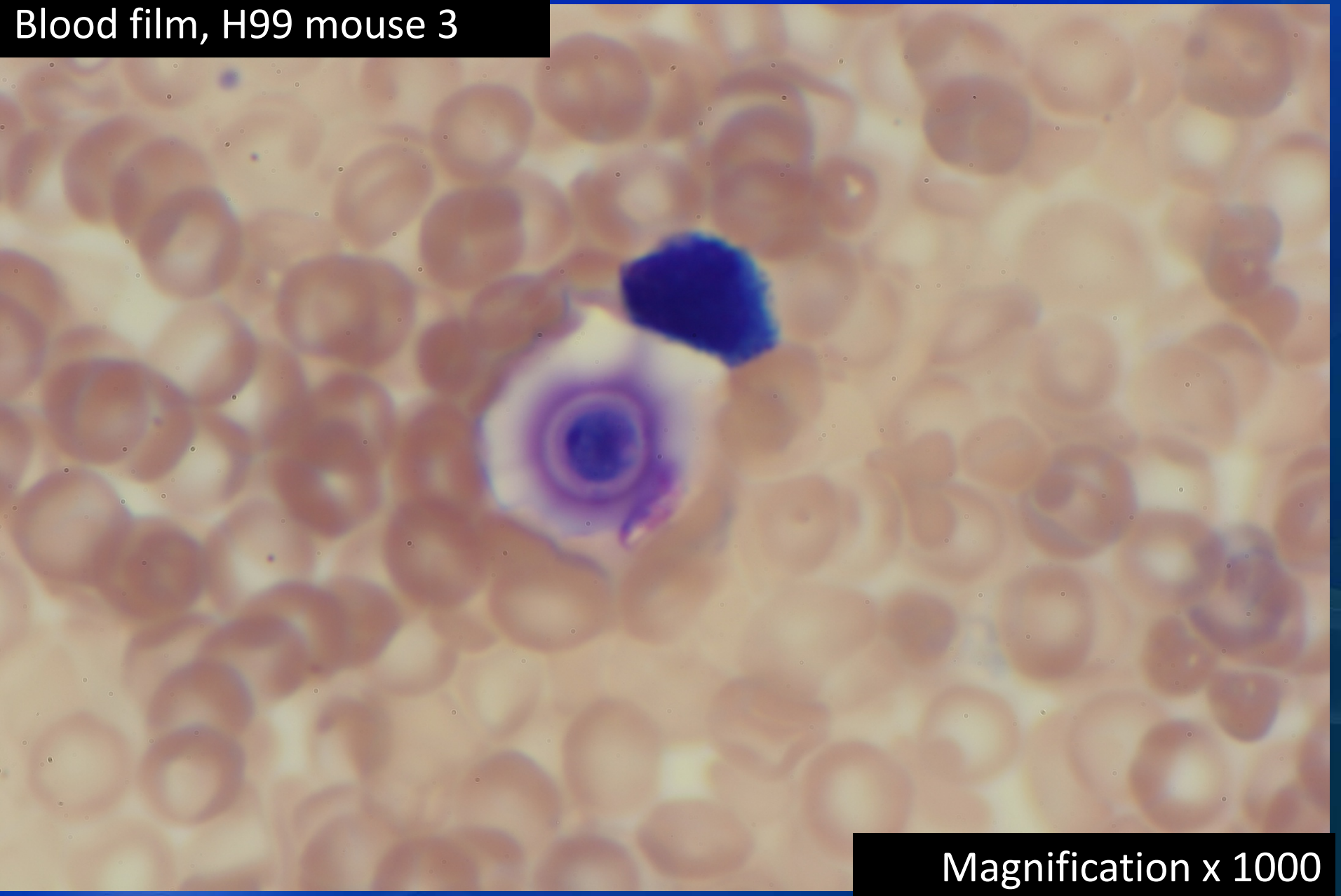
-Extremely flexible model
    -Variety of routes of infection (IV, IT, IN, IP, IC)
    -Variety of endpoints (imm. resp., culture, survival)
    -Variety of mouse strains (in/out-bred, KO mice)
-Used for both antifungal drug studies and pathogenesis.

Specific aim: To develop preliminary data characterizing murine response to cryptococcal disease by means of whole blood gene expression.

