Yeast Transcriptome and \textit{In Vivo} Hypoxia Detection Reveals \textit{Histoplasma capsulatum} Response to Low Oxygen Tension

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Received 8 April 2015; Revised 27 June 2015; Accepted 13 July 2015

Abstract

Although there is growing understanding of the microenvironmental conditions fungal pathogens encounter as they colonize their host, nothing is known about \textit{Histoplasma capsulatum}'s response to hypoxia. Here we characterized hypoxia during murine histoplasmosis using an \textit{in vivo} hypoxia detection agent, Hypoxyprobe-2 (HP-2); and analyzed \textit{H. capsulatum}'s transcriptional profile in response to \textit{in vitro} hypoxia. Immunohistopathology and flow cytometry analyses revealed distinct regions of hypoxia during infection. Granuloma cells, enriched with macrophages and T-cells isolated from infected livers were 66–76% positive for HP-2, of which, 95% of macrophages and 55% of T-cells were hypoxic. Although inhibited, \textit{H. capsulatum} was able to survive under \textit{in vitro} hypoxic conditions (<1% O\textsubscript{2}), and restored growth when replaced in normoxia. Next-generation sequencing (RNA-seq) analysis after 24 hours of hypoxia demonstrated a significant increase in \textit{NIT50} (swirm domain DNA binding protein), a predicted ABC transporter (\textit{ABC}), NADPH oxidoreductase (\textit{NADP/FAD}), and guanine nucleotide exchange factor (\textit{RSP/GEF}); and other genes with no known designated function. Computational transcription factor binding site analysis predicted human sterol regulatory element binding protein (SREBP) binding sites upstream of \textit{NIT50}, \textit{ABC}, \textit{NADP/FAD} and \textit{RSP/GEF}. Hypoxia resulted in a time-dependent increase in the \textit{H. capsulatum} homolog of SREBP, here named \textit{Srb1}. \textit{Srb1} peaked at 8 hours and returned to basal levels by 24 hours. Our findings demonstrate that \textit{H. capsulatum} encounters and survives severe hypoxia during infection. Additionally, the hypoxic response may be regulated at the level of transcription, and these studies contribute to the understanding of hypoxic regulation and adaptation in \textit{H. capsulatum}.

Key words: Histoplasma, hypoxia, RNA-seq, Hypoxyprobe.
**Introduction**

*Histoplasma capsulatum* is a dimorphic fungal pathogen that thrives in its mycelial phase in soil containing high concentrations of bird or bat guano, and every year hundreds of thousands of individuals who live in areas of endemicity are infected.\(^1\)\(^2\) Though found worldwide, histoplasmosis is primarily a disease of North and Central America that is generally asymptomatic in healthy persons but may be fatal in immunosuppressed individuals.\(^3\)\(^4\)\(^5\) Once inhaled, *H. capsulatum* must overcome numerous *in vivo* microenvironmental challenges to cause disease. The immediate host response is the stimulation of the macrophage’s micbicidal properties: oxidative stress, acidic pH, degradative stress caused by hydrolytic enzymes present in the lysosome, and nutrient (zinc and iron) deprivation.\(^6\)\(^7\) Despite the harsh intracellular environment of the macrophage, *H. capsulatum* has evolved various mechanisms to evade destruction by the macrophage.\(^8\) Phagocytosed yeasts can travel via the lymph nodes to the liver and spleen and cause disseminated infection. There activated macrophages and other host components surround the infected cells forming a circular wall of aggregated inflammatory cells or granuloma.\(^8\)\(^9\) The granuloma functions to inhibit *H. capsulatum* growth and replication by restricting access to oxygen and nutrients and exposing the fungi to acidic pH and other immune effectors.\(^10\)\(^11\) However, the fungus within the granuloma may also benefit from this isolated microenvironment. The granuloma may provide shelter from destruction by the host, and can serve as the source from which surviving pathogens emerge during reactivation of latent infection.\(^12\)\(^13\) Along these lines, investigating *H. capsulatum* growth *in vivo* and the surrounding microenvironment at the infection site is an important area of *H. capsulatum* research.

Our understanding of the dynamic microenvironment encountered by the fungus during infection, and the mechanisms it utilizes to survive and cause disease are limited. The availability of oxygen during infection and within the *H. capsulatum*-induced granuloma has never been evaluated. Oxygen levels vary significantly throughout the mammalian body, depending on tissue type and inflammatory response and most mammalian cells induce a hypoxic response at an oxygen level of less than 6% (pO\(_2\) of 40mmHg).\(^17\)\(^18\)\(^19\) There is a substantial amount of heterogeneity in oxygen levels inside the human body (21% O\(_2\) in the atmosphere, 14% O\(_2\) in the alveolar air, 12% O\(_2\) in the arterial blood and 5.3% O\(_2\) in venous blood), with a mean of 3% O\(_2\) in tissues.\(^14\)\(^15\)\(^16\) Regions of extremely low oxygen tension (<1%) are common in tumors, wounds, necrotic tissue and inflamed/infected tissues.\(^18\)\(^19\)\(^20\) At sites of inflammation, the available oxygen is significantly reduced compared to surrounding tissues, due to a discontinuous supply of blood caused by blood vessels that are congested with phagocytes or the pathogen itself.\(^22\)

Evidence that pathogenic fungi must rapidly adapt to changing oxygen levels during infection has increased over the last 10 years. Tolerance to hypoxia has been studied in a number of fungi, including *Aspergillus fumigatus*, *Cryp
tococcus neoformans*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans*, and Blastocladiella emersonii, as well as in *Mycobacterium tuberculosis* at oxygen concentrations of less than 1%.\(^12\)\(^18\)\(^20\)\(^22\)\(^23\) In these studies hypoxia was shown to be a key regulator of fungal pathogenesis and elongation, bacterial phagocytosis and latency. In the nonpathogenic ascomycete fission yeast *S. pombe*, a pathway homologous to the mammalian Sterol-response elementary binding protein (SREBP), Sre1 was shown to be crucial for hypoxic adaptation.\(^25\)\(^30\)\(^39\) In fungi, hypoxia resulted in an increased expression of genes encoding enzymes that form part of oxygen-dependent pathways such as sterol and lipid biosynthesis.\(^29\)\(^40\) Changes in cell wall morphology and gene expression, increased glycolytic enzymes, reduction in respiratory genes and fermentation have also been reported.\(^23\)\(^41\)\(^46\) Since then orthologs and homologs have been identified in the fungal pathogens *A. fumigatus* (SrbA) and *C. neoformans* (Sre1). SREBP homologs were shown to be necessary for sterol biosynthesis, resistance to azole antifungals, survival during hypoxia and virulence.\(^22\)\(^25\)

