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DISSERTATION

Titel der Dissertation

The role of histone deposition pathways in *Candida albicans* stress resistance and virulence

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1 Summary

The opportunistic fungal pathogen *Candida albicans* is the most frequent cause of *Candida*-derived infections. *Candida spp*. can cause diseases ranging from mucocutaneous to life-threatening systemic infections in immunocompromised patients. During the infection process, *C. albicans* encounters various environmental stress conditions including reactive oxygen species (ROS) produced by innate immune cells such as neutrophils dedicated to kill the pathogen. Furthermore, treatment with antifungal agents also represents severe stress to the pathogen. Two mechanisms are therefore essential for *C. albicans* to be able to survive under these conditions: the fungus has to be able to repair damaged cellular components efficiently, and it has to respond rapidly by changing its transcriptional program. Changes in chromatin structure are involved in both mechanisms. Disassembly and reassembly of histones is required for efficient repair of DNA damage. Furthermore, transcriptional induction and repression are intimately linked to the histone density at the corresponding loci.

The conserved histone H4 specific acetyltransferase Hat1 is known to be involved in the incorporation of histones at sites of DNA damage in different species. This work demonstrates that *C. albicans* Hat1 is required for efficient repair of not just exogenous but also endogenous DNA damage. Cells lacking Hat1 rapidly accumulate DNA lesions and show severe morphological defects. Thus, *C. albicans* is the first organism known to require histone H4 processing by Hat1 for endogenous DNA damage repair.

Interestingly and unexpectedly, loss of Hat1 does not increase susceptibility to DNA-damaging ROS, but it renders cells hyperresistant to oxidizing agents. Strikingly, we could identify Hat1 as a negative regulator of oxidative stress genes. Furthermore, misregulation of oxidative stress genes and resistance to H_2O_2 are mimicked by inactivation of the CAF-1 histone chaperone complex. Interestingly, genome-wide transcriptional profiling revealed specific roles for Hat1 in transcriptional repression, both in concert with as well as independently of CAF-1. Hyperresistance to oxidative stress also improves survival of *hat1*Δ/Δ cells to neutrophil killing. Remarkably, loss of Hat1 leads to defects in killing infected mice although the mutant can persist in infected kidneys. Besides its role in oxidative stress resistance, lack of Hat1 also leads to increased tolerance to azole antifungals. This function is shared with the HIR histone chaperone complex. Thus, this work indicates for the first time a dual function for Hat1 in DNA damage repair as well as in the regulation of stress resistance via distinct chromatin assembly pathways, the latter of which seems to be highly specific for *C. albicans*.

2 Zusammenfassung

Der opportunistische humanpathogene Pilz *Candida albicans* ist die Hauptursache für *Candida* Infektionen. *Candida spp*. können Krankheiten auslösen, die von mukokutanen bis hin zu lebensbedrohenden systemischen Infektionen in immunsupprimierten Patienten reichen. Während des Infektionsprozesses ist *C. albicans* diversen Stressbedingungen, wie reaktiven Sauerstoffspezies (ROS) produziert von Zellen des angeborenen Immunsystems (z.B. Neutrophilen), ausgesetzt, welche die Aufgabe haben Pathogene zu töten. Darüber hinaus bewirken antifungale Wirkstoffe auch Stress beim Pathogen. Zwei Mechanismen sind essenziell für das Überleben von *C. albicans* unter diesen Bedingungen: Der Pilz muß geschädigte Zellkomponenten effizient reparieren und sein transkriptionelles Programm schnell ändern können. Mit beiden Mechanismen verbunden sind Änderungen in der Chromtinstruktur. Für eine effiziente Reparatur von DNA Schäden ist die Entfernung und der Wiedereinbau von Histonen notwendig. Auch transkriptionelle Induktion und Repression sind eng verknüpft mit der Histondichte am jeweiligen Lokus.

Die konservierte Histon H4 spezifische Azetyltransferase Hat1 spielt eine Rolle im Einbau von Histonen bei DNA Schäden in verschiedenen Spezies. Diese Arbeit zeigt, dass *C. albicans* Hat1 nicht nur für die Reparatur von exogenen sondern auch von endogenen DNA Schäden benötigt wird. Fehlt Hat1, akkumulieren die Zellen schnell DNA Schäden und zeigen schwere morphologische Defekte. Damit ist *C. albicans* der erste bekannte Organismus, der die Histon H4 Prozessierung durch Hat1 für die Reparatur von endogenen DNA Schäden benötigt.

Interessanterweise erhöht der Verlust von Hat1 nicht die Suszeptibilität gegenüber DNA schädigenden ROS, sondern macht die Zellen resistenter gegenüber oxidierenden Agenzien. Hat1 konnte als negativer Regulator von oxidativen Stressgenen identifiziert werden. Darüber hinaus führt die Inaktivierung des CAF-1 Histon-Chaperons auch zu Misregulierung von oxidativen Stressgenen und H_2O_2 Resistenz. Eine genomweite Transkriptionsanalyse zeigte spezifische Funktionen für Hat1 in der Transkriptionsrepression, sowohl gemeinsam mit als auch unabhängig von CAF-1. Die oxidative Stressresistenz erhöht auch das Überleben von *hat1*Δ/Δ Zellen gegenüber einem Angriff durch Neutrophile. Weiters können *hat1*Δ/Δ Zellen infizierte Mäuse weniger effizient töten, obwohl die Mutante in infizierten Nieren persistieren kann. Neben der Rolle in der oxidativen Stressresistenz führt der Verlust von Hat1 auch zu erhöhter Toleranz gegenüber der antifungalen Wirkstoffgruppe der Azole. Diese Funktion teilt sich Hat1 mit dem HIR Histon-Chaperon. Diese Arbeit zeigt damit zum ersten Mal, dass Hat1 eine duale Funktion sowohl in der Reparatur von DNA Schäden als auch in der Regulation von Stressresistenz gemeinsam mit verschiedenen Histon-Chaperonen hat. Letztere Funktion scheint dabei spezifisch für *C. albicans* zu sein.

3 Introduction

3.1 Introduction to fungi

Fungi are a highly diverse group of eukaryotic organisms which are composed of a rigid chitinous cell wall. Some 180000 fungal species have been described so far, although about 1.5 million species are estimated to exist on earth (Mueller & Schmit, 2007). The fungal kingdom evolved over more than 500 million years and is divided into seven proposed phyla: *Dikarya* (*Ascomycota* and *Basidiomycota*), *Microsporidia*, *Chytridiomycota*, *Neocallimastigomycota*, *Blastocladiomycota* and *Glomeromycota* (Hibbett *et al.*, 2007). Well-known macroscopic fungi such as morels and mushrooms represent only a minor fraction of all fungal species. The majority of fungi form multicellular filaments and are called moulds. A rather small group of fungi, the yeasts, grow as single cells. Notably, this group includes economically important and scientifically well-studied species such as the "baker's yeast" *Saccharomyces cerevisiae* (Carlile *et al.*, 1994). Due to their huge morphological diversity fungi are found in almost every environment and occupy different ecological niches. Symbiontic, commensal as well as pathogenic forms are known.

3.2 Opportunistic fungal pathogens and Candida *species*

Several fungal species are known which spend at least part of their life cycle in a pathogenic form. Some of them are pathogenic to plants or animals and are therefore economically important. In addition, there are about 300 fungal species known to cause diseases in humans (Taylor *et al.*, 2001). They can lead to superficial, cutaneous, subcutaneous or systemic infections, as well as allergic disease and claim about 1.5 million lives per year (Brown *et al.*, 2012). Furthermore, opportunistic mycoses represent an increasing health problem. They are caused by normally commensal fungi belonging to the natural microbial flora. However, under specific circumstances (e.g. immunosuppressed conditions) they change to a pathogenic life form and cause infections in the host. Widespread members of this group of fungi belong to the genus *Aspergillus*, *Cryptococcus* or *Candida*. Especially systemic infections with *Candida* and *Aspergillus* species are associated with extremely high mortality rates (Horn *et al.*, 2009, Nace *et al.*, 2009, Neofytos *et al.*, 2009, Mean *et al.*, 2008, Bicanic *et al.*, 2005, Warris & Verweij, 2005, Wenzel & Gennings, 2005). Furthermore, *Candida* species are responsible for most of the fungal infections worldwide and represent the fourth-leading cause of hospital-acquired infections. They account for 8-10% of all nosocomial blood stream infections with mortality rates of up to 40% (Pfaller & Diekema, 2007, Wenzel & Gennings, 2005). Normally, *Candida spp*. are commensals colonising the gastrointestinal and genitourinary tracts and to a lesser extent the skin. However under immunosuppressive conditions (e.g. after cancer

chemotherapy, HIV infection or in neonates) *Candida* can penetrate epithelia and cause systemic infections (Mavor *et al.*, 2005). Of the roughly 200 *Candida* species described only a few are of medical importance. Besides *Candida albicans*, which is the main cause of candidemia with 48-58% of all cases*, C. glabrata, C. parapsilosis, C. tropicalis*, *C. dubliniensis* and *C. krusei* are also known to cause infections in humans (Pfaller *et al.*, 2008). Interestingly, *C. albicans* has been rarely isolated from the environment and is therefore considered as being obligatory associated with mammalian hosts. Thus, understanding the regulation of the commensal to pathogen transition is necessary to understand its pathogenicity and to develop efficient therapies.

3.3 Virulence factors of Candida albicans

3.3.1 Morphology

C. albicans is a diploid organism and is therefore able to grow in a yeast form as well as in a hyphal form depending on the environmental conditions. The yeast form consists of round to ellipsoid single cells that divide by budding. Chromosome separation takes place at the mother-daughter junctions, which is defined by a septum (Fig. 3-1) (Whiteway & Bachewich, 2007). In contrast, upon induction of hyphal growth a germ tube is formed. Chromosome separation takes place within this germ tube at the site where the septum is formed. Subsequently, one nucleus migrates back into the mother cell while the other nucleus moves farther into the elongating germ tube (Fig. 3-1) (Whiteway & Bachewich, 2007). Cells within the elongating hyphae are only divided by septa. In addition to these two growth patterns, *C. albicans* can also exist in other morphologies such as pseudohyphae, which are elongated cells that stay attached together and are divided by constrictions at the septa (Fig. 3-1) (Whiteway & Bachewich, 2007, Sudbery *et al.*, 2004). Furthermore, Chlamydospores are a thick walled morphology formed primarily under suboptimal growth conditions (Staib & Morschhäuser, 2006).

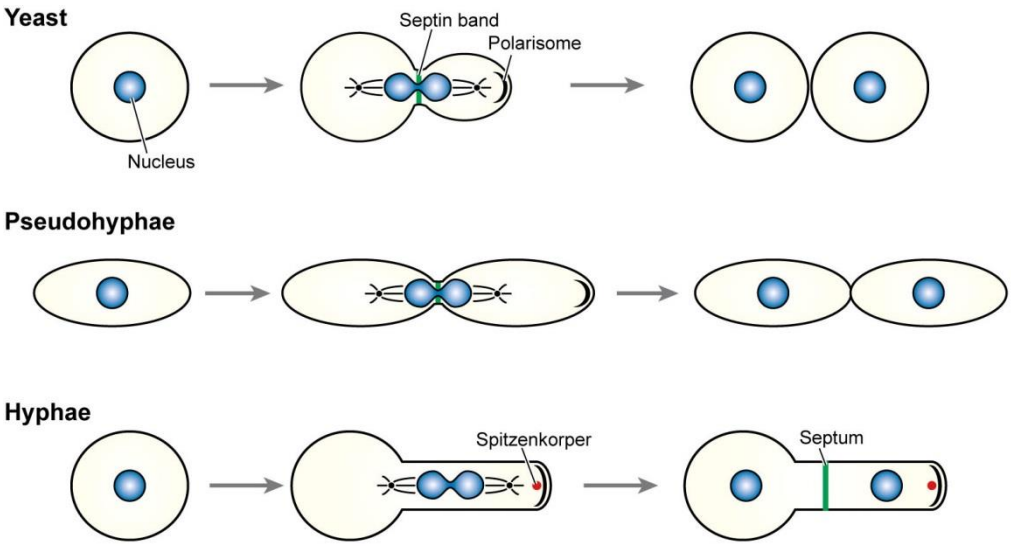

Figure 3-1: Morphological forms of *C. albicans*. Yeast cell proliferation takes place via budding followed by separation of the mother and the daughter cells. Pseudohyphal cells are elongated and stay attached after cell division. In both cases polarized growth is governed by the polarisome and nuclear division takes place at the mother-daughter junction which is defined by a septum. In true hyphae polarized growth is directed via the polarisome as well as a structure called Spitzenkörper and nuclear division takes place within the elongating germ tube. Modified from Whiteway & Bachewich, 2007.