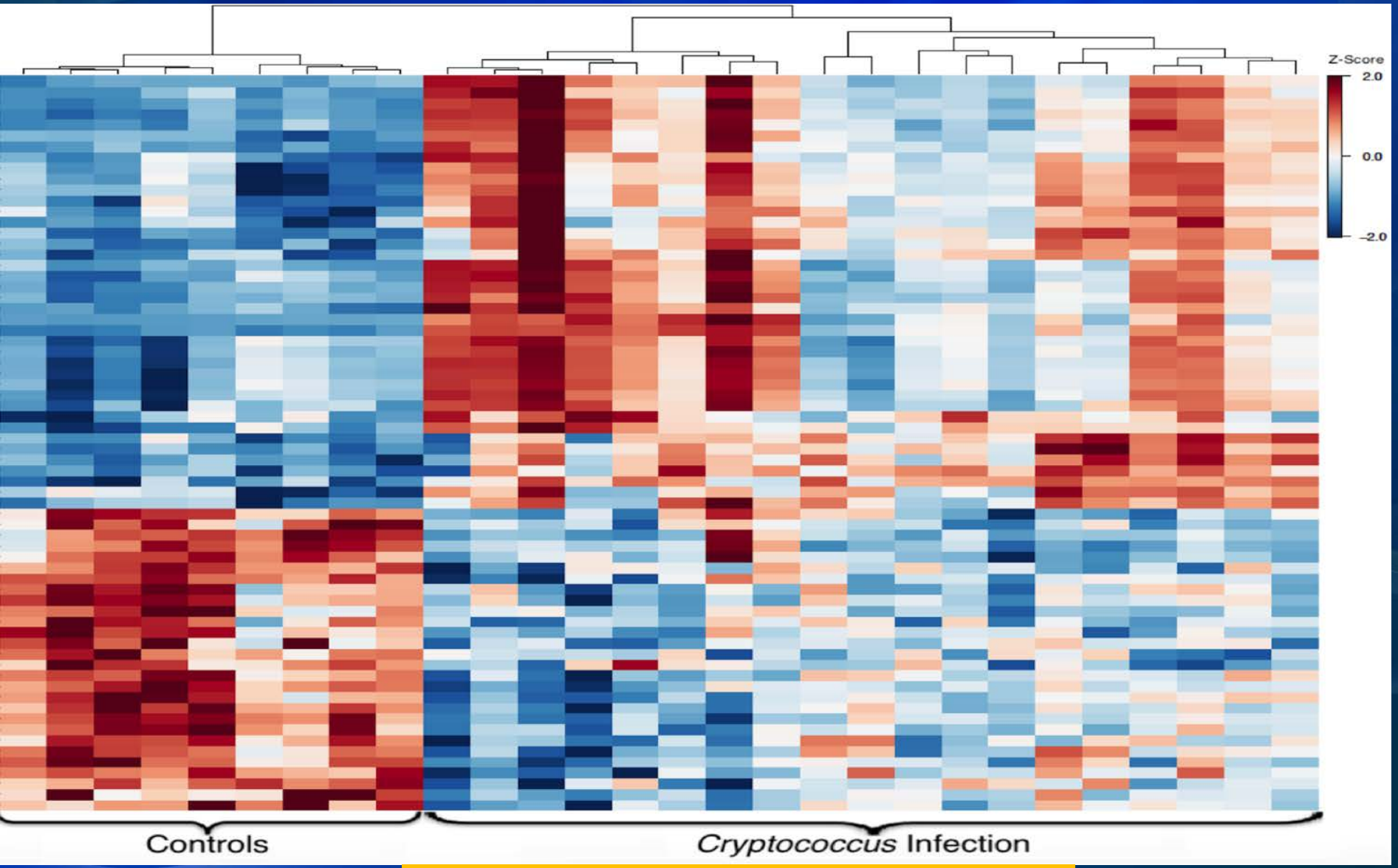
Goals

- To determine if there is an specific host blood gene expression "signature" for *C.neoformans* (H99) and *C.gattii* (VGII strain) infections.
- To validate the cryptococcal blood signature using a separate cohort of mice (candida or bacterial pneumonia).