In order to advance our understanding of *H. capsulatum* pathogenesis, determining the extent to which hypoxia contributed to the fungal microenvironment was crucial. In this study, we report that (1) distinct regions of tissue hypoxia exists during murine disseminated histoplasmosis (2) *H. capsulatum* has the ability to survive oxygen levels of <1%, and (3) *H. capsulatum* induces the transcription of specific genes during hypoxic stress including a homolog of the mammalian sterol regulatory element binding protein (SREBP). In addition, we present insights into the *H. capsulatum* infection model and the interplay between the pathogen and the localized tissue response of the host which are critical for the understanding the progression of infection.
Materials and methods

Strains and culture conditions

All *Histoplasma capsulatum* strains utilized in this study were derived from the ATCC *capsulatum* strain G217B (ATCC # 26032). *H. capsulatum* was grown in liquid or solid Histoplasma Macrophage Medium (HMM), at 37°C and 5% CO₂. Solid medium contained 0.8% Agarose (Lonza SeaKem ME, Pittsburgh, PA, USA) and supplemented with 25 µM FeSO₄. Liquid cultures were continuously shaken for 48–72 hours at 200 rpm until mid-log phase. Normoxic conditions were considered general atmospheric levels within the incubator (~21% O₂). Hypoxic conditions were achieved using a controlled atmospheric chamber (BioSpherix C-Chamber, Salem, NC USA) with O₂ and CO₂ levels controlled by an external controller. The chamber was maintained at 37°C and kept at ~1% O₂ utilizing a gas mixture with 94% N₂ and 5% CO₂.

RNA isolation and quantitative real-time PCR

Hypoxic and normoxic yeast cells were harvested by centrifugation and subjected to RNA extraction. Total RNA was extracted from fresh cell pellets using a MasterPure Yeast RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) following the manufacturer’s instructions, and treated with DNase I. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (ThermoScientific, Florence, KY, USA). Complementary DNA (cDNA) was synthesized from total RNA using SuperScript II Reverse Transcriptase (Life Technologies, Grand Island, NY, USA) and oligo dT primers (purchased from Integrated DNA Technologies, Coralville, IA). Approximately 1 ng of cDNA was used as template in quantitative real-time polymerase chain reaction (qRT-PCR) reactions containing SYBR Green dye (Life Technologies, Grand Island, NY). Gene expression was measured on Applied Biosystems 7500 FAST PCR machine (Applied Biosystems, Foster City, CA, USA). At least one primer of each qRT-PCR primer pair was designed to span an intron, and all primers were tested for the inability to amplify genomic DNA. For each primer set, standard curves were generated using 5-fold sequential dilutions of cDNA to account for differences in priming efficiencies. For each sample, values obtained using normalized RNA levels, calculated using the standard curve method for relative quantification, using GAPDH as the endogenous reference in *H. capsulatum* and HPRT in murine cells. Oligonucleotide primers used for qRT-PCR study are listed in Table 1 and Supplementary material.

RNA-seq analysis

RNA collected after 24 hours of hypoxia or normoxia was subjected to mRNA sequencing. RNA-seq was performed by the Cincinnati Children’s Medical Center DNA Sequencing and Genotyping Core. Using Qubit high sensitivity assay kit (Invitrogen, Carlsbad, CA), 150 to 500 ng of total RNA was quantified via spectrofluorometric measurement. cDNA libraries were prepared using TruSeq stranded mRNA library preparation kit (Illumina). Briefly, Poly (A) mRNA was isolated and fragmented, then reverse transcribed to generate cDNA. One of 24 adapters containing a different 8 base molecular barcode for high level multiplexing was ligated onto each sample. Following ligation of adapters, 15 cycles of PCR amplification was performed to enrich for cDNA with adapters on both ends. Completed libraries were sequenced on an Illumina HiSeq2500 in Rapid Mode, generating 20 million or more high quality 75 base long paired end reads per sample.

RNA-Seq analysis was based on the TopHat/Cufflinks pipeline. First, the spliced alignment tool TopHat, which efficiently aligns reads spanning known or novel splice junctions, was used to align short RNA-seq reads to the *Histoplasma capsulatum* genome (Taxonomy ID: 5036). Each sample was then independently processed with Cufflinks in order to generate an initial transcriptome and estimate abundance. The Cuffmerge tool was used to merge the private transcriptomes into a single reference, and at the same time annotate known genes and extend private transcripts. This common transcriptome was then used in a second pass with Cufflinks, which quantifies each transcript and gene (known or novel) in each sample. The reference annotation used was based on the UCSC known Genes table.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
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<tr>
<td>Srb1</td>
<td>GTAGCAGCCGAACACATCTGGAATGAGACCTTGGGCGATACG</td>
</tr>
<tr>
<td>NIT50</td>
<td>CGCCACCTCAACAACACCAAGCCTTTCA</td>
</tr>
<tr>
<td>RSP/GEF</td>
<td>CGTCACTCATCAATCCACCGGAAATCCTTGGG</td>
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<tr>
<td>ABC</td>
<td>GCGACAAATAAGCAACACAGACAGCACAAATCATACCC</td>
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<tr>
<td>NADP/FAD</td>
<td>TGTCACTGTCCACAGAATCCC</td>
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<tr>
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<tr>
<td>Erg2</td>
<td>TTCGACAACCCAGGAACCCC</td>
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<tr>
<td>Erg3</td>
<td>GGATTATGCGCAAGCCTTAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CAGGACACGTCCACAGATTTATG</td>
</tr>
</tbody>
</table>

Table 1. List of primers used for qRT-PCR analysis.
and included the long non-coding RNAs described in Cali-
ble et al. This method allowed the accurate quantification
of expression of all transcripts, known or novel.

Results were confirmed utilizing qRT-PCR. Data shown
are averages of the 3 independent experiments and are
normalized to the levels of transcript present on wild-type
under normoxic conditions.