Importantly, the ability to undergo morphogenetic transitions is considered a key virulence factor of *C. albicans*, since mutants that are locked in one morphological form show reduced virulence in different models (Banerjee *et al.*, 2008, Zheng & Wang, 2004, Hwang *et al.*, 2003b, Braun *et al.*, 2000, Lo *et al.*, 1997). The switch from the yeast to the hyphal form is controlled by environmental stimuli. Prominent triggers of the hyphal form are growth in nutrient poor media such as Lee's medium and an elevated temperature of 37°C in combination with the presence of *N*-acetyl-glucosamine or serum (Whiteway & Bachewich, 2007). The transcriptional regulation of the transition between the yeast and the hyphal form is well explored. Different signalling pathways control the expression of hyphae-specific genes in response to environmental signals via a set of transcriptional regulators (Biswas *et al.*, 2007). Deletion of the genes encoding the transcription factors Efg1 and Cph1 blocks hyphal induction in response to different stimuli (Lo *et al.*, 1997). Rim101 is another transcription factor required for alkali-induced filamentation (Davis *et al.*, 2000). Furthermore, the transcriptional regulator Tec1 is required for serum-induced hyphal growth (Schweizer *et al.*, 2000). Additionally, several other transcription factors (Czf1, Flo8, Hap5, Efh1, Ace2, Mcm1, Ash1 and Cph2) that control filamentation under specific conditions were discovered (Whiteway & Bachewich, 2007).

In addition to the yeast-to-filament transition *C. albicans* can also switch between the so-called white form resembling the mating-incompetent form and the opaque form, which is the mating-competent form (Lohse & Johnson, 2009, Slutsky *et al.*, 1987). These two yeast-like morphologies differ in terms of host niche colonization (Lachke *et al.*, 2003), interaction with

immune cells (Lohse & Johnson, 2008) as well as virulence (Kvaal *et al.*, 1999). Switching to the opaque form can only occur in cells homozygous for the *mating type locus* (*MTL*). However, most *C. albicans* cells are *MTL* heterozygous (*MTL**a***/*MTLα*) and therefore express the heterodimeric **a**1/α2 repressor. Thus, they are unable to switch, because this repressor inhibits expression of *WOR1* encoding the primary transcription factor that controls white-opaque switching (Huang *et al.*, 2006, Zordan *et al.*, 2006, Miller & Johnson, 2002). Additional transcriptional regulators of white-opaque switching are Efg1, Czf1 and Wor2. They form a transcriptional feedback loop which controls the switching process (Zordan *et al.*, 2007). In addition, different chromatin modifiers are able to modulate this transcriptional circuitry since they can influence the switching process (Stevenson & Liu, 2011, Hnisz *et al.*, 2009, Klar *et al.*, 2001, Srikantha *et al.*, 2001).

3.3.2 Oxidative stress resistance

C. albicans encounters severe oxidative stress during the infection process due to host-derived reactive oxygen species (ROS) produced by innate immune cells to clear pathogens (Miramon *et al.*, 2012, Youseff *et al.*, 2012, Frohner *et al.*, 2009). Therefore, *C. albicans* has developed defence mechanisms to detoxify ROS and survive oxidative stress conditions. This includes expression of oxidative stress-induced genes encoding for ROS-degrading enzymes. The major transcriptional regulator controlling these genes under conditions of low as well as high hydrogen peroxide concentrations is Cap1 (Enjalbert *et al.*, 2006). Cells lacking Cap1 are hypersensitive to oxidative stress caused by diamide or H_2O_2 (Alarco & Raymond, 1999). Furthermore, expression of *CAP1* increases in the presence of human neutrophils and *cap1*Δ/Δ cells are more efficiently killed by this cell type (Fradin *et al.*, 2005). Genome-wide transcription analysis and localization studies showed that Cap1 is in addition to the regulation of oxidative stress genes also involved in controlling carbohydrate and energy metabolism, protein degradation, drug resistance, phospholipid transfer and nitrogen utilisation (Znaidi *et al.*, 2009, Wang *et al.*, 2006).

In addition to Cap1 the high osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway is also involved in the adaptation to oxidative stress caused by high peroxide concentrations in *C. albicans* (Enjalbert *et al.*, 2006). Several genes in this pathway are required for resistance to oxidative stress. For example the absence of the response regulator protein Ssk1 causes sensitivities to different oxidants as well as neutrophil killing and results in reduced virulence in a murine infection model (Du *et al.*, 2005, Chauhan *et al.*, 2003). Furthermore, lack of the Hog1 MAP kinase, the Pbs2 MAP kinase kinase or the response regulator Skn7 also increases the sensitivity to oxidative stress.

Various antioxidant enzymes are induced upon oxidative stress via the pathways mentioned above to counteract oxidative damage. This group of enzymes include superoxide dismutases (SODs),

glutathione reductases, thioredoxin and the catalase (Wang *et al.*, 2006, Martchenko *et al.*, 2004, Hwang *et al.*, 2003a, Nakagawa *et al.*, 2003, Hwang *et al.*, 2002, Lamarre *et al.*, 2001, Wysong *et al.*, 1998). *C. albicans* harbours a total of six SODs. Sod1 (Cu-Zn-SOD) and Sod3 (Mn-SOD) are cytoplasmic, whereas Sod2 (Mn-SOD) localizes to the mitochondria (Hwang *et al.*, 2003a, Hwang *et al.*, 2002, Lamarre *et al.*, 2001). Furthermore, Sod4-6 are Cu-Zn-SODs and supposed to be surface-located (Sorgo *et al.*, 2010, Frohner *et al.*, 2009, Martchenko *et al.*, 2004). Deletion of *SOD1* causes hypersensitivities to menadione and to killing by macrophages. Furthermore, it decreases virulence in a murine infection model (Hwang *et al.*, 2002). In addition, especially Sod5 and to some extend Sod4 are required for detoxification of macrophage and neutrophil derived ROS (Miramon *et al.*, 2012, Frohner *et al.*, 2009). Lack of Sod5 causes hypersensitivity to killing by murine macrophages and defects in virulence in a mouse model (Frohner *et al.*, 2009, Martchenko *et al.*, 2004). Furthermore, *SOD5* is induced upon phagocytosis by human neutrophils and lack of Sod5 leads to reduced survival of neutrophil attack (Miramon *et al.*, 2012, Fradin *et al.*, 2005).

C. albicans harbours several genes encoding enzymes with glutaredoxin activities. For example, *GRX2* encodes a putative glutathione reductase and deletion of this gene increases the susceptibility to killing by neutrophils as well as to intracellular superoxide stress and causes virulence defects (Miramon *et al.*, 2012, Chaves *et al.*, 2007).

Only a single catalase gene (designated as *CAT1*, *CTA1* or *CTT1*) can be found in the genome of *C. albicans*. This catalase is required for resistance to H_2O_2, as well as for protection against damage by human neutrophils. Furthermore, deletion of *CAT1* causes defects in virulence in a murine infection model (Nakagawa *et al.*, 2003, Wysong *et al.*, 1998).

3.4 Antifungal drugs

Treatment of fungal infections is usually difficult due to constantly arising antifungal resistance as well as high mortality rates and often lead to high costs for the health care system (Wilson *et al.*, 2002). Therefore, the discovery of new drug targets and the development of novel antifungal drugs is absolutely required to ensure effective treatment of fungal infections in the future. Currently available antifungal drugs target mainly the plasma membrane or cell wall biogenesis (Fig. 3-2).

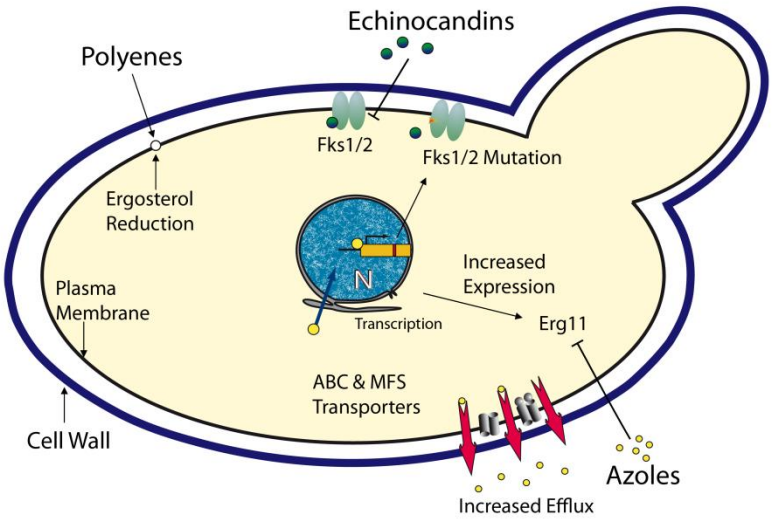

Figure 3-2: Cellular targets of antifungal drugs and resistance mechanisms in *C. albicans*. The targets of azoles, echinocandins and polyenes, as well as mechanisms increasing drug resistance of *C. albicans* are depicted in the cartoon. Modified from Tscherner *et al.*, 2011. N=nucleus;

Azoles are among the most widely used drugs for the treatment of different fungal infections. Their cellular target is the lanosterol 14-α-demethylase, which is encoded by the *ERG11* gene (Vanden Bossche *et al.*, 1995). Inhibition of Erg11 blocks ergosterol biosynthesis, an essential fungal membrane component, and leads to accumulation of a toxic sterol intermediate (Lupetti *et al.*, 2002). Increased efflux of azoles via plasma membrane transporters represents the main resistance mechanism of *C. albicans* against this class of antifungals. Upregulation of two classes of multidrug transporters, the ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS) transporters, can lead to increased azole resistance. The main ABC transporters involved in azole resistance in *C. albicans* are encoded by the *CDR1* and *CDR2* genes (Sanglard *et al.*, 1997, White, 1997, Prasad *et al.*, 1995, Sanglard *et al.*, 1995). Furthermore, the MFS transporter Mdr1 also contributes to azole resistance (Franz *et al.*, 1998, White, 1997). In addition to transporter-mediated efflux, mutation, overexpression or increase in gene dosage of the azole target Erg11 can also lead to reduced azole susceptibility in *C. albicans* (Selmecki *et al.*, 2006, Perea *et al.*, 2001, Franz *et al.*, 1998, Lopez-Ribot *et al.*, 1998). Furthermore, loss of function of Erg3, the sterol $\Delta^{5,6}$-desaturase, causes reduced azole susceptibility because the accumulation of toxic sterol intermediates upon inhibition Erg11 is blocked (Kelly *et al.*, 1997).

Echinocandin antifungals target the 1,3-β-D-glucan synthase, the enzyme responsible for the synthesis of 1,3-β-D-glucan, which is an essential component of the fungal cell wall (Douglas, 2001). Currently approved echinocandin drugs (caspofungin, micafungin and anidulafungin) are non-

competitive inhibitors of Fks1, the catalytical subunit of the 1,3-β-D-glucan synthase (Douglas *et al.*, 1997). Fks1 inhibition disrupts the integrity and structural organization of the cell wall leading to cell death (Perlin, 2007). As with the azoles, mutation of the echinocandin target has been reported to cause reduced susceptibility to this family of antifungals (Balashov *et al.*, 2006, Kurtz *et al.*, 1996). Notably, ectopic overexpression of the ABC transporter Cdr2 can also decrease caspofungin susceptibility implying that efflux-mediated tolerance to echinocandins exists in *C. albicans* (Schuetzer-Muehlbauer *et al.*, 2003).

Polyenes are antifungals with fungicidal activity and have been used for decades to treat different fungal infections. They exert their function by intercalating into ergosterol-containing membranes (mainly the plasma membrane) leading to the formation of pores which results in the leakage of cellular components and ultimately cell death. Unfortunately, severe side effects to the host (i.e. nephrotoxicity) make a long term usage of this class of antifungal difficult (Ellis, 2002). Although resistance to this class of antifungals is not a major problem, reduced susceptibility to amphotericin B, the most prominent polyene, has been reported in clinical isolates of different *Candida spp.* including *C. albicans* (Forastiero *et al.*, 2013, Sterling & Merz, 1998). A reduction of the ergosterol content in the plasma membrane seems to correlate with a decrease in the susceptibility to amphotericin B (Sharma *et al.*, 2014, Nolte *et al.*, 1997).