John Perfect, Aimee Zaas, Marisol Betancourt


Blood film, H99 mouse 3
Magnification x 1000

Peripheral blood gene expression patterns separate infected from uninfected mice



Holcomb et al IDSA Week New Orleans

Cryptococcosis: Why the central nervous system target? The site of infection is important

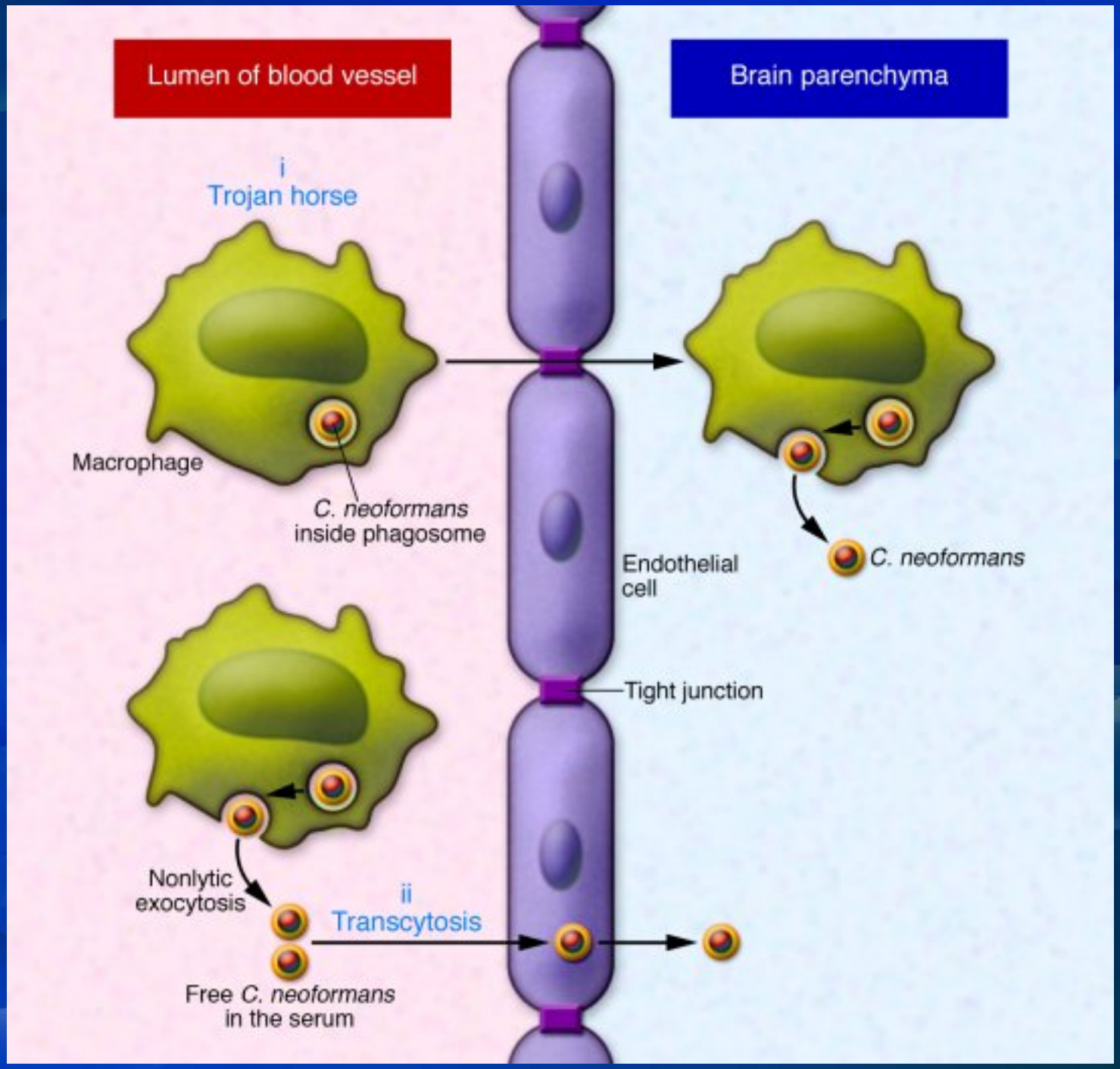
- Entry
- Survival
- Mechanism for survival