Mouse infection

Mouse infection

Male C57BL/6 mice (6–8 weeks old purchased from Na-
tional Cancer Institute, NIH, Bethesda, MD) were infected
intranasally (i.n.) with 2 × 10⁶ midexponential phase
H. capsulatum yeast cells resuspended in 50 µl PBS; or
via intraperitoneal (i.p.) infection with 5 × 10⁵ H. cap-
sulatum yeast cells in 0.5 ml PBS as described previ-
yously. Fungal Burden: At days 7, 14, 21, and 28 following H. cap-
sulatum infection, lungs, livers, and spleens were harvested
from infected mice. Tissue samples were homogenized in
RPMI 1640 Medium (Life Technologies, Grand Island,
NY, USA). Serial dilutions of the homogenate were plated
on HMM solid medium supplemented with 25 µg/ml gentamycin; and cultured for 7–10 days to determine
limit of detection is 1 × 10⁵ CFU. Histology. Small pieces
of liver, lung and spleen were harvested and fixed in
from infected mice. Tissue samples were homogenized in
RPMI 1640 Medium (Life Technologies, Grand Island,
NY, USA). Serial dilutions of the homogenate were plated
on HMM solid medium supplemented with 25 µM FeSO₄
and gentamicin; and cultured for 7–10 days to determine
colony-forming units (CFU) recovered from each organ.
Fungal burden was expressed as the mean CFU per whole
organ or per gram of tissue where specified ± SEM. The
limit of detection is 1 × 10⁵ CFU. Histology. Small pieces
of liver, lung and spleen were fixed in 10% phosphate-
buffered formalin, embedded in paraffin, and sectioned
5 µm. Hematoxylin and eosin (H&E) was done for mor-
phological detection of granulomas. Thin sections were stained
with Grocott methenamine silver (GMS) by using standard
histological techniques, to detect H. capsulatum in granu-
losumatous areas of the liver. Microscopic examinations were
performed on an Olympus BX51 microscope and Olympus
DP72 imaging system. Hypoxyprobe-2. Mice were injected
i.p. with hypoxyprobe at a dose of 60 mg/kg weight of the
mouse (Hypoxyprobe Inc., Burlington, MA, USA). After 60
to 90 min, mice were sacrificed using increasing concentra-
tions of CO₂ and cervical dislocation. Following the manu-
facturer’s instructions, tissue sections were washed and in-
cubated with the mouse monoclonal antibody FITC-Mab1
(Hypoxyprobe-1 Plus Kit, Hypoxyprobe Inc., Burlington,
MA, USA) diluted 1:75 in antibody diluent. After another
wash, sections were incubated for 30 min at room tem-
perature with Rabbit Anti-FITC (Hypoxyprobe-1 Plus Kit,
Hypoxyprobe Inc.,) diluted at 1:75. Microscopic examina-
tions were performed on Olympus BX52 microscope and
Olympus DP2-BSW imaging system. For each time point, a
total of 3 to 4 mice were examined and experiments were
repeated in triplicate.

All mice were housed under pathogen-free conditions,
and protocols were approved by the Institutional Animal
Care and Use Committee. The mice were housed in a barrier
facility, maintained in sterile conditions in microisolator
cages until use, and supplied with sterilized food and water.

Isolation of liver granulomas

Granuloma infiltrating cells from 3 to 4 pooled infected livers
were isolated using a previously published protocol. Livers were homogenized with a gentleMACS Dissociator
(Miltenyi Biotec, CA, USA), and liver granulomas were al-
lowed to settle by virtue of their higher density. Settled gran-
ulomas were washed in RPMI 1640 medium (Life Tech-
nologies, Grand Island, NY, USA), digested with collagen
type IV (Sigma-Aldrich, St. Louis, MO, USA), at 37°C
for 40 minutes with shaking. Granulomas were disrupted
using a syringe and filtered through 70 µm nylon strainer to
remove any tissue debris. Live leukocyte count was deter-
mained by Trypan Blue staining. PCR and Primers: mRNA
from granuloma infiltrating cells was isolated using Qiagen
AllPrep DNA/RNA Mini Kit (Valencia, CA, USA), using
the manufacturer’s instructions. cDNA synthesis and RT-
PCR was performed using TaqMan Gene Expression Assay
(Life Technologies, Grand Island, NY, USA).

FACS-based hypoxia detection and granuloma staining

C57BL/6 mice were injected i.p. at with pimonidazole hy-
drochloride 60 mg per kg body weight (Hypoxyprobe,
Burlington, MA, USA) as described above. Ninety min-
utes later the animals were sacrificed and the liver har-
vested and disassociated into a single-cell suspension us-
ing collagenase IV (Sigma-Aldrich, St. Louis, MO). To
characterize HP-2 expression, cells were incubated with
Cytofix/Cytoperm (BD Biosciences, San Diego, CA, USA),
flushed in Permeabilization buffer (BD Biosciences, San
Diego, CA, USA), and incubated for 1 h with FITC-
conjugated anti-hypoxyprobe antibody (Hypoxyprobe,
Burlington, MA, USA) (HP2–100, 1:100). The following
mAbs were purchased from BD Biosciences: PE-conjugated
Mac3 and CD4, allophycocyanin-conjugated CD11b and
CD3, peridin-chlorophyll protein-conjugated CD8α and
CD11c. For surface staining, splenocytes and granuloma
cell suspensions were washed with 1% BSA in HBSS
(pH 7.4) and were stained at 4°C for 30 min. Cells were
characterized using a FACSCalibur Flow Cytometer (BD
Biosciences, Germany) and FCS Express Software.
 Statistical analyses

All statistical analyses were performed using GraphPad Prism for Windows. All experiments were performed in triplicate and all bars represent mean ± S.E.M. Significance was calculated using a Student’s t-test, one-way or two way analysis of variance (ANOVA) with Bonferroni’s Multiple Comparison Test or Newman-Keuls post hoc test for comparison of groups greater than two. A P-value of less than .05 as considered statistically significant as follows: ***<.001 **P < .01 *P < .05.

Results

H. capsulatum survives predominantly within granulomas as infection progresses

To identify the hypoxic regions at the site of infection, we used a previously established intraperitoneal (i.p) infection model to induce disseminated H. capsulatum infection. Intraperitoneal infection resulted in rapid development of systemic, disseminated infection, and the subsequent development of small granulomas in the liver. These granulomas were similar in size and morphology to granulomas formed following respiratory H. capsulatum infection.

Histological analysis of H&E-stained thin liver sections of H. capsulatum inoculated mice showed granulomas with a visible influx of immune cells. The morphology of the granuloma was consistent in size and shape with previously published data. Some leukocyte migration and cellular infiltration was visible early at 7 d.p.i. (Fig. 1A). Higher magnification images (Fig. 1A, bottom row) showed leukocyte extravasation and aggregation and the appearance of many well-formed granulomas by day 14, which persist through 21 days (Fig. 1A, top bottom). Analysis of the size and number of granulomas supported previous studies that demonstrated a high number of granulomas at 14 d.p.i. that was maintained at 21 d.p.i., but decreased in average size. At 28 days post-infection, a few small well-formed granulomas continued to persist in the liver.

Silver staining (GMS) was used to determine the cellular location of H. capsulatum yeasts within the liver during infection. At 7 d.p.i. H. capsulatum yeast cells were visible next to blood vessels in the liver parenchyma without a structured granulomatous response (Fig. 1B). At later time points, H. capsulatum was visible almost exclusively in the focal regions of well-formed granulomas in the liver (Fig. 1B).