3.5 Chromatin assembly and functions

In all eukaryotes, the genetic information is stored in a nucleoprotein complex known as chromatin. The repeating unit of chromatin is the nucleosome, which is composed of 146 base pairs of DNA wrapped around an octamer of the four core histones H2A, H2B, H3 and H4 (Luger *et al.*, 1997). Post-translational chemical modifications of histones at their N-terminal tail or at the globular core can alter the biological activity of nucleosomes. A wide variety of histone modifications are known including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation and ADP ribosylation (Kouzarides, 2007). Furthermore, each core histone has multiple sites that can be modified. This creates a huge number of possible combinations for every histone within a nucleosome and these combinations can lead to distinct biological outcomes (Kurdistani *et al.*, 2004). However, the majority of histones are not modified at every site. For example, the N-terminal tail of histone H4 can be acetylated at four lysine residues (K5, K8, K12 and K16), but in *S. cerevisiae* only 12% of total H4 is acetylated at all four sites. In fact, the majority is acetylated only at one lysine residue (Smith *et al.*, 2003). Acetylation of the ε-amino group of lysine residues is one of the best-studied histone modifications. The enzymes catalysing this reaction are referred to as histone acetyltransferases (HATs) (Brownell & Allis, 1996). Like most histone modifications, acetylation of

lysines can be highly dynamic and it is removed by histone deacetylases (HDACs). Furthermore, acetylation neutralizes the positive charge of lysine and can therefore modify histone-DNA interactions (Shahbazian & Grunstein, 2007, Tse *et al.*, 1998). A dense chromatin structure has a negative impact on the rate of transcription. Therefore, loosening the histone-DNA interaction can yield in transcriptional activation. Thus, acetylation of histones within chromatin is traditionally considered as an activating mark, whereas deacetylation is rather known as repressing modification (Tse *et al.*, 1998).

3.5.1 Type A histone acetyltransferases

In addition to acetylation of histones within chromatin, free histones can also be acetylated which is often connected to their incorporation into nucleosomes. One of the best studied modifications present on free histones is acetylation of histone H3 lysine 56 (H3K56ac) by the fungal-specific Rtt109 HAT (Recht *et al.*, 2006). This lysine residue is located in the globular core of H3 and newly synthesized histone H3 carries this modification. Recently, H3K56ac was also found in higher eukaryotes and the modification is catalysed by CBP/p300 (Das *et al.*, 2009). Acetylation of this lysine residue facilitates incorporation of histone H3 into chromatin. Therefore, Rtt109 can act as a repressor by increasing histone density and chromatin compaction (Klopf *et al.*, 2009). Thus, acetylation of free histones is often not correlated with gene activation, but rather with repression. Recently, another HAT acetylating free histone H4 at lysine residues 79 and 91, which are also located within the globular core, was discovered. It is called *HAT4* and can also facilitate H4 incorporation into chromatin, which is required to maintain genome stability (Yang *et al.*, 2011).

3.5.2 The NuB4 complex

Another acetylation mark present on newly synthesized histones is acetylation of histone H4 at lysine 5 (H4K5ac) and 12 (H4K12ac) (Sobel *et al.*, 1995). These modifications are carried out by the Hat1 histone acetyltransferase. This enzyme is conserved throughout the eukaryotic kingdom and functions in complex with a subunit called Hat2 in *S. cerevisiae* and RbAp46/RbAp48 in higher eukaryotes, respectively (Parthun *et al.*, 1996). Newly synthesized histones are acetylated by Hat1 immediately after their synthesis in the cytoplasm (Fig. 3-3). Therefore, Hat1 is considered a classic type B HAT, since it shows partial cytoplasmic localization and processes free non-nucleosomal histones (Poveda & Sendra, 2008, Barman *et al.*, 2006). In contrast, type A HATs show strict nuclear localization and acetylate nucleosomal histones. Upon acetylation in the cytoplasm the Hat1/Hat2 complex stays associated with histone H4/H3 in the nucleus (Poveda *et al.*, 2004). However, it is not known if Hat1 is translocated into the nucleus together with the histones. Interestingly, H4K5ac and

H4K12ac can influence the import process (Alvarez *et al.*, 2011, Ejlassi-Lassallette *et al.*, 2010, Glowczewski *et al.*, 2004). In *S. cerevisiae*, the histone chaperone Hif1 (Hat1 interacting factor 1) binds to Hat1/Hat2 in the nucleus to form the so-called NuB4 (nuclear type B histone acetyltransferase specific for H4) complex (Parthun, 2007, Poveda *et al.*, 2004). Although Hif1 is less conserved than Hat1 and Hat2, putative orthologues in different species have been identified (Dunleavy *et al.*, 2007).

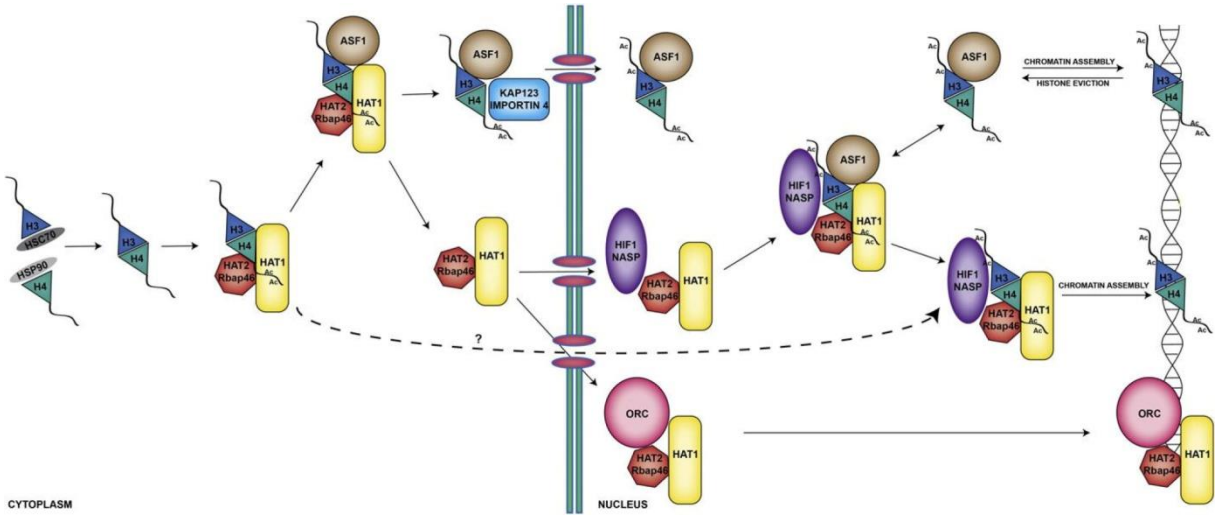

Figure 3-3: Processing of newly synthesized histones by Hat1. The Hat1/Hat2 complex binds and acetylates newly synthesized histone H4 in the cytoplasm. Nuclear import of histone H3/H4 dimers occurs at least partially via the histone chaperone Asf1 and import factors. Weather the Hat1/Hat2 complex stays associated with histones during import or it is imported separately is not known. In the nucleus Hat1 is involved in the incorporation of histones via distinct pathways. It facilitates histone deposition at sites of DNA damage together with the histone chaperones Hif1 and Asf1. Furthermore, Hat1 and Hat2 also interact with the origin recognition complex. Taken from Parthun, 2012.

The NuB4 complex is thought to be involved in histone deposition at heterochromatic regions as well as at sites of DNA damage. In *S. cerevisiae*, Hat1 localizes to double strand breaks and facilitates histone incorporation in the final repair steps (Ge *et al.*, 2011, Qin & Parthun, 2006). Furthermore, deletion of Hat1 in *S. cerevisiae* leads to heterochromatic silencing defects (Mersfelder & Parthun, 2008). Importantly, these defects as well as DNA damage sensitivity are only observed in combination with mutations in the N-terminal tail of histone H3. This redundancy seems to be specific for *S. cerevisiae*, since deletion of Hat1 *in Schizosaccharomyces pombe* or in chicken DT40 cells does not require histone H3 mutations to yield sensitivities to DNA-damaging agents (Benson *et al.*, 2007, Barman *et al.*, 2006).

Interestingly, *S. cerevisiae* Hat1 was also found to be associated with the origin recognition complex (ORC). Deletion of *HAT1* or *HAT2* produces synthetic growth defects when combined with mutated alleles of *ORC2* and *ORC5*. Furthermore, Hat1 localizes to origins of replication specifically in S-phase implying a role for this type B HAT in DNA replication (Suter *et al.*, 2007). Hat1 stays

associated with histone H4 upon acetylation of K5 and K12, which raises the possibility of additional functions for this HAT than just histone acetylation. In fact, the NuB4 complex could also function as a histone chaperone and contribute to the shielding of highly concentrated positive charges of histones to avoid inappropriate interactions. In agreement with this hypothesis, *S. cerevisiae* Hat1 is indeed an acidic protein with an isoelectric point of 5.1 and it was found in various complexes containing different histone chaperones such as Asf1, CAF-1 or HIR (Yang *et al.*, 2013, Fillingham *et al.*, 2008, Ahmad *et al.*, 2001). Furthermore, the importance of H4K5ac and H4K12ac in the incorporation of histones into chromatin varies among different organisms and studies. Mutation of H4K5 and K12 to arginine mimicking the unacetylated state had no effect on histone deposition onto a 2 micron plasmid or in an *in vitro* nucleosome assembly assay (Ma *et al.*, 1998). In contrast, histone turnover is impaired in *S. cerevisiae* upon mutation of histone H4K5 and K12 (Verzijlbergen *et al.*, 2011). In addition, H4K5ac and H4K12ac can promote histone deposition in *Physarum polycephalum* (Ejlassi-Lassallette *et al.*, 2010). Therefore, whether H4K5 and H4K12 acetylation is directly required for histone incorporation or if the main function of NuB4 rather resembles a histone chaperone still remains to be determined.

3.5.3 Chromatin assembly

Formation of nucleosomes requires a process called chromatin assembly. During this process newly synthesized histones or histones disassembled from intact chromatin are deposited onto DNA to form nucleosomes. This is an essential step in various biological processes, including regulation of transcription, replication and DNA damage repair (Chen *et al.*, 2008, Franco *et al.*, 2005, Tsukuda *et al.*, 2005, Schwabish & Struhl, 2004, Kaplan *et al.*, 2003, Saunders *et al.*, 2003). The incorporation of histones into chromatin is facilitated by several histone chaperones and ATP-dependent chromatin remodellers (Eberharter & Becker, 2004, Loyola & Almouzni, 2004). A list of histone chaperones with associated functions is shown in Table 3-1.

Table 3-1: Summary of histone chaperones. Histone H3/H4 chaperones and the processes in which they are involved are listed. Modified from Das *et al*., 2010.

Histone chaperone	Interaction partners	Functions	References
Asf1	Hat1, HIRA, CAF-1, RFC, MCMs	Transcriptional regulation, Replication, Repair, Transcriptional silencing, Promotes histone acetylation	(Campos *et al.*, 2010, Barman *et al.*, 2008, Groth *et al.*, 2007, Mousson *et al.*, 2007, Tsubota *et al.*, 2007, Recht *et al.*, 2006, Schneider *et al.*, 2006, Tyler *et al.*, 2001)
CAF-1	Hat1, Rtt106, Asf1, HP1, PCNA	Replication, Repair, Transcriptional silencing	(Zhang *et al.*, 2012, Barman *et al.*, 2008, Huang *et al.*, 2005, Tyler *et al.*, 2001, Murzina *et al.*, 1999, Shibahara & Stillman, 1999, Enomoto *et al.*, 1997, Kaufman *et al.*, 1997, Gaillard *et al.*, 1996, Smith & Stillman, 1989)
HIR/HIRA	Hat1, Asf1, Swi/Snf	DNA synthesis independent nucleosome assembly, Transcriptional repression, Transcriptional silencing	(Yang *et al.*, 2013, Fillingham *et al.*, 2009, Blackwell *et al.*, 2004, Ray-Gallet *et al.*, 2002, Sharp *et al.*, 2001, Dimova *et al.*, 1999, Kaufman *et al.*, 1998, Spector *et al.*, 1997, Sherwood & Osley, 1991)
Rtt106	CAF-1	Replication, Transcriptional silencing, Transcription repression	(Fillingham *et al.*, 2009, Imbeault *et al.*, 2008, Li *et al.*, 2008, Huang *et al.*, 2007, Huang *et al.*, 2005)
FACT	Spt6, RPA, MCMs	Replication, Repair, Transcription, Recombination	(Reinberg & Sims, 2006, Lindstrom *et al.*, 2003)
Spt6	FACT, RNAPII	Transcription elongation, Repression of intragenic transcription	(Hierlmeier *et al.*, 2013, Mayer *et al.*, 2012, Kaplan *et al.*, 2003, Lindstrom *et al.*, 2003, Hartzog *et al.*, 1998)
Vps75	Rtt109	Transcriptional regulation, Repair, Telomere length maintenance, Promotes histone acetylation	(Selth *et al.*, 2009, Jessulat *et al.*, 2008, Han *et al.*, 2007, Selth & Svejstrup, 2007, Tsubota *et al.*, 2007)

3.5.4 Replication-coupled chromatin assembly

During S phase the entire genome of a cell is duplicated and this process requires extensive disassembly of chromatin and subsequent incorporation of recycled, as well as newly synthesized histones onto nascent DNA. It is believed that histones are randomly distributed to the two daughter strands for the majority of the genome. This would lead to an equal distribution of post-transcriptional modifications on the replicated DNA and therefore re-establishment of the pre-existing chromatin structure. The remaining histones have to be newly synthesized during S phase (Avvakumov *et al.*, 2011). The coupling of DNA synthesis with chromatin assembly is known as replication-coupled nucleosome assembly. Several histone chaperones as well as histone-modifying enzymes are involved in this process (Fig. 3-4).