Genes associated with Blood-brain entry

- CPS1, URE1, MPR1, FXN1, RUB1
- Estimated 100-150 genes associated with efficient transmigration

Eric Tseng et al

PLoS One 7:*e* 45083


Lumen of blood vessel
Brain parenchyma
i
Trojan horse
Macrophage
C. neoformans
inside phagosome
Endothelial
cell
C. neoformans
Tight junction
Nonlytic
exocytosis
ii
Transcytosis
Free C. neoformans
in the serum

Genes necessary for survival in CSF human

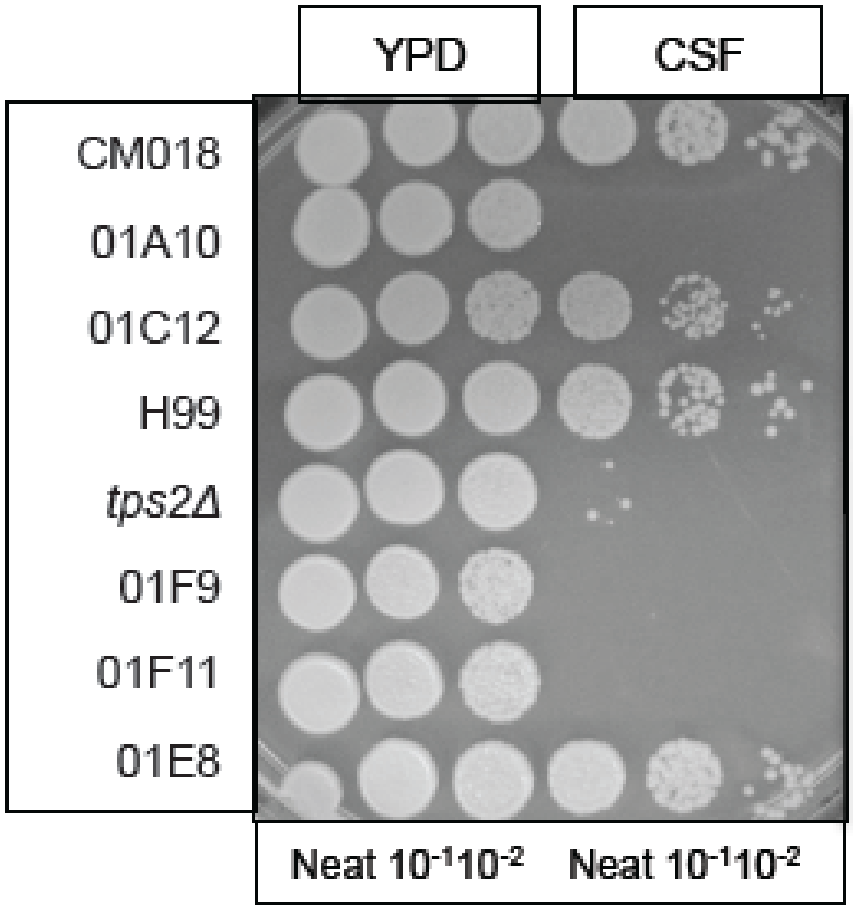
Multiple genes (ENA1, RUB1,PIK1, 28 transcription factors) associated with cryptococcal survival in human CSF