We defined the kinetics of i.p H. capsulatum infection and confirmed the primary location of H. capsulatum yeasts by quantitative plating of the livers (separated into granuloma and nongranuloma liver sections) and spleens of sacrificed animals, and calculating the organism burden. The highest fungal load was detected at 7 d.p.i. and was undetectable by 21 d.p.i. (Suppl. Fig. 1). At 7 d.p.i. the H. capsulatum load was 7.2 × 10^6 CFU per spleen, 1.06 × 10^7 CFU in the nongranuloma section of the liver and 2.5 × 10^6 CFU within the liver granuloma. The fungal burden in both organs decreased by 50% at 14 d.p.i. While H. capsulatum load in non-granuloma liver tissue was higher than within the granuloma at 7 d.p.i., by 14 d.p.i. the fungus was contained within granulomas and remained undetected in non-granuloma liver tissue. Most of the infection was controlled by 21 d.p.i. and was below the level of detection of the assay by 28 d.p.i. (Suppl. Fig. 1).

In summary, the data confirmed that H. capsulatum can induce the formation of small granulomas during i.p. infection of C57B/6 mice. Yeasts were dispersed throughout the liver early on during infection, but primarily contained within the granuloma at later time points.

H. capsulatum induced granulomas in the liver are hypoxic

To test directly for the presence of hypoxic conditions in the granulomas of H. capsulatum infected mice, we used the tissue hypoxia marker pimonidazole hydrochloride (Hypoxyprobe-2 or HP-2). This nitroheterocyclic chemical becomes activated by hypoxia (pO2 ≤ 10mmHg or 1%) and forms intracellular adducts with thiol groups on proteins, peptides, and amino acids. Through a horseradish peroxidase-linked reaction the presence of tissue pimonidazole adducts results in the chromogenic production of a brown color. We hypothesized that animals infected with H. capsulatum would develop regions of hypoxia within the granulomas of infected livers.

The development of hypoxia in vivo was tested in the intraperitoneal (i.p) infection model. Interestingly, we found regions within both the H. capsulatum-induced liver granulomas and the surrounding liver parenchyma were HP-2 positive, and varied in intensity as the disease progressed (Fig. 1C). Hypoxia staining remained around the granuloma periphery at day 7, and by day 14 hypoxia could be readily detected at the center of the granuloma (brown). HP-2 staining significantly expanded to other regions of the liver by days 21 and 28. Surprisingly, at 28 d.p.i the presence of HP-2 staining not only increased in intensity, but also in distribution throughout the entire liver. Cytoplasmic HP-2 staining can normally be seen in tubular epithelial cells in the outer medulla of the kidney sections of mice and served as a positive control. Uninfected liver sections harvested at all four time points and isotype control staining of the same granulomas in subsequent liver sections did not
Figure 1. Morphology of Histoplasma-induced granuloma and hypoxic environments at the site of H. capsulatum infection in murine livers. In a murine model of disseminated histoplasmosis mice were euthanized on indicated days after inoculation with H. capsulatum strain G217B (A) Photomicrographs shown are of H&E-stained thin liver sections at days 7, 14, 21, and 28 after H. capsulatum yeast infection of C57B/6 mice. Total magnification is ×125 (top row) or ×500 (bottom row). Inflammatory lesions/ granulomas are indicated by arrowheads. (B) Photomicrographs at ×500 total magnification of GMS stained thin liver sections at days 7, 14, 21, and 28 post H. capsulatum infection of C57B/6 mice. H. capsulatum cells are indicated by arrows. (C) Prior to sacrifice, Hypoxyprobe-2 (60 mg/kg) was intraperitoneally injected into all mice and allowed to circulate for 90 minutes. Photomicrographs at ×125 total magnification (top row) and zoomed in (bottom row) of liver sections at days 7, 14, 21, and 28 after infection of C57B/6 mice. (Negative control = uninfected mouse liver, Positive control = infected mouse kidney). (Data represents n = 3 using 3–4 mice per group).
stain for HP-2, demonstrating the specificity of the HP-2 and antibodies utilized (Fig. 1c).

As a secondary method, we determined the presence of HP-2 protein adducts within *H. capsulatum* infected granulomatous lesions using flow cytometry. Using a previously published protocol,11 granulomatous lesions were extracted and infiltrating cells were isolated after settling and digestion of granulomas from the dispersed liver tissue. This approach has been applied successfully to isolate of *H. capsulatum*-induced granulomas and also granulomas induced from other infectious agents including *Mycobacterium tuberculosis*.11,56 Single cell preparations were made from both the isolated granulomas and the non-granuloma fraction of the infected liver, and the whole infected spleen and cell preparations were analyzed at the time points with the largest number of granulomas, 14 and 21 d.p.i.

The typical granuloma is primarily comprised of a large number of macrophages, some T-Cells and few dendritic cells. Flow cytometry using macrophage and T-cell surface markers was used to identify the major cell types in our granuloma preparation. Granuloma cell preparations were made from three to four pooled livers from infected mice.

Mac3+CD11b+CD11c− macrophages, CD3+CD4+ and CD3+CD8+ T-cells dominated the *H. capsulatum*-induced granuloma compared to non-granuloma liver cells (Fig. 2). The day 14 granuloma consisted of 23 ± 3% macrophages, 34% CD3+CD4+ T-cells, and 19% CD3+CD8+ T-cells (Fig. 2b). The later stage, well-formed, day 21 granulomas were dominated by macrophages with a distribution of 49 ± 10% macrophages, 21% CD3+CD4+ T-cells and 19% CD3+CD8+ T-cells (Fig. 2b). Surprisingly, granulomas isolated at 14 d.p.i. contained a higher abundance of CD3+CD4+ T-cells than macrophages or CD3+CD8+ T-cells; while macrophages were the predominant cell type during the later phase of granuloma development at 21 d.p.i. The proportion of CD3+CD4+ T-cells from both the granuloma and spleen were elevated compared to uninfected control, at both stages of infection (Fig. 2a). In contrast, the percentage of CD3+CD8+ T-cells in splenocytes and granuloma cells remained unchanged compared to uninfected splenocytes and non-granuloma liver cells, respectively.

When compared to nongranuloma liver fractions, there was an increase in HP-2 staining within *H. capsulatum*-induced granulomas. HP-2+ cells represent 66% of the total granuloma cell fraction at 14 d.p.i and 76% at 21 d.p.i. In the non-granuloma liver sections, HP-2+ cells accounted for 36% and 47% of the cells at 14 and 21 d.p.i., respectively (Fig. 3a-c). In uninfected control mice significantly more HP-2 positive cells were detected in the liver compared to the spleen (Fig. 3c-d). The average percentage HP-2+ cells in the granuloma and non-granuloma liver tissues during infection was significantly higher than the percentage in uninfected liver. In contrast, there was no change in the number of HP-2 positive splenocytes at either time point of infection when compared to splenocytes isolated from uninfected animals (Fig. 3d).