During DNA replication the helicase complex unwinds double-stranded DNA to facilitate DNA polymerase passage. This might lead to disruption of nucleosomes and release of histones. The FACT histone chaperone complex is recruited to the helicase and binds evicted H2A-H2B dimers (Gambus *et al.*, 2006). Subsequently, the Asf1 histone chaperone takes over H3-H4 dimers (Groth *et al.*, 2007). The presence of these histone chaperones is essential for helicase activity (Groth *et al.*, 2007, Tan *et al.*, 2006). Furthermore, Asf1 is also involved in the processing of newly synthesized histones, which are acetylated at various lysine residues prior to incorporation into chromatin. This histone chaperone facilitates acetylation of H3K56 by Rtt109 (Recht *et al.*, 2006). Another histone chaperone, Vps75, can enhance the activity of Rtt109 towards H3K9, H3K23 and H3K27 (Burgess *et al.*, 2010, Berndsen *et al.*, 2008, Fillingham *et al.*, 2008). In addition, Asf1 interacts with the NuB4 complex in yeast, which is responsible for the acetylation of newly synthesized histone H4 at lysine 5 and 12 (Parthun *et al.*, 1996, Kleff *et al.*, 1995).

Following acetylation, newly synthesized as well as recycled histones are transferred to the histone chaperones CAF-1, which consists of three subunits (Cac1, Cac2 and Cac3 in yeast and p48, p60 and p150 in higher eukaryotes) and Rtt106. Interestingly, H3K56ac enhances the affinity of CAF-1 and Rtt106 for histone H3 thereby promoting chromatin assembly (Li *et al.*, 2008). Finally, both histone chaperones are able to deposit H3-H4 dimers onto newly replicated DNA (Li *et al.*, 2008, Shibahara & Stillman, 1999).

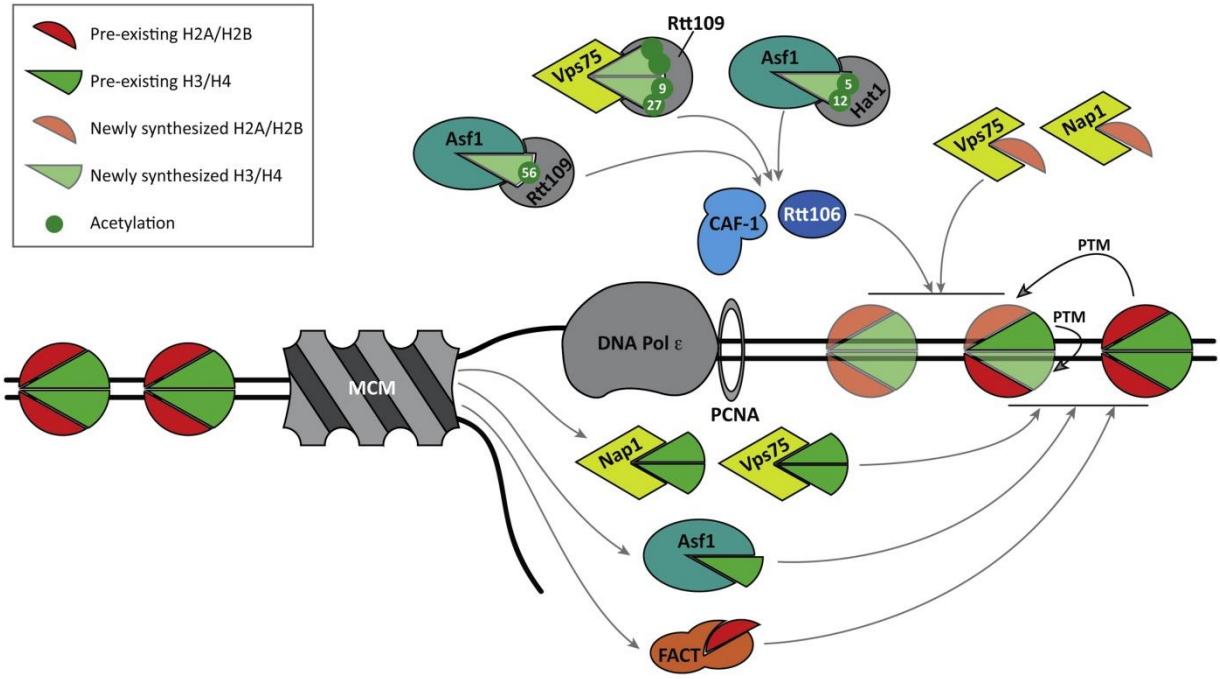

Figure 3-4: Histone turnover during replication. Histones evicted upon passage of the MCM helicase are bound by histone chaperones and redeposited behind the replication fork. The same histone chaperones are also responsible for deposition of newly synthesized histones acetylated by type A histone acetyltransferases. Posttranslational modifications (PTM) are restored upon chromatin reassembly. Numbers correspond to lysine residues modified by the corresponding HATs. Taken from Avvakumov *et al*., 2011.

3.5.5 Transcription and chromatin assembly

At the onset of transcription of any gene, the transcription machinery has to assemble along the promoter region. Subsequently, the whole apparatus moves across the coding sequence to produce the corresponding mRNA. Chromatin can represent a significant steric challenge to the transcription machinery by blocking initiation as well as elongation thereby modifying transcription kinetics. The combined action of histone-modifying enzymes, histone chaperones and ATP-dependent chromatin remodellers leads to alteration or removal of key nucleosomes at the promoter and facilitates initiation of transcription (Li *et al.*, 2007). For example, Asf1 is required for proper induction of the *PHO5* and *PHO8* genes in yeast by facilitating the eviction of histones at promoter regions (Korber *et al.*, 2006, Adkins *et al.*, 2004). In fact, it was also found that this histone chaperone is recruited to promoters of active genes and influences histone H3 exchange (Rufiange *et al.*, 2007, Schwabish & Struhl, 2006). Therefore, Asf1 is considered to function in replication-coupled, as well as in replication-independent nucleosome assembly. Interestingly, Asf1-mediated histone H3K56 acetylation by Rtt109 is also required for proper disassembly at the *PHO5* promoter (Williams *et al.*, 2008).

The yeast HIR histone chaperone complex, which contains the subunits Hir1, Hir2, Hir3 and Hpc2, is also involved in the regulation of transcription initiation (Prochasson *et al.*, 2005, Spector *et al.*, 1997). The orthologue of yeast Hir1 and Hir2 in higher eukaryotes is called HIRA (Wilming *et al.*, 1997, Lamour *et al.*, 1995). This histone chaperone is required for efficient repression of histone genes outside of S phase and renders nucleosomes resistant to remodelling by the SWI/SNF complex (Prochasson *et al.*, 2005, Spector *et al.*, 1997). Furthermore, it is able to enhance recruitment of the RSC remodelling complex, which leads to transcriptional repression (Ng *et al.*, 2002). Interestingly, it was shown that depletion of Hir1 as well as Asf1 delays reassembly of histones at promoters of different genes (Schermer *et al.*, 2005). In addition, lack of the histone chaperone Spt6 abolishes reassembly and leads to defects in transcriptional repression (Adkins & Tyler, 2006). Interestingly, the Rtt106 histone chaperone is recruited by both Asf1 and the HIR complex to the *HTA1* and *HTB1* histone gene promoters and facilitates transcriptional repression (Fillingham *et al.*, 2009). These data indicate that different histone chaperones together with associated histone modifications act at promoter regions to influence transcription initiation or rapid repression in response to specific stimuli.

The majority of these histone chaperones is also involved in chromatin remodelling within coding regions during transcription. For example, Spt6 together with the FACT complex facilitates disassembly of histones in front of RNA polymerase II as well as reassembly behind the polymerase (Li *et al.*, 2007). Furthermore, Asf1-mediated H3K56 acetylation facilitates incorporation of histones at coding regions of transiently induced genes (Klopf *et al.*, 2009). In addition, deletion of HIR subunits in yeast leads to synthetic lethality with different components of the elongation machinery (Nourani *et al.*, 2006, Formosa *et al.*, 2002). The HIRA complex in higher eukaryotes can incorporate histones at coding regions (Henikoff & Ahmad, 2005). Thus, various histone chaperones and associated modifications are required to promote chromatin remodelling at promoter and coding regions and facilitate proper regulation of transcription (Fig. 3-5).

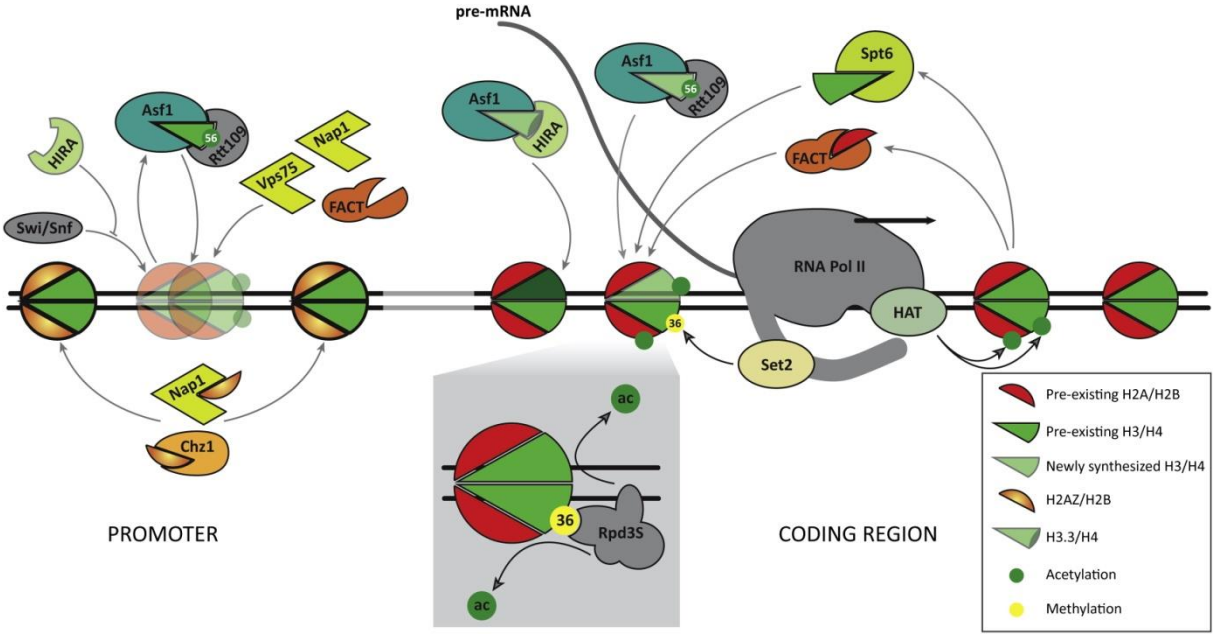

Figure 3-5: Chromatin remodelling during transcription. Histones evicted from the promoter upon transcriptional induction are bound by Asf1 and acetylated by Rtt109. HIRA is able to interfere with binding of the Swi/Snf chromatin remodeller thereby repressing transcription. In addition, the precise role of other histone chaperones in the remodelling of promoter chromatin is currently unknown. Within coding regions histones evicted in front of the RNA polymerase are bound and redeposited by Spt6 and FACT. Eviction is facilitated by acetylation via distinct HATs. Furthermore, Asf1 together with Rtt109 or HIRA is involved in the incorporation of newly synthesized histones in the wake of the passing polymerase. Reassembled histone H3 is methylated by the Set2 methyltransferase. This modification is bound by the Rpd3S HDAC complex, which removes acetylation marks and creates a repressive chromatin structure to avoid inappropriate transcription initiation. Numbers indicate modified histone residues. Taken from Avvakumov *et al*., 2011.