Anthony Lee et al

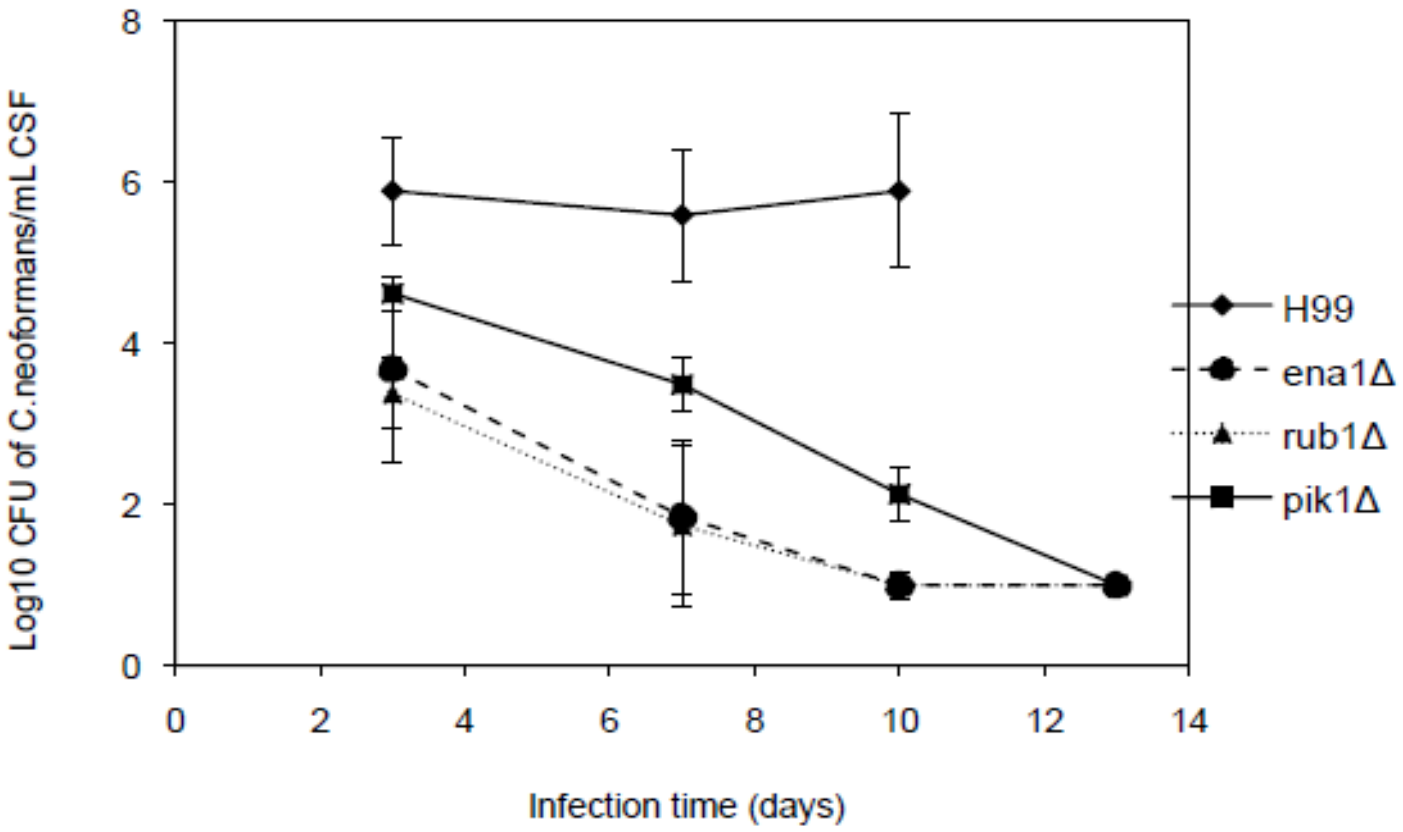
Infect. Immun. 78:4213-4225,2010

Jung and Bahn et al

Nat. Commun. 6:6757,2015


YPD
CSF
CM018
01A10
01C12
H99
tps2Δ
01F9
01F11
01E8
Neat 10-1 10-2
Neat 10-1 10-2