Macrophages represented not only the most predominant inflammatory cell type within the granuloma but also the greatest proportion of hypoxic cells (Fig. 4a). At 14 and 21 d.p.i. more than 95% of macrophages were positive for the hypoxia marker HP-2, while an average of 55% of T-cells isolated from granulomas were HP-2 positive (Fig. 4b). In comparison, the histogram represents the percentage of HP-2+ cells isolated from uninfected liver and spleen, 19.9% and 0.5% respectively (Fig. 4c).

To further confirm the presence of hypoxia, RNA was isolated from liver preparations to determine the transcript levels of HIF-1α, a mammalian hypoxia responsive gene. Using qRT-PCR, liver granulomas showed a significant increase in HIF-1α mRNA expression at 14, 21, and 28 d.p.i compared to non-granuloma liver tissue (Fig. 5a). In addition, HIF-1α downstream effectors VEGF, PGK1 and LDHA expression were significantly elevated within the granuloma compared to the surrounding hepatic tissue (Fig. 5b). Although regions of hypoxia were not detected via HP-2 staining of spleen sections, HIF-1α mRNA expression in infected spleens were higher compared to uninfected spleens though not statistically significant (Fig. 5c).

Histoplasmosis is primarily a pulmonary disease that invades the lungs before it disseminates to distal organs; therefore, it was important to test hypoxia in the lungs of infected mice. We used an intranasal model of infection to measure the degree of HIF-1α expression within the lungs of infected mice. HIF-1α mRNA levels were elevated 100-fold at 7 d.p.i. and continued to increase at 21 d.p.i. compared to uninfected animals (Fig. 5d). Taken together, these data confirm that *H. capsulatum* encounters hypoxic microenvironments (O2 concentration ≤ 1%) during murine histoplasmosis. The degree of hypoxia, fungal subcellular location and granuloma formation suggests that the interaction between the fungus and the host immune response plays an essential in the generation of the hypoxic microenvironment.

**H. capsulatum** is capable of growth at low oxygen tensions *in vitro*

To determine whether *H. capsulatum* can survive prolonged periods of hypoxia, we used a hypoxia chamber with O2 maintained at ≤ 1%. Log phase *H. capsulatum* yeast cells were placed under conditions of normoxia or hypoxia for
Figure 2. *H. capsulatum*-induced granuloma is enriched with macrophages and T-cells. Flow cytometric analysis of splenocyte and granuloma infiltrating cells during the course of *H. capsulatum* infection. (A) Dot plots (left column) represent expression of macrophage surface markers (Mac-3$^+$ CD11b$^+$ and CD11c$^-$) on forward and side scatter gated cells from granuloma suspension, non-granuloma liver cells and spleen 14 d.p.i. Middle column and right columns represent T-cell surface staining in the indicated tissues. Middle column represents CD3$^+$ and CD4$^+$ surface staining and right column represents CD8$^+$ and CD4$^+$ surface staining. Values on dot plots represent the percentage of the gated cells in the indicated regions from one representative experiment of three. (B) Plotted values represent the average percentage of cells expressing macrophage, and T-cell surface markers for 3–4 mice per time point. Three independent experiments were conducted. Error bars represent ± SEM.
Figure 3. *H. capsulatum* induced granuloma is hypoxic. Flow cytometry analysis of HP-2 intracellular staining of granuloma-infiltrating cells, non-granuloma liver cells and splenocytes. (A) and (B), 14 d.p.i and 21 d.p.i., respectively. Histograms represent HP-2 expression within forward and side scatter gated cells, and values are mean fluorescent intensity (MFI). Plotted MFI values (right column) represent the average MFI per condition indicated compared to uninfected control mice. (C) Values represent average percentage of HP-2 expression within each liver tissue fraction at 14 and 21 d.p.i. from 3 to 4 mice per time point compared to uninfected control livers. (D) Plotted values represent the average percentage of HP-2+ splenic cells at 14 and 21 d.p.i. compared to uninfected control spleens. All plots and histograms are representative of three independent experiments. Error bars represent ± SEM.
Figure 4. Macrophages within the granuloma are hypoxic. HP-2 antibodies were used in combination with macrophage surface markers (Mac-3$^+$ CD11b$^+$ and CD11c$^-$) and T-cell surface markers (CD3$^+$) to detect the degree of HP-2 expression in each cell fraction within the granuloma. (A) HP-2 gating on Mac-3$^+$ CD11b$^+$ and CD11c$^-$ (B) Histogram (left column) represents the average percentage of HP-2$^+$ cells in the granuloma-infiltrating cells, non-granuloma liver cells and splenocytes at 21 d.p.i. Middle column represents the percentage of HP-2$^+$, Mac-3$^+$, CD11b$^+$ and CD11c$^-$. Right column represents the percentage of HP-2$^+$ CD3$^+$ cells. (C) Histogram represents the percentage of HP-2$^+$ cells in the liver and spleen of an uninfected mouse. All plots and histograms are representative of three independent experiments. Error bars represent ± SEM.
Figure 5. Expression of mammalian HIF-1α during H. capsulatum infection. HIF-1α mRNA expression was compared in two different H. capsulatum infection models, disseminated infection (i.p) and pulmonary infection (i.n.). Bar graph represents HIF-1α expression (A) and downstream HIF-1α effector gene expression (B) during i.p. H. capsulatum infection in liver granulomas of 3 pooled mouse livers. (C) Whole spleen HIF-1α expression post i.p. infection. (D) Bar graph represents HIF-1α expression in the lung during i.n. H. capsulatum infection. Values are normalized to HPRT and shown relative to uninfected mice in 2 independent experiments.

48 hours and plated to determine the number of colony forming units (CFU) every 12 or 24 hours (Fig. 6a). As expected, under normal in vitro oxygen conditions within an incubator, H. capsulatum doubled every 12–14 hours; however, when placed under hypoxia, the CFU plateaued and remained constant up to 48 hours (Fig. 6a). H. capsulatum also maintained 80%–94% viability under hypoxia.

As a second approach to verify the survival of H. capsulatum under hypoxia, we used culture turbidity (optical density) as a measure of yeast concentration. We found that hypoxia inhibited H. capsulatum growth, however, upon transfer back to normoxic conditions growth was restored (Fig. 6b).