3.5.6 Chromatin remodelling during DNA damage repair

For the repair of different types of DNA damages cells use a strategy referred to as "Access-Repair-Restore" (Fig. 3-6) (Smerdon, 1991). In the first steps after damage detection, chromatin is disassembled to permit the repair machinery access to lesions. Furthermore, cell cycle arrest is triggered by the DNA damage checkpoint to provide sufficient time for the actual repair of the DNA damage. Finally, the pre-existing chromatin structure has to be restored to release repair proteins and deactivate the DNA damage checkpoint (Chen *et al.*, 2008). During the repair of double-strand breaks (DSBs), histone H2A (H2AX in higher eukaryotes) is phosphorylated in regions flanking the site of damage. After repair of the DNA lesion the yeast Swr1 chromatin remodeller and the FACT histone chaperone complex are able to remove phosphorylated H2A (Rossetto *et al.*, 2010, Heo *et al.*, 2008). Furthermore, the histone chaperone Asf1, while being dispensable for the actual repair process, is absolutely required for subsequent chromatin reassembly at DSBs. Deletion of yeast *ASF1* triggers persistent activation of the DNA damage checkpoint, even though the lesion is successfully repaired (Chen *et al.*, 2008). Asf1 is known to trigger histone H3K56 acetylation by the histone

acetyltransferase Rtt109 (Recht *et al.*, 2006). Interestingly, mutation of H3K56 mimicking the acetylated state can rescue the *asf1*Δ phenotype indicating that Asf1-mediated K3K56 acetylation by Rtt109 is required for chromatin assembly during DNA damage repair (Chen *et al.*, 2008). Furthermore, these data show that restoration of the pre-existing chromatin structure is necessary for completion of the repair process and re-entering the cell cycle. Interestingly, the histone chaperone complex CAF-1 is also involved in the repair of UV-induced DNA lesions indicating that chromatin assembly is essential for the repair of different types of DNA damage.

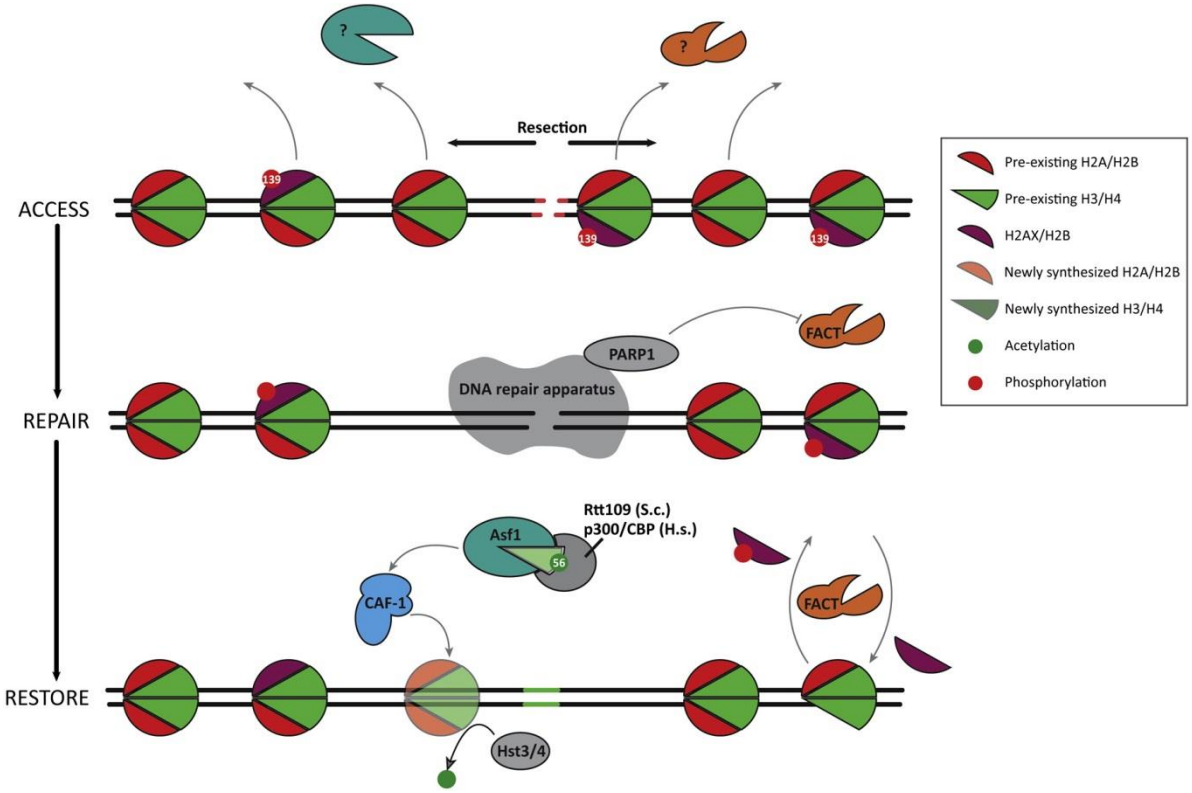

Figure 3-6: Chromatin remodelling during the repair of DNA double strand breaks. Histone H2AX (histone H2A in *S. cerevisiae*) is phosphorylated in the surrounding of a DNA lesion. During subsequent DNA resection histones are evicted from chromatin and most likely bound by hitherto unknown histone chaperones. During the repair process PARP1 is responsible for poly-ADP-ribosylation and thereby inactivation of FACT. After repair of the lesion the histone chaperones CAF-1 and Asf1 together with Rtt109 are responsible for incorporation of newly synthesized histones to restore the chromatin structure. In addition, FACT replaces phosphorylated with unmodified H2AX to avoid further binding of repair factors. Numbers indicate modified histone residues. Taken from Avvakumov *et al.*, 2011.

3.5.7 *Candida albicans* HATs and histone chaperones

In *C. albicans*, Rtt109 is the only type A HAT investigated so far. As its *S. cerevisiae* orthologue it is the only enzyme responsible for acetylation of histone H3K56. Furthermore, the absence of Rtt109 in *C. albicans* leads to spontaneous accumulation of DNA damage and slow growth (Lopes da Rosa *et al.*, 2010). Importantly, DNA damage is known to be a trigger for cell elongation in *C. albicans*

and the *rtt109*Δ/Δ mutant therefore also shows a constitutive filamentation phenotype (Lopes da Rosa *et al.*, 2010, Shi *et al.*, 2007). Genotoxic stress resistance is essential for *C. albicans*, since reactive oxygen species (ROS) produced by innate immune cells to kill the pathogen can induce DNA damage. Indeed, *rtt109*Δ/Δ cells are more susceptible to hydrogen peroxide and to macrophage-generated ROS. Furthermore, cells lacking Rtt109 are efficiently cleared from infected mice (Lopes da Rosa *et al.*, 2010). In addition, Rtt109 is also involved in the regulation of white-opaque switching (Stevenson & Liu, 2011). Interestingly, deletion of *CAC2* and *HIR1* simultaneously mimics lack of Rtt109 and leads to defects in maintaining the opaque cell type (Stevenson & Liu, 2013). H3K56ac is known to be removed upon incorporation of histone H3 into chromatin. The enzyme responsible for deacetylation of H3K56 in *C. albicans* is Hst3. It is an essential gene and encodes a NAD^+-dependent sirtuin HDAC. Strikingly, deletion of *RTT109* restores the viability of *hst3*Δ/Δ cells indicating that accumulation of H3K56ac is indeed lethal to the cells. Furthermore, the NAD^+-dependent HDAC inhibitor nicotinamide is able to inhibit growth of *C. albicans* as well as other pathogenic fungi (Wurtele *et al.*, 2010). These data indicate that chromatin assembly associated histone acetylation is essential for *C. albicans* stress resistance and virulence.

3.6 Innate immune system and oxidative burst

The innate immune system represents the first defence line against invading pathogens. This defence consists of different cell types including neutrophils, macrophages and dendritic cells carrying pattern recognition receptors (PRRs), which are able to recognise pathogens via so-called pathogen-associated molecular patterns (PAMPs) (Netea *et al.*, 2008, Romani, 2004). The response of the innate immune system to invading pathogens includes phagocytosis as well as secretion of microbicidal compounds to kill the pathogen. Furthermore, innate immune cells also produce cytokines to modulate the subsequent adaptive immune response (Netea *et al.*, 2008, Hoebe *et al.*, 2004).

Phagocytic cells of the innate immune system such as neutrophils and macrophages are able to kill fungal pathogens. One of the most important mechanisms to do this is the oxidative burst. After recognition by a phagocytic cell the pathogen is engulfed, which leads to its encapsulation by a membrane envelope and finally the formation of the so-called phagosome. Phagocytosis triggers the production of ROS to kill the pathogen (Babior, 2002). The enzyme responsible for the production of ROS is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. It is a membrane-bound multicomponent complex. After its assembly during phagocytosis it generates superoxide anions (O_2^-), which can be converted to hydrogen peroxide (H_2O_2) by SODs (Schrenzel *et al.*, 1998). Hydroxyl radicals (HO°) can arise upon interaction of H_2O_2 with O_2^-. Furthermore, hypochlorous acid (HOCl) is

produced in the presence of Cl^- by an enzyme called myeloperoxidase (MPO), which is present in neutrophils but not in macrophages (El-Benna *et al.*, 2005). This system is required for an efficient host defence against fungal pathogens, since patients suffering from chronic granulomatous disease (CGD), which is a defect in one of the subunits of the NAPDH oxidase, are more susceptible to bacterial and fungal infections (Warris *et al.*, 2003, Aratani *et al.*, 2002, Johnston, 2001, Hohn & Lehrer, 1975).

4 Aims of this Work

The most frequent human fungal pathogen *C. albicans* encounters various stress conditions during the infection process. To survive these conditions, the pathogen has to be able to efficiently repair damaged cellular components such as genomic DNA, and it must rapidly adjust its transcriptional program to the changing environment. Modification of the chromatin structure by disassembly and reassembly of nucleosomes is required for both repair of DNA damage in the context of chromatin and transcription regulation. The histone H4 specific acetyltransferase Hat1, which is the catalytic subunit of the NuB4 complex in *S. cerevisiae*, is involved in the deposition of histones at sites of DNA damage in various species.

Thus, we hypothesised that the NuB4 complex might be required for efficient DNA damage repair, oxidative stress resistance and virulence in *C. albicans*.

In the first part of this work, the role of *C. albicans* Hat1 in the repair of endogenous, as well as exogenous DNA damage should be investigated. Furthermore, conservation of the NuB4 complex should be determined by construction and characterisation of deletion mutants of candidate NuB4 component homologues. Another aim was to identify binding partners and to characterize the enzymatic activity of Hat1 by constructing strains carrying tagged alleles for subsequent immunoprecipitation. Finally, sensitivities of the mutants to oxidative stress resistance and antifungal drug tolerance should be determined.

In the second part of this work, the oxidative stress and azole resistance phenotypes of *hat1*Δ/Δ cells should be investigated in detail. Deletion strains of different histone chaperones should be constructed to specify histone deposition pathways involved in the observed resistance phenotypes. Furthermore, another aim was to elucidate the molecular mechanism causing H_2O_2 resistance in *hat1*Δ/Δ cells. Therefore, the effect of *HAT1* deletion should be investigated on a genome-wide scale using RNA-seq. Finally, the consequences of Hat1 inactivation on virulence should also be examined using a mouse model of systemic candidiasis.