Rabbit Cryptococcal meningitis



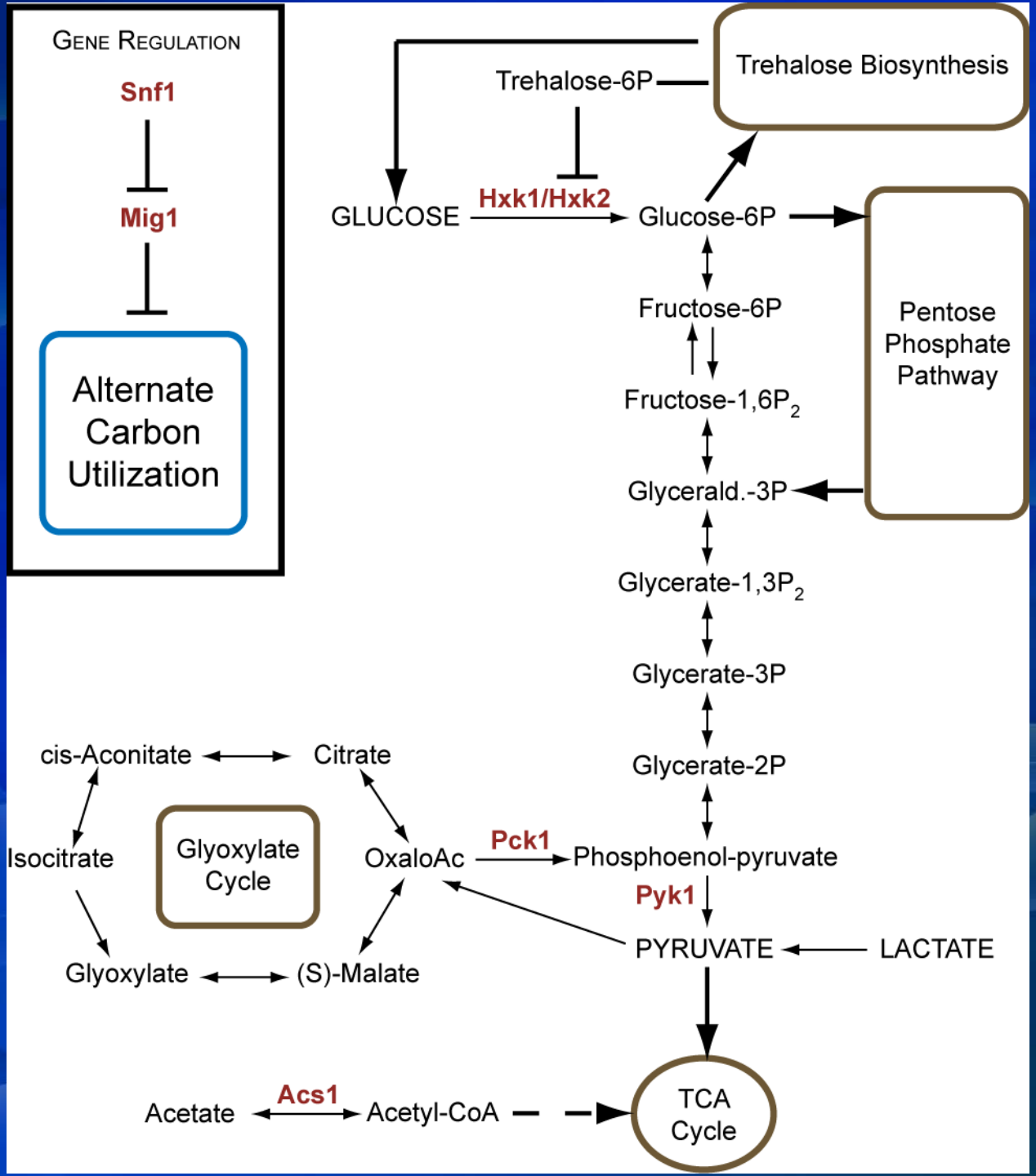
- K.O. Library of Transcription Factors (~250)
- 28 will CSF phenotype for CSF survival
- Δ YAP and ΔYTF double mutant has severe virulence defect

Mechanisms of CNS survival (back to basics)

Metabolism/Energy important (one must eat to fight!)