The lack of an increase or decrease in yeast cells under conditions of low oxygen tension could be explained by either inhibition of H. capsulatum replication or a rate of death under hypoxia equaling the rate of replication. Thus, replication was tested using ten-fold serial dilutions on solid agar plates, starting from 10^6 H. capsulatum yeast cells, spot-plated onto solid agar plates and cultured under normoxia or in a hypoxia chamber (O_2 ≤1%) for 8 days (Fig. 6c). The number of colonies were equal under both growth conditions however, hypoxia resulted in visibly smaller colonies. These results show that H. capsulatum not only survives hypoxia but also replicates. However, although replication occurs, it is severely hindered as evidenced by the barely discernible colonies after 8 days of hypoxic stress.

Transcriptional profile of H. capsulatum in response to hypoxia

In order to determine whether the transcriptome of H. capsulatum contributed to its ability to survive hypoxia, we conducted next generation sequencing to compare the transcriptional profile of H. capsulatum (G217B) grown under hypoxic (>1% O_2) conditions for 24 hours, to yeast grown under normoxic conditions (~21% O_2). We found that in addition to upregulating 95 genes and downregulating 106 genes many with no putative assigned function, RNA-seq revealed 6 hypoxia responsive gene transcripts (HRG) to be statistically higher under hypoxia compared to normoxia.
Sequence alignments using BLASTN identified putative homologs for 4 of the gene transcripts. A 23 fold increase in the expression of $NIT50$, a previously identified $H.\ capsulatum$ DNA binding protein; a 21 fold increase in a predicted ABC transporter that shares 70% identity with a $\text{Trichophyton rubrum}$ homolog; a 14 fold increase in an NADP/FAD oxidoreductase that shares 67% identity with an $A.\ fumigatus$ homolog; and a 10 fold increase in an RSP/GEF that shares 87% identity with a $\text{Blastomyces dermatitidis}$ homolog (Table 2). Surprisingly, RNA-seq analysis did not reveal significant changes in $H.\ capsulatum$ homologs of $Srb1$ or the oxygen-sensing, prolyl hydroxylase $Ofd1$, known to be an integral part of the hypoxic response pathway in other fungi. RNA-seq results were confirmed by
Table 2. Expression profile of genes upregulated under hypoxia compared to normoxia after 24 hours.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Fold Change</th>
<th>Closest Homolog</th>
<th>% ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HISTO_GY.Contig460.Fgenesh.Aspergillus.198.final_new</td>
<td>23.472</td>
<td>NIT50 (Histoplasma capsulatum)</td>
<td>100%</td>
<td><em>H</em> e nitrosative stress induced transcript 50 (NIT50)</td>
</tr>
<tr>
<td>HISTO_EA.Contig33-snap.100.final_new</td>
<td>21.030</td>
<td>ABC transporter (Trichophyton rubrum)</td>
<td>70%</td>
<td>Membrane Transporter. Transports metabolic products, lipids and sterols, and drugs (ABC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ABC multidrug transporter (Aspergillus fumigatus)</td>
<td>63% 50%</td>
<td></td>
</tr>
<tr>
<td>HISTO_ZT.Contig1089-snap.139.final_new</td>
<td>14.752</td>
<td>RSP (Blastomyces dermatitidis)</td>
<td>84%</td>
<td>Rho guanyl nucleotide exchange factor (RSP/GEF)</td>
</tr>
<tr>
<td>HISTO_EA.Contig13.Fgenesh.histc.158.final_new</td>
<td>10.626</td>
<td>FAD binding domain protein (Aspergillus fumigatus)</td>
<td>67%</td>
<td>NADP-cytochrome P450 reductase; involved in ergosterol biosynthesis; associated and coordinately regulated with Erg11p (NADP/FAD)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HISTO_ZQ.Contig188.genewise.5.final_new</td>
<td>7.51775</td>
<td>Hc 18S ribosomal RNA gene (Histoplasma capsulatum)</td>
<td>100%</td>
<td>Small subunit ribosomal RNA gene</td>
</tr>
<tr>
<td>HISTO_KK.Contig134-snap.6.final_new, HISTO_KK.Contig134.eannot.1136.final_new</td>
<td>5.9657</td>
<td>CFEM domain protein (Histoplasma capsulatum)</td>
<td>100%</td>
<td>Fungal specific cysteine rich domain (CFEM) protein. CFEM domain found in some proteins with proposed roles in fungal pathogenesis, eight cysteine-containing domain present in fungal extracellular membrane proteins.</td>
</tr>
</tbody>
</table>

qRT-PCR (Fig. 7). HRG expression was significantly increased compared to normoxia after 4 hours of hypoxia. While ABC and RSP/GEF gene transcripts continued to increase in expression after 8 and 24 hours of hypoxia; the NIT50 and NADP/FAD gene transcripts peaked at 8 hours and did not continue to increase after 24 hours.

RNA-seq identified few genes upregulated after 24 hours of hypoxia. However, when mRNA levels of these genes were evaluated at earlier time points (4 and 8 hours) by qRT-PCR, HRG expression was significantly higher compared to expression at 24 hours of hypoxia. Thus it was imperative for us to identify other genes induced early during hypoxia that were not detected at 24 hours.

### Identification of *Srb1* in *H. capsulatum*

Analysis of the *H. capsulatum* genome databases (Histobase, [http://histo.ucsf.edu/gb2/gbrowse/HcG217B/](http://histo.ucsf.edu/gb2/gbrowse/HcG217B/), and the Broad Institute, [http://www.broadinstitute.org/annotation/genome/histoplasma_capsulatum/GenomesIndex.html](http://www.broadinstitute.org/annotation/genome/histoplasma_capsulatum/GenomesIndex.html)) identified one putative SREBP homolog in *H. capsulatum*, named NIT53. Encoded by a single copy gene (HISTO_HS.Contig68.Fgenesh.Aspergillus.243.final_new) in *H. capsulatum* strain G217B, NIT53 is noncharacterized gene identified by Nittler et al., shown to be upregulated during the stationary phase of growth.28 BLASTP search using the *A. fumigatus* SREBP homolog, SrbA sequence and the genome database of *H. capsulatum* resulted in the
same NIT53 gene. The work reported in this manuscript refers to this NIT53 gene, herein designated as Srb1.