5 Results

5.1 Publication: The histone acetyltransferase Hat1 facilitates DNA damage repair and morphogenesis in Candida albicans

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The histone acetyltransferase Hat1 facilitates DNA damage repair and morphogenesis in *Candida albicans*

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Summary

Chromatin assembly and remodelling is an important process during the repair of DNA damage in eukaryotic cells. Although newly synthesized histone H4 is acetylated prior to nuclear import and incorporation into chromatin during DNA damage repair, the precise role of acetylation in this process is poorly understood. Here, we identify the histone acetyltransferase 1 (Hat1) catalysing the conserved acetylation pattern of histone H4 preceding its chromatin deposition in the fungal pathogen *Candida albicans*. Surprisingly, Hat1 is required for efficient repair of not just exogenous but also endogenous DNA damage. Cells lacking Hat1 rapidly accumulate DNA damages and switch from yeast-like to pseudohyphal growth. In addition, reduction of histone H4 mimics lack of Hat1, suggesting that inefficient H4 supply for deposition into chromatin is the key functional consequence of Hat1 deficiency. Thus, remarkably, we demonstrate that *C. albicans* is the first organism known to require histone H4 processing for endogenous DNA damage repair and morphogenesis. Strikingly, we also discover that *hat1*Δ/Δ cells are hypersusceptible to caspofungin due to intracellular reactive oxygen species induced by this drug. Hence, we propose that targeting this class of histone acetyltransferases in fungal pathogens may have potential in antifungal therapy.

Introduction

The nucleosome, which is composed of 146 base pairs of DNA wrapped around an octamer of the four core histones H2A, H2B, H3 and H4, is the basic unit of eukaryotic chromatin (Luger *et al.*, 1997). Assembly of histones into nucleosomes is an essential step in many biological processes, including regulation of transcription, replication and repair of DNA damage (Kaplan *et al.*, 2003; Saunders *et al.*, 2003; Schwabish and Struhl, 2004; Franco *et al.*, 2005; Tsukuda *et al.*, 2005; Chen *et al.*, 2008). Newly synthesized histones are transiently acetylated immediately after their synthesis in the cytoplasm, which is essential for their subsequent incorporation into chromatin (Ruiz-Carrillo *et al.*, 1975; Sobel *et al.*, 1995; Chen *et al.*, 2008; Yang *et al.*, 2011). During chromatin maturation the acetylation marks are removed (Annunziato and Seale, 1983). Acetylation of histones requires two types of histone acetyltransferases (HAT), referred to as type A and type B (Brownell and Allis, 1996). Nuclear type A HATs utilize only nucleosomal histones as substrates, whereas the type B HATs are defined by their specificity for free non-nucleosomal histones and their partial cytoplasmic localization (Brownell and Allis, 1996).

The founding member of type B HATs is the histone acetyltransferase Hat1 (Kleff *et al.*, 1995). In *Saccharomyces cerevisiae*, Hat1 forms a complex with a subunit called Hat2 to bind and acetylate newly synthesized histone H4 at lysine 5 and 12 (H4K5ac and H4K12ac), which is a highly conserved acetylation pattern at the N-terminus of H4 (Sobel *et al.*, 1995). Histone H3 then joins and the entire complex is shuttled into the nucleus (Kleff *et al.*, 1995; Parthun *et al.*, 1996; Ai and Parthun, 2004; Campos *et al.*, 2010). In addition, the type B HAT Rtt109 acetylates histone H3 lysine 56 to yield H3K56ac (Driscoll *et al.*, 2007). In *S. cerevisiae*, the histone chaperone Hif1 recognizes Hat1/Hat2 to form the NuB4 complex (nuclear type B histone acetyltransferase specific for H4) (Ai and Parthun, 2004; Poveda *et al.*, 2004; Parthun, 2007). NuB4 is involved in the incorporation of histone H4-H3 into chromatin at sites of DNA damage, as well as in heterochromatic regions, although the exact mechanism remains elusive (Kelly *et al.*, 2000; Ai and Parthun, 2004; Qin and Parthun, 2006). Interestingly, H3K56ac is required for efficient chromatin reassembly following DNA damage repair (Chen *et al.*, 2008). Acetylation of histone H4 by Hat1 presumably functions in a similar way to facilitate restoration of proper chromatin structure during the final steps of DNA repair (Qin and Parthun, 2002; 2006; Ge *et al.*, 2011). Importantly, newly synthesized histone H4 acetylated by Hat1 represents only a minor fraction of the total cellular

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histone H4 pool (Poveda *et al*., 2004; Barman *et al*., 2008; Poveda and Sendra, 2008). The vast majority of histones are embedded in chromatin.

Candida albicans is an obligatory diploid fungal pathogen able to undergo reversible morphological transitions between three different growth forms known as yeast, pseudohyphae and true hyphae (Gow *et al*., 2002; Saville *et al*., 2003). Notably, the ability to undergo morphogenetic transitions is considered a key virulence factor of *C. albicans*, since a variety of mutants locked in one of these forms showed reduced virulence in different infection models (Lo *et al*., 1997; Braun *et al*., 2000; Hwang *et al*., 2003; Zheng and Wang, 2004; Banerjee *et al*., 2008). In addition, *C. albicans* can switch between the so-called white and opaque form (Slutsky *et al*., 1987), two yeast-like morphologies displaying distinct phenotypes related to host niche colonization (Lachke *et al*., 2003), interaction with immune cells (Lohse and Johnson, 2008), virulence (Kvaal *et al*., 1999), as well as mating that occurs exclusively in opaque cells (for a recent review see Lohse and Johnson, 2009).

C. albicans encounters genotoxic stress during infection due to host-derived reactive oxygen species (ROS) produced by immune cells, which damage DNA (Vazquez-Torres and Balish, 1997; Salmon *et al*., 2004) and kill invading pathogens such as *Candida* spp. (Frohner *et al*., 2009), as well as *Histoplasma capsulatum* (Youseff *et al*., 2012). Notably, a loss of the DNA damage repair machinery favours a constitutively pseudohyphal morphology and reduces virulence in *C. albicans* (Leng *et al*., 2000; Andaluz *et al*., 2006; Legrand *et al*., 2007; Shi *et al*., 2007; Hao *et al*., 2009). Moreover, inhibition of the DNA damage repair machinery in *C. albicans*, as well as treatment with genotoxic drugs promotes white-opaque switching (Bennett and Johnson, 2005; Alby and Bennett, 2009). Interestingly, the *C. albicans* Rtt109 HAT, which generates H3K56ac (Lopes da Rosa *et al*., 2010), is also involved in DNA repair (Wurtele *et al*., 2010), white-opaque switching (Stevenson and Liu, 2011) and pathogenesis (Lopes da Rosa *et al*., 2010).

Hat1 homologues have been implicated in DNA damage repair in different organisms (Qin and Parthun, 2002; Barman *et al*., 2006; Benson *et al*., 2007). Here, we aimed to answer the question if Hat1 and other components of the NuB4 complex affect susceptibility to genotoxic stress in this important human pathogen. We demonstrate an essential role for the *C. albicans* Hat1/Hat2 complex in DNA damage repair and morphogenesis through the acetylation of histone H4. Interestingly, the lack of Hat1 debilitates the repair of both exogenous and endogenous DNA lesions, leads to accumulation of DNA damages, and triggers the switch to pseudohyphal growth. Furthermore, we show that genetic inactivation of the Hat1/Hat2 complex causes hypersensitivity to the widely used antifungal caspofungin (CASP) and triggers white-to-opaque switching. *C. albicans* is to our knowledge the first organism known to require histone H4 processing by a type B HAT for efficient repair of endogenous DNA damage and morphogenesis. Our data also strengthen emerging notions that the specific inhibition of chromatin remodelling by drugs (Simonetti *et al*., 2007; Agbor-Enoh *et al*., 2009; Hnisz *et al*., 2010; Wurtele *et al*., 2010) may prove of therapeutic relevance in infectious diseases, including those caused by fungal pathogens.

Results

Deletion of C. albicans *NuB4 components leads to constitutive pseudohyphal growth*

The *C. albicans* orf19.779 encodes the orthologue of *S. cerevisiae* Hat1. Furthermore, a BLAST search (http://www.ncbi.nlm.nih.gov/blast) identified two additional genes, orf19.2146 and orf19.7185, sharing homology with *ScHAT2*. In addition, orf19.6843 might represent the third component of the NuB4 complex (Dunleavy *et al*., 2007). Thus, we employed the SAT flipper technique (Reuss *et al*., 2004) to generate homozygous deletion strains for all candidate components of the putative NuB4 complex. Interestingly, homozygous *hat1*Δ/Δ deletion cells displayed a slow growth phenotype with constitutively wrinkled colony morphologies, indicating an active filamentation programme even under yeast-promoting conditions (Fig. 1A), when compared with the wild-type control or the heterozygous *HAT1*/*hat1*Δ strain. Liquid cultures of the *hat1*Δ/Δ strain contained a mixture of yeast cells and elongated cells, which formed pseudohyphae with typical constrictions at the septa (Fig. 1B and C). Importantly, reintegration of *HAT1* at its endogenous locus fully restored smooth colony morphology, as well as normal yeast growth. Out of the two putative Hat2 orthologues only deletion of orf19.2146 resulted in altered colony and cell morphology similar to the lack of *HAT1* indicating that this ORF is indeed the orthologue of *Sc*Hat2 (Fig. 1A and B). Thus, we refer to it as *HAT2* in this report. As for orf19.7185, no morphological changes were observed after deleting orf19.6843. Thus, this ORF may not encode the orthologue of *Sc*Hif1. Alternatively, *Ca*Hif1 is not required for the function of Hat1 in maintaining the yeast morphology (data not shown). Interestingly, deletion of both Hat1 and Hat2 mimicked the phenotype of the respective single deletions, suggesting that Hat1 and Hat2 function in a complex to regulate cell morphology in *C. albicans* (Fig. 1A and B). Furthermore, to characterize the pseudohyphae produced in the absence of Hat1, we determined transcription levels of hyphae-specific genes under yeast-promoting conditions. We detected a strong upregulation of the typical

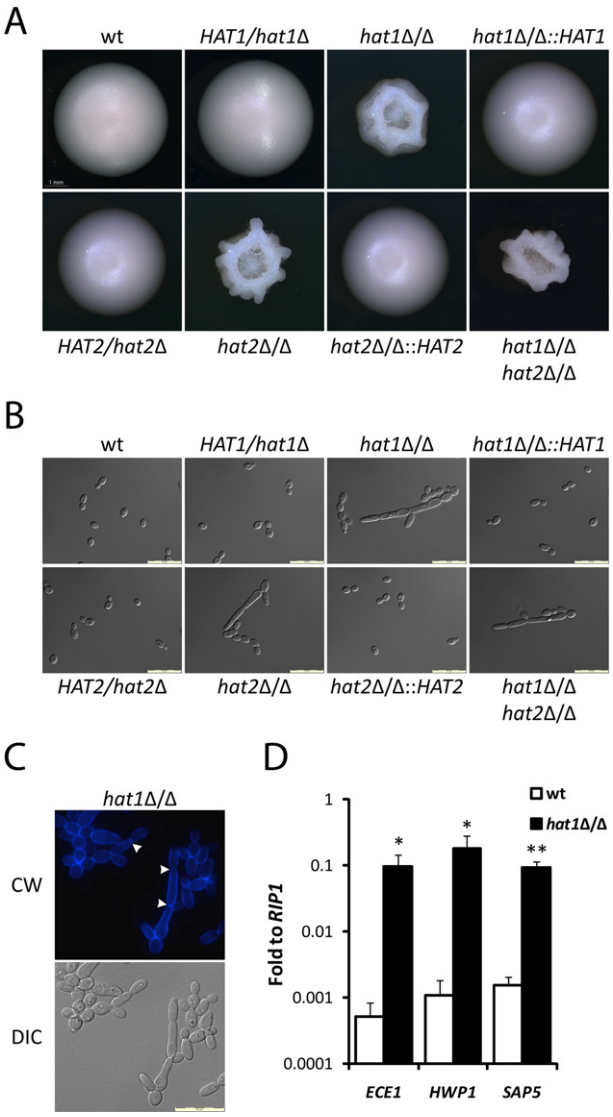


Fig. 1. Loss of *C. albicans HAT1* or *HAT2* induces pseudohyphal growth and hyphae-specific genes.
A. *hat1*Δ/Δ and *hat2*Δ/Δ strains form wrinkled colonies on YPD plates at 30°C indicating constitutive filamentous growth. Images were taken after 3 days of incubation. Scale bar corresponds to 1 mm.
B. Cells lacking Hat1 show elongated cell morphology under yeast-promoting conditions (YPD 30°C). Deletion of *HAT2* or *HAT1* and *HAT2* mirrors lack of *HAT1*. Scale bar corresponds to 20 μm.
C. Elongated cells of the *hat1*Δ/Δ strain form pseudohyphae with constrictions at the cell junctions. Cells were grown as in (B). Cell wall was stained with Calcofluor White (CW) and arrowheads indicate constrictions. Scale bar corresponds to 20 μm.
D. Filamentation of *hat1*Δ/Δ cells is accompanied by induction of hyphae-specific genes. RNA was isolated from four independent cultures of cells grown in YPD at 30°C. Transcript levels of *ECE1*, *HWP1* and *SAP5* were analysed by RT-qPCR and normalized to *RIP1* expression. Data are shown as mean + SD. $*P < 0.05$ and $**P < 0.01$ relative to wild-type cells (Student's *t*-test).

hyphae-induced genes such as *ECE1*, *HWP1* and *SAP5*, demonstrating a fully active hyphal transcriptional programme (Fig. 1D). Thus, *C. albicans* cells lacking the NuB4 components Hat1 and Hat2 are defective in maintaining the yeast morphology and constitutively grow in a pseudohyphal morphology. Furthermore, the constitutive filamentation of *hat1*Δ/Δ cells is accompanied by a hyphae-specific transcription programme.