Michael Price et al

mBIO 2:*e* 0013-11,2011

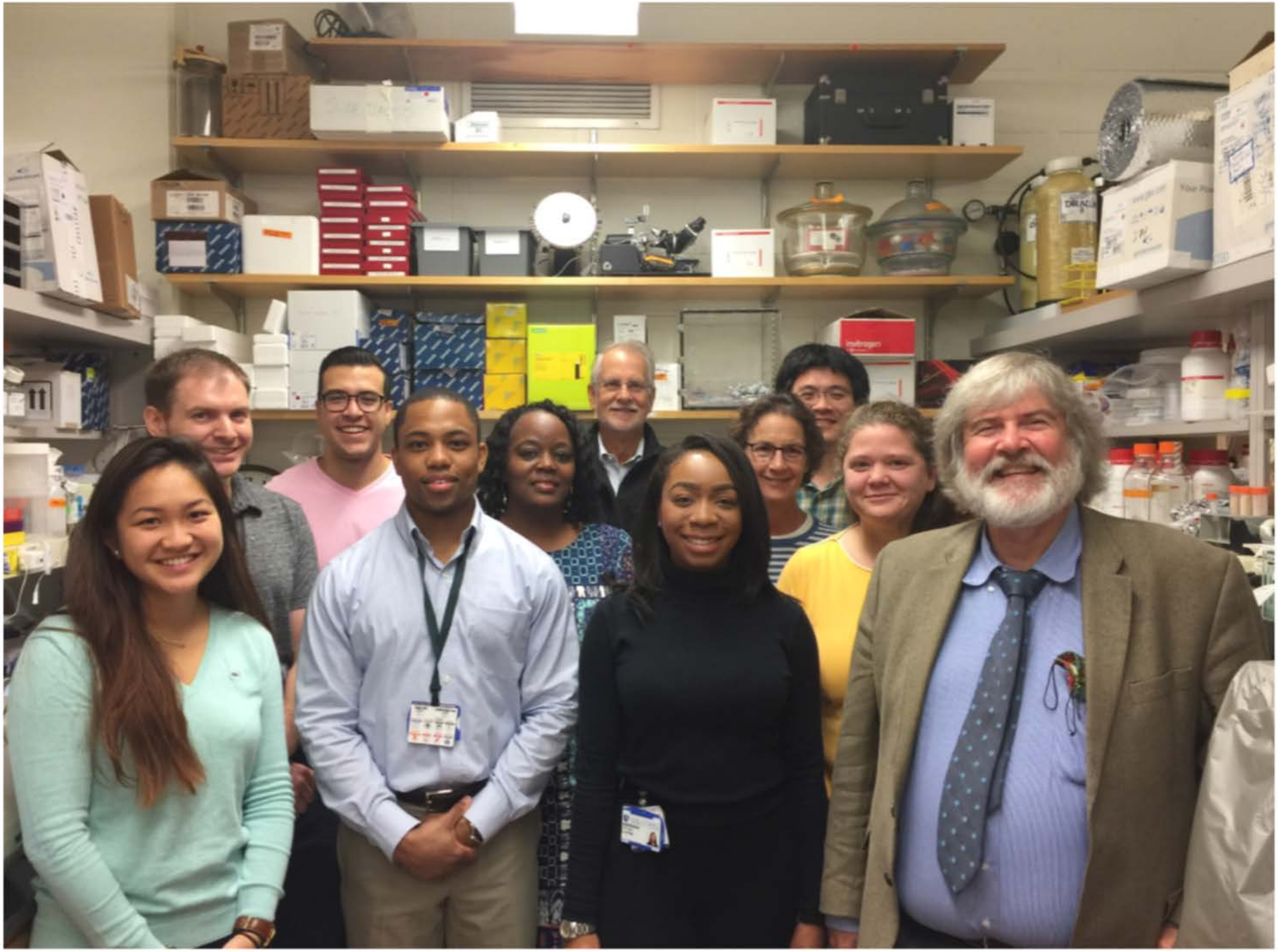

Gene Regulation
Snf1
Mig1
Alternate Carbon Utilization
Trehalose Biosynthesis
Trehalose-6P
GLUCOSE
Hxk1/Hxk2
Glucose-6P
Pentose Phosphate Pathway
Fructose-6P
Fructose-1,6P2
Glycerald.-3P
Glycerate-1,3P2
Glycerate-3P
Glycerate-2P
cis-Aconitate
Citrate
Isocitrate
Glyoxylate Cycle
OxaloAc
Pck1
Phosphoenol-pyruvate
Pyk1
PYRUVATE
LACTATE
Glyoxylate
(S)-Malate
Acetate
Acs1
Acetyl-CoA
TCA Cycle

Summary
Cryptococcus
(Sugar-Coated Killer)

- It is important, deadly, and its mysteries are being unwrapped
- In treatment the goal is efficiently killing the sugar-coated yeast (Fungicidal targets)
- Pathogenic principles set:

  (a) Weak spots are many but relationships are complex and dynamic thus keep close to the host; control for change; analyze and choose wisely from the massive bioinformatic data being generated

  (b) It's the host; it's the yeast…….it's a two way street!



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