The predicted SREBP protein is composed of 1076 amino acids, which has an identity of 47% with A. fumigatus SrbA and 57% identity to S.pombe Sre1. Closer analysis using InterProScan revealed its conserved protein domains. Similar to all SREBPs, it contains a basic helix-loop-helix (bHLH) leucine zipper DNA binding domain in the N-terminus and a unique tyrosine (Y) for arginine (R) residue substitution within the bHLH region (Fig. 8a lower panel). This single amino acid residue substitution changes the DNA-binding properties of SREBPs by prohibiting their binding exclusively to E-box (5-CANNTG-3) DNA motifs in target gene promoters, and unique sterol regulatory elements (StREs) (5-ATCACCCAC-3). Another noteworthy characteristic of the Srb1 sequence, shared with other SREBPs, is the presence of the same domain unknown function (DUF2014). This domain is found exclusively at the C terminal of a family of ER membrane bound transcription factors called sterol regulatory element binding proteins (SREBP) (Fig. 8a upper panel). Multiple sequence alignment of Srb1, SREBP and bHLH nucleotides from different species was used to construct a phylogenetic tree. Using observed divergence analysis re A. fumigatus SrbA and Srb1 clustered together with 100% bootstrap support (Fig. 8b). Thus, sequence analysis indicated that Srb1 is the putative SREBP in H. capsulatum.

The lack of increase in Srb1 gene expression after 24 hours of hypoxia was confirmed by qRT-PCR (Fig. 9a). Both Srb1 and Ofd1 genes are known hypoxia responsive transcriptional regulators in other fungi. Although there was no detectable increase in Srb1 expression at 24 hours post hypoxia treatment we hypothesized that as a transcription factor, Srb1 may increase early during hypoxia, modulate numerous molecular pathways and induce the expression of downstream targets before returning to baseline levels.

To test this hypothesis, we measured the mRNA abundance of Srb1 in response to hypoxia over time. Srb1 expression increased as early as 2 hours post-hypoxia treatment and continued to gradually increase. Srb1 expression peaked after 8 hours of hypoxia to levels of more than 1000 fold compared to normoxia. At 12 hours Srb1 mRNA levels were no longer significantly elevated with only a 5 fold increase compared to normoxia (Fig. 9b). This suggests that an increase in Srb1 expression is important early during hypoxia but not after 12 hours.

RNA-seq analysis after 24 hours of hypoxia did not reveal significant changes in other genes predicted to be hypoxia responsive. As Srb1 expression peaked at 8 hours and was not induced after 24 hours of hypoxia, we predicted that genes that encode the ergosterol biosynthesis enzymes will be induced in a similar manner. Using qRT-PCR other predicted genes were evaluated at early time points for changes under hypoxia. There was a gradual increase in Erg2 and Erg3, two oxygen requiring components of the ergosterol biosynthesis pathway. Both genes peaked at 12 hours post hypoxia treatment, by which stage Srb1 is no longer significantly elevated (Suppl. Fig. 2).

We used computational transcription factor binding site analysis to examine the upstream regions of genes upregulated under hypoxia. Using the de novo bioinformatics tools: TESS Transcription Element Search System and TFSearch analysis of promoter regions of HRG, human SREBP binding sites were identified (Tables 3 and 4). Manual search of the A. fumigatus SrbA binding sites within the promoter regions of HRG also confirmed the potential for binding to SREBPs (Table 4). Consequently, these results strongly suggest that Srb1 plays a role during early exposure to hypoxia and may be important in the regulation of hypoxia responsive genes. H. capsulatum contains a putative SREBP homolog and components of the SREBP pathway.
Figure 8. Bioinformatic analysis of the _H. capsulatum_ homolog of SREBP. Figure 9. Bioinformatic analysis of the _H. capsulatum_ homolog of SREBP. (A) InterProScan revealed srb1 conserved protein domains. (B) Phylogenetic tree analysis of Srb1, SREBP homologs and other proteins containing basic helix loop helix domains (bHLH) obtained from Genbank. Phylogenetic analysis of the nucleotide sequences was calculated using the DNAman software package (Lynnon, Quebec, Canada). One-thousand bootstrap replications were performed and the values (expressed as percentages) are shown at the nodes. Bar shows a genetic distance of 0.05.

Discussion

*H. capsulatum* has the ability to survive within the granuloma of an infected host in an apparent latent state, and subsequently reactivate to cause disease. The _H. capsulatum_-induced granuloma is the result of the interplay between the fungus and the host cells at the infection site and consists of mainly lymphocytes, macrophages and dendritic cells. C57B/6 mice were infected with a sub-lethal dose of _H. capsulatum_ yeasts in order to characterize hypoxia during granulomatous reaction in histoplasmosis. We determined the presence of hypoxia _in vivo_ and the data proves that _H. capsulatum_ encounters hypoxic microenvironments (oxygen concentrations <1%) during experimental *Histoplasmosis*. Although seemingly obvious, our studies are the first to demonstrate low oxygen levels within the granuloma of _H. capsulatum_ infected tissue.

While oxygen is essential for the growth and survival of most eukaryotic organisms, its concentration in the atmosphere differs from the soil. The atmosphere is composed of 79% nitrogen, 21% oxygen and 0.03% CO2. While the soil near the surface has a similar concentration of gases, as the depth increases diffusion of the gases decreases, leading to a gradual decrease in oxygen content. As a result, oxygen levels in the soil begin to decrease to <5% at a depth of 8–12 inches.
capsulatum is generally found in the top 6 inches of soil, however, it has been found in blackbird roosts at a depth of more than 12 inches.1,62,63 This suggests that H. capsulatum has mechanisms to survive low oxygen tension not only during infection, but also within its ecological niche and our in vitro hypoxia model proved that H. capsulatum can survive extended periods of O2 concentrations of <1%.

To define hypoxia within the H. capsulatum induced granuloma, we used the hypoxia marker, pimonidazole hydrochloride (HP-2), which has traditionally been used as a hypoxia-imaging agent in clinical studies to detect reduced oxygen concentrations in human and animal tumors. In our study, we observed that regions of the liver of mice infected with H. capsulatum are hypoxic, as evidenced by the formation of stable adducts between reduced pimonidazole and host proteins at the site of infection. However, the extent of hypoxia differed as the infection progressed. While tissue hypoxia was demonstrated within the infected granulomas, after 21 d.p.i hypoxia was not limited exclusively to the granuloma, but was dispersed throughout the liver tissue and in peri-granuloma regions of the liver. In addition, 66–76% of the total granuloma infiltrating cells were positive for the hypoxia detection agent HP-2. Macrophages represented the cell type with the greatest proportion of hypoxic cells within the granuloma at 95% HP-2+ compared to 55% of T-cells. These results together with the proportional increase in macrophages as the disease progressed, suggests that the activity of the host cells may contribute to the development of hypoxic regions during histoplasmosis.

In contrast, splenocytes showed no difference in HP-2 staining during infection, but had significantly high levels of HIF-1α mRNA at 14, 21, and 28 days post infection. While HP-2 only detects regions of hypoxia of less than 1%, HIF-1α can be induced at oxygen concentrations of <6%.64 Thus, during colonization and subsequent dissemination, H. capsulatum is exposed to a dynamic range of oxygen levels during infection and granulomas play an integral, but not exclusive role in the development of the hypoxic microenvironment H. capsulatum encounters.