Lack of HAT1 leads to genotoxin sensitivity and increase in DNA damage followed by filamentation

Hat1 is involved in the repair of DNA damages in different organisms. In *C. albicans*, a defective DNA damage repair machinery can lead to accumulation of DNA damage and pseudohyphal growth (Andaluz *et al*., 2006; Shi *et al*., 2007; Lopes da Rosa *et al*., 2010). Thus, we asked if the morphogenesis defect upon deletion of *HAT1* is due to impaired DNA damage repair. First, we determined the consequence of deleting *HAT1* on the sensitivity to various DNA-damaging agents. Interestingly, *hat1*Δ/Δ and *hat2*Δ/Δ cells showed a pronounced hypersensitivity to methylmethane sulphonate (MMS), ethylmethane sulphonate (EMS) and 4-nitroquinoline *N*-oxide (4-NQO) (Fig. 2A). Determination of cfu after transient MMS treatment confirmed that the lack of *HAT1* and *HAT2* or deletion of both genes had a similar effect on MMS sensitivity (Fig. 2B). Furthermore, the genetic removal of *HAT1* also caused hypersensitivity to UV irradiation (Fig. 2C). Importantly, reintegration of *HAT1* or *HAT2* fully restored the wild-type phenotype, indicating that the sensitivities observed are specifically caused by the absence of the respective proteins. Since these stress conditions cause distinct kinds of DNA lesions, we reasoned that Hat1 and Hat2 play a general role in the repair of different types of DNA damage.

Next, we assessed transcription levels of several orthologues of DNA damage-induced genes and verified DNA damages in the absence of genotoxic stress by determining H2A serine 129 phosphorylation (H2A-S129), a hallmark modification present in the proximity of double-strand breaks (Shroff *et al*., 2004). Interestingly, *hat1*Δ/Δ cells overexpressed several DNA damage-induced marker genes such as *RAD6*, *RAD17* or *MEC1* (Fig. 2D), and showed markedly increased levels of H2A-S129 phosphorylation, indicating the accumulation of DNA damages in the absence of Hat1 (Fig. 2E). Because of the heterogeneous cell morphology phenotype of *hat1*Δ/Δ cells, we investigated the occurrence of DNA damage at a single cell level using fluorescence microscopy. Therefore, we constructed strains expressing fully functional (Fig. S1A) GFP-tagged Rad52 alleles in both wild-type and *hat1*Δ/Δ backgrounds. Rad52 forms foci at DNA lesions repaired by homologous recombination, which can be visualized by fluorescence microscopy in tagged strains (Lisby *et al*.,

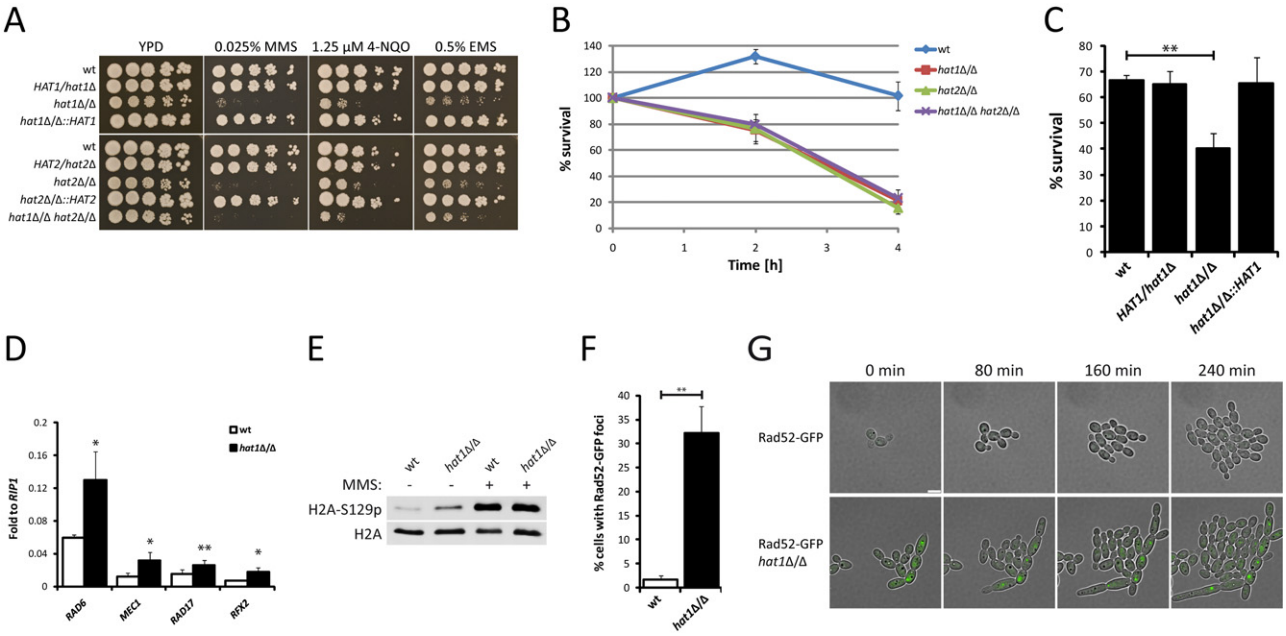


Fig. 2. Lack of Hat1/Hat2 causes genotoxin sensitivity and accumulation of DNA damage followed by filamentation.
A. Lack of Hat1 leads to hypersensitivity to agents causing DNA damage. The removal of *HAT2* or *HAT1* and *HAT2* mimics lack of *HAT1*. To investigate sensitivity to genotoxic drugs, plates contained 0.025% methylmethane sulphonate (MMS), 0.5% ethylmethane sulphonate (EMS) or 1.25 μM 4-nitroquinoline *N*-oxide (4-NQO). Fivefold serial dilutions of indicated strains were spotted on agar plates and incubated at 30°C for 3 days.
B. Exponentially growing cells were cultured in MMS-containing YPD for the indicated times at 30°C. Cultures were diluted and plated onto YPD plates for cfu counting. Colonies were counted after 3 days of incubation at 30°C to determine viability. Data are shown as mean ± SD from three independent experiments.
C. The UV sensitivity of indicated strains was tested by plating on YPD plates and irradiating with 4 mJ. Colonies were counted after 3 days of incubation at 30°C. The survival was calculated relative to un-irradiated plates. Data are shown as mean + SD from three independent experiments.
D. Constitutively activated DNA damage response in *hat1*Δ/Δ cells was detected by measuring expression levels of DNA damage-inducible genes using RT-qPCR. RNA was isolated from three to four independent cultures of cells grown in YPD at 30°C. Transcript levels were normalized to the expression level of *RIP1*. Data are shown as mean + SD.
E. Increased phosphorylation of histone H2A at serine 129 upon loss of Hat1 was detected by immunoblot analysis. Treatment with MMS (0.02% for 2 h) further increased H2A-S129 phosphorylation. Detection of total histone H2A indicates equal loading.
F. Rad52 was C-terminally tagged with GFP in wild-type and *hat1*Δ/Δ backgrounds. Rad52-GFP foci were counted in logarithmically growing cells in SC medium at 30°C. Data are shown as mean + SD from three independent experiments. At least 250 cells were counted in each experiment.
G. Rad52-GFP foci formation is followed by cell elongation in *hat1*Δ/Δ cells. CellASIC Y04C plates were used to perform live cell imaging with cells carrying a *RAD52*-GFP allele in a wild-type (Rad52-GFP) or *hat1*Δ/Δ background (Rad52-GFP *hat1*Δ/Δ). Cells were grown in SC medium at 30°C and pictures were taken at the indicated time points. Scale bar corresponds to 5 μm.
C–F. $*P < 0.05$ and $**P < 0.01$ relative to wild type (Student's *t*-test).

2001). Indeed, loss of *HAT1* sharply increased the number of cells containing Rad52-GFP foci even in the absence of any genotoxic stress (Fig. 2F), confirming the H2A-S129 phosphorylation data (Fig. 2E). Furthermore, we aimed to determine whether accumulating DNA lesions are triggering the constitutive filamentation of *hat1*Δ/Δ cells. Therefore, we subjected Rad52-GFP strains grown in SC medium to live cell imaging. Interestingly, the vast majority of elongating cells (92%, $n = 50$) that developed a pseudohyphal morphology also contained Rad52-GFP foci and thus must carry accumulated DNA damages before cell elongation (Fig. 2G). As expected, wild-type cells neither elongated nor formed Rad52-GFP foci under these conditions (Fig. 2G). These results clearly demonstrate that accumulation of DNA damages in the absence of Hat1 triggers pseudohyphal growth.

C. albicans *Hat1 is required for efficient repair of DNA damage*

The observed accumulation of DNA lesions in the absence of Hat1 could have two different explanations. First, DNA damages occurring during normal growth cannot be repaired efficiently and thus accumulate in the cell. This is consistent with what was shown for different DNA damage repair mutants in *C. albicans* (Shi *et al.*, 2007; Lopes da Rosa *et al.*, 2010). Another possible explanation could be that damages occur more frequently in the absence of

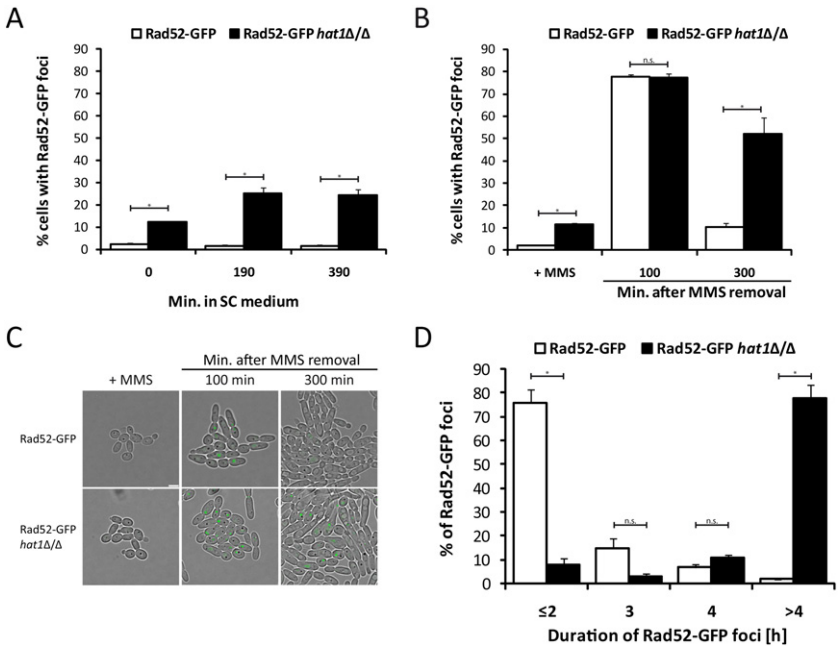


Fig. 3. MMS treatment of *hat1*Δ/Δ cells results in persistent Rad52 foci.
A. The fraction of *hat1*Δ/Δ cells with Rad52-GFP foci remains constant over time. Live cell imaging was performed using cells harbouring a *RAD52*-GFP allele in a wild-type (Rad52-GFP) or *hat1*Δ/Δ background (Rad52-GFP *hat1*Δ/Δ). Cells were grown in SC medium at 30°C. Pictures were taken at the indicated time points and the fraction of cells carrying foci was quantified. At least 50 cells were inspected for each time point. Data are shown as mean + SD from two independent experiments.
B. Removal of Rad52-GFP foci is delayed in *hat1*Δ/Δ cells. Live cell imaging was performed as described in (A). After 2 h in SC medium a pulse MMS treatment (0.02% MMS for 90 min) was done. Afterwards, the medium was changed back to SC lacking MMS and the fraction of cells containing Rad52-GFP foci was determined at the indicated time points (+ MMS: start MMS treatment). At least 50 cells were examined for each time point. Data are shown as mean + SD from two independent experiments.
C. Microscopy of cells from the experiment described in (B).
D. The lifetime of Rad52-GFP foci is prolonged upon deletion of *HAT1*. Live cell imaging was performed as described in (B). After a transient MMS treatment (0.02% MMS for 90 min), cells were grown in medium lacking MMS and the lifetime of Rad52-GFP foci was determined. Fifty cells containing Rad52-GFP foci were examined for each strain. The graph shows the fraction of cells in which Rad52-GFP foci lasted for the indicated period of time. Foci that appeared within 120 min after MMS removal were examined. Data are shown as mean + SD from two independent experiments.
A–D. *$P < 0.05$ relative to the wild-type background (Student's *t*-test).