We also identified a SREBP homolog (Srb1) that is induced during hypoxia in a time dependent manner. In A. fumigatus, S. pombe, and C. neoformans, SREBP homologs are essential for survival under hypoxia, fungal virulence and sterol biosynthesis and mammalian SREBPs regulate the transcription of genes required for cholesterol and fatty acid synthesis.30,65 Given the limited information known about direct fungal SREBP target genes, we sought to examine the Srb1-mediated transcriptome of H. capsulatum in response to hypoxia. Our bioinformatic analysis provides some evidence that Srb1 may be important regulating the expression of the hypoxia responsive gene transcripts NIT50, ABC, RSP, and NADP/FAD. Current studies in our lab are centered on identifying the function of the Srb1 pathway in H. capsulatum, whether it’s necessary for survival under hypoxia, and how it contributes to H. capsulatum virulence.

Many organisms have developed a complex set of adaptive responses to deal with the stress associated with limited oxygen supply to ultimately enhance their survival. H. capsulatum is not an exception and in order to

Table 3. TESS analysis of HRG regions revealed putative SREBP binding sites in the promoter regions.

<table>
<thead>
<tr>
<th>NIT50</th>
<th>ABC</th>
<th>RSP/GEF</th>
<th>NADP/FAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SREBP-1</td>
<td>USF</td>
<td>HNF-3</td>
<td>MCBF</td>
</tr>
<tr>
<td>GATA-1</td>
<td>SREBP-1</td>
<td>FOXF-2</td>
<td>GATA-1</td>
</tr>
<tr>
<td>AP-1</td>
<td>Sox-5</td>
<td>SREBP-1</td>
<td>MGF</td>
</tr>
<tr>
<td>CREB</td>
<td>AML-1a</td>
<td>SEF-4</td>
<td>SREBP-1</td>
</tr>
<tr>
<td>Yeast (S. cerevisiae)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCR1</td>
<td>ADR1</td>
<td>GCR1</td>
<td>MAT1ap</td>
</tr>
<tr>
<td>ADR1</td>
<td>MATα1</td>
<td>ADR1</td>
<td>MIG1</td>
</tr>
<tr>
<td>GCN4</td>
<td>HSF</td>
<td>MATα1</td>
<td>GCR1</td>
</tr>
<tr>
<td>HSF</td>
<td>GCN4</td>
<td>HSF</td>
<td>GCR1</td>
</tr>
</tbody>
</table>

Figure 9. Normalized Srb1 expression under hypoxia compared to normoxia. Srb1 expression is elevated under hypoxia in a time dependent manner. (A) Srb1 expression measured by qRT-PCR after 24 hours of hypoxia. No significant difference observed. (B) Srb1 expression over 24 hours of hypoxia. Values are normalized to GAPDH and shown relative to normoxia. Results are mean and SEM of three experiments.
successfully colonize and cause disease, it must be able to adapt to rapidly changing environments. During murine histoplasmosis, we observe in vivo hypoxic microenvironments and begin to delineate how the dimorphic fungus adapts to low oxygen tension and continues to colonize the host. These results, together with other published data in A. fumigatus supports the hypothesis that hypoxia adaptation is an important aspect of pathogenic fungi survival. The impact of hypoxia on H. capsulatum and the host, and the extent to which it occurs during histoplasmosis, remains an important area of investigation.

Acknowledgments

We would like to thank Dr. George Deepe (University of Cincinnati, Cincinnati, OH) for providing advice, reagents, and equipment. This work was supported by Merit Review Award # 5I01BX000335 from the United States (U.S.) Department of Veterans Affairs Biomedical Laboratory Research and Development Program from the Office of Research and Development.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Supplementary Material

Supplementary material is available at Medical Mycology online (http://www.mmy.oxfordjournals.org/).

References


Table 4. Transcription factor binding sites search reveals SREBP predicted binding sites in hypoxia responsive genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcription Factor</th>
<th>Position</th>
<th>Sequence Hit</th>
<th>Search Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIT50</td>
<td>SREBP-1</td>
<td>502(+)</td>
<td>AAGCACCTCAC</td>
<td>KATCACCCCCCAC</td>
</tr>
<tr>
<td></td>
<td>SREBP-1</td>
<td>118(+)</td>
<td>TTTCCCCAC</td>
<td>KATCACCCCCCAC</td>
</tr>
<tr>
<td></td>
<td>SREBP-1</td>
<td>833(+)</td>
<td>CGTCATCCCCAA</td>
<td>KATCACCCCCCAC</td>
</tr>
<tr>
<td></td>
<td>SrβA (A. fumigatus)</td>
<td>44(−)</td>
<td>ATATACACATA</td>
<td>ATCATAGCAT (ataatacga)</td>
</tr>
<tr>
<td>ABC</td>
<td>SREBP-1</td>
<td>930(−)</td>
<td>CACACGTCGAC</td>
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</tr>
<tr>
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<td>SREBP-1</td>
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<td>ACCACGTGACC</td>
<td>NATACAGTGAY</td>
</tr>
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<td></td>
<td>SrβA (A. fumigatus)</td>
<td>318(−)</td>
<td>ATCATACATC</td>
<td>ATCATACAG (atcatacag)</td>
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<tr>
<td>RSP</td>
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<td>KATCACCCCCCAC</td>
</tr>
<tr>
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<td>SREBP-1</td>
<td>982(−)</td>
<td>CTCAGGCTCATA</td>
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</tr>
<tr>
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<td>SrβA (A. fumigatus)</td>
<td>575(−)</td>
<td>ATATCATGAT</td>
<td>ATCATACAG (atcatacag)</td>
</tr>
<tr>
<td>NADP/FAD</td>
<td>SREBP-1</td>
<td>434(−)</td>
<td>GGCCGCGTGA</td>
<td>KATCACCCCCCAC</td>
</tr>
<tr>
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<td>228(+)</td>
<td>GATCCGCTGAT</td>
<td>NATACAGTGAY</td>
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<td></td>
<td>SrβA (A. fumigatus)</td>
<td>333(−)</td>
<td>TTGATACGAT</td>
<td>ATCATACAG (atgatacag)</td>
</tr>
</tbody>
</table>

Note: TFSEARCH ver.1.3 (c) 1995 Yutaka Akiyama (Kyoto Univ.) searches highly correlated sequence fragments versus TFMATRIX transcription factor binding site profile database. High scoring values of >85 reported here are predicted to bind with high affinity.


38. Hughes BT, Nwosu CC, Espenshade PJ. Degradation of (SREBP) precursor requires the (ERAD) components (Ub7) and (Hrd1) in fission yeast. *J Biol Chem* 2009; 284: 20512–20521.