Hat1 when compared with wild-type cells. Thus, to investigate, if Hat1 is indeed required for the efficient execution of DNA damage repair via homologous recombination, we measured the dynamics of Rad52-GFP foci in cells after providing a pulse exposure to MMS. This approach has been successfully used in *S. cerevisiae* to demonstrate the importance of H3K56ac in the completion of DNA damage repair (Wurtele *et al.*, 2011). Thus, we performed live cell imaging using the CellASIC microfluidic plate system, which allows for quick changes of growth medium at specific time points (Lee *et al.*, 2008). Cells grown in SC medium were treated with 0.02% MMS for 90 min. Importantly, *hat1*Δ/Δ cells do not loose viability in the presence of MMS under these experimental conditions (Fig. S1B). After treatment, the medium was changed back to SC medium lacking MMS, and the fraction of cells containing Rad52-GFP foci, as well as their lifetime was determined. Importantly, without MMS treatment, the fraction of *hat1*Δ/Δ cells containing Rad52-GFP foci never exceeded 25% (Fig. 3A). The reduced number of cells with Rad52-GFP foci at the beginning of the experiment can be explained by the fact that large elongated cells containing Rad52-GFP foci can hardly enter the culture chamber of the microfluidic plate. In contrast, 100 min after MMS removal, some 78% of wild-type cells and 77% of *hat1*Δ/Δ cells contained foci (Fig. 3B and C). Interestingly, after 300 min, the fraction of wild-type cells containing Rad52-GFP foci had declined to 10%, whereas foci remained in 52% of the *hat1*Δ/Δ cells (Fig. 3B and C). Strikingly, lack of Hat1 also dramatically increased the lifetime of individual Rad52-GFP foci when compared with wild-type cells. The majority of foci in wild-type cells disappeared within 2 h. In striking contrast, 78% of the foci formed in *hat1*Δ/Δ cells persisted for longer than 4 h (Fig. 3D). Taken together, these results demonstrate that Hat1 is indeed required for efficient repair of DNA damage, at least for DNA lesions whose repair requires homologous recombination via Rad52.

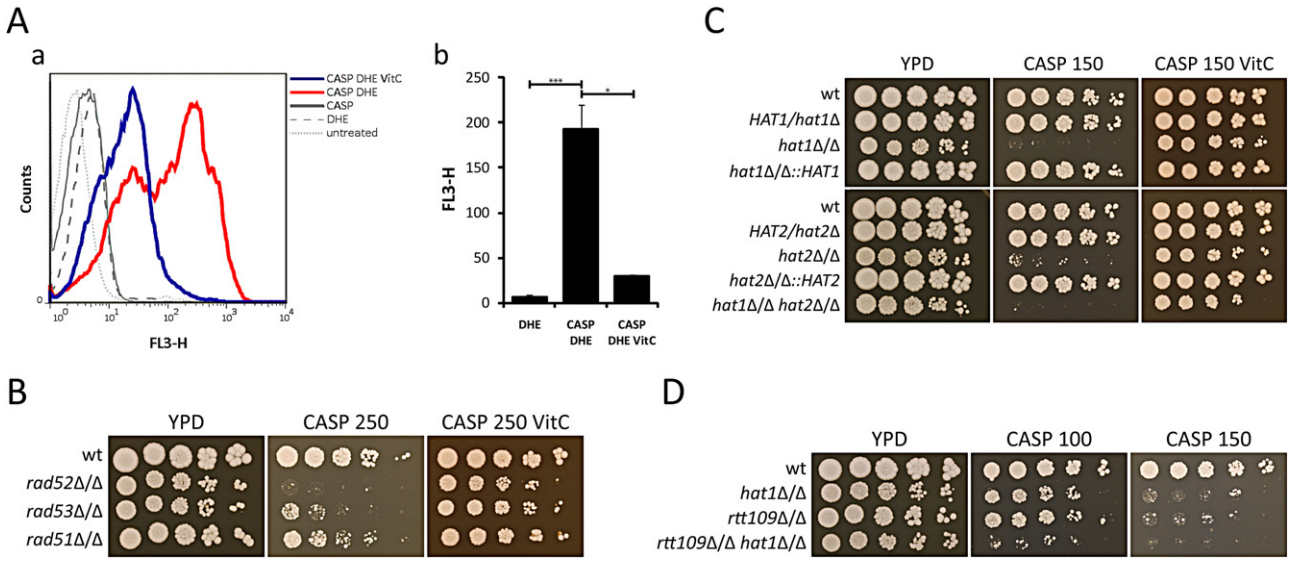
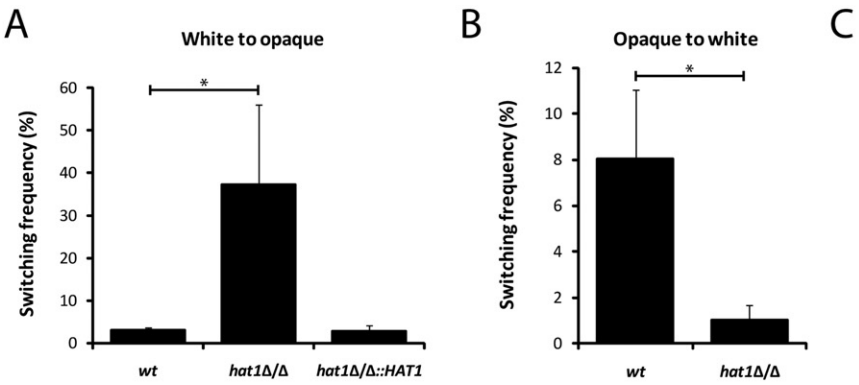


Fig. 4. Lack of DNA damage repair proteins or NuB4 components causes ROS-mediated CASP hypersensitivity.
A. Intracellular ROS production in response to CASP treatment was measured by FACS using dihydroethidium (DHE). Logarithmically growing cells were loaded with 20 μM DHE for 1 h, treated with 250 ng ml^{-1} CASP for 2.5 h and analysed by FACS. Twenty-five mM vitamin C (VitC) was added together with DHE to quench ROS as indicated (a). To quantify the ROS production, the mean fluorescent intensity of each sample was determined. Data are shown as mean + SD from at least two independent experiments (b). *$P < 0.05$ and ***$P < 0.001$.
B. Deletion of DNA damage repair genes encoding Rad52, Rad53 and Rad51 renders cells sensitive to CASP, which can be rescued by addition of vitamin C as antioxidant. Fivefold serial dilutions of indicated strains were spotted onto YPD plates containing 250 ng ml^{-1} CASP and 25 mM VitC as indicated. Plates were incubated at 30°C for 3 days.
C. Lack of Hat1 or Hat2 leads to CASP hypersensitivity, which can be rescued by addition of vitamin C. Experiment was performed as described in (B). Plates contained 150 ng ml^{-1} CASP and 25 mM VitC as indicated.
D. Lack of Rtt109 increases CASP sensitivity of *hat1*Δ/Δ cells. Experiment was performed as described in (B). Plates contained 100 or 150 ng ml^{-1} CASP as indicated.

Deletion of NuB4 components leads to CASP sensitivity

Echinocandins are antifungal drugs frequently used to treat *C. albicans* infections (Pfaller and Diekema, 2007; Perlin, 2011). They inhibit the Fks1 subunit of the β-(1,3)-D-glucan synthase and therefore block normal fungal cell wall biosynthesis (Douglas *et al.*, 1997). Interestingly, CASP, a prototype echinocandin, also triggers an oxidative stress response in *C. albicans* (Kelly *et al.*, 2009). Furthermore, the lack of the Rtt109 histone acetyltransferase leads to CASP sensitivity, which can be rescued by addition of antioxidants. The authors propose that this phenotype is caused by CASP-induced ROS that induce DNA damages (Wurtele *et al.*, 2010). Therefore, we asked if CASP treatment indeed triggers ROS accumulation and if Hat1 mediates tolerance to this drug. To determine if CASP causes ROS accumulation, we measured intracellular ROS levels by FACS analysis using dihydroethidium (DHE) and dihydrorhodamine 123 (DHR). Both DHE and DHR get oxidized by superoxide, yielding in a fluorescent product emitting at 567 nm and 540 nm respectively (Emmendorffer *et al.*, 1990; Zhao *et al.*, 2003). Interestingly, with both dyes we detected ROS accumulation upon CASP treatment (Figs 4A and S2A). Furthermore, addition of the antioxidant vitamin C reduced ROS levels triggered by CASP (Fig. 4A). To determine if CASP-induced ROS accumulation leads to DNA damage, we tested mutants lacking DNA damage repair proteins for CASP sensitivity. Interestingly, deletion of *RAD51*, *RAD52* and *RAD53* caused a marked CASP hypersensitivity, which was completely rescued by adding vitamin C, implying that CASP causes DNA damage through accumulating ROS (Figs 4B and S2B). Therefore, we also tested strains lacking *HAT1* and *HAT2* for their CASP susceptibilities. As expected, both single mutants and the double mutant were clearly CASP-hypersensitive, and growth was fully restored by vitamin C (Fig. 4C). Importantly, constitutive ROS levels in *hat1*Δ/Δ cells are only slightly increased. Moreover, adding vitamin C to the culture medium did not rescue the slow growth phenotype (Fig. S3). Therefore, increased ROS production is not responsible for the growth defect of the *hat1* mutant. Furthermore, we confirmed the CASP sensitivity phenotype of *hat1*Δ/Δ cells in a liquid assay (Fig. S2C). Finally, because Hat1 and Rtt109 may function in parallel pathways by acetylating histone H4 and H3, respectively, we constructed double-deletion strains and investigated their CASP sensitivity. Interestingly, deletion of both *HAT1* and *RTT109* clearly exacerbated CASP



C

Strain	Mating frequency White	Opaque
wt	$< 2.86*10^{-7}$	$8.00*10^{-3}$
*hat1*Δ/Δ	$3.08*10^{-6}$	$8.00*10^{-3}$
*hat1*Δ/Δ::*HAT1*	$< 3.03*10^{-7}$	$1.25*10^{-2}$

Fig. 5. Deletion of *HAT1* enhances phenotypic W/O switching towards the opaque form.
A. Quantitative white to opaque switching assays were performed with the indicated strains. The percentages represent the fraction of colonies that showed alterations of the original phenotype. All strains were *MTL***a/a** strains. Data are shown as mean + SD from three independent experiments.
B. Quantitative opaque to white switching assays were performed as described in (A). Data are shown as mean + SD from three independent experiments.
A and B. $*P < 0.05$.
C. To verify the mating-competent opaque and the mating-incompetent white phases quantitative mating assays were performed with an opaque phase *MTL*α/α tester strain. At least two independent experiments per genotype were performed yielding qualitatively similar results. Values are shown of one representative experiment.

hypersensitivity of the corresponding single mutants, suggesting that these two HATs function independently to mediate echinocandin tolerance (Fig. 4D). Furthermore, our data strongly suggest that interfering with the function of the NuB4 complex renders *C. albicans* hypersensitive to the antifungal drug CASP.

Deletion of HAT1 induces white to opaque switching

The accumulation of DNA damages in *C. albicans* triggers phenotypic switching from the white phase to the mating-competent opaque phase (Alby and Bennett, 2009). Therefore, we investigated a possible role of Hat1 in this process in more detail. White-opaque (W/O) switching can only occur in a mating-type homozygous background (Miller and Johnson, 2002). *C. albicans* has a diploid genome harbouring a mating type-like locus (*MTL*) with two alleles, '**a**' and 'α'. Thus, we generated mating-competent *MTL***a**/**a** homozygous *hat1*Δ/Δ deletion strains and performed quantitative switching assays. Interestingly, lack of Hat1 led to a dramatic increase in the W/O switching frequency towards the opaque from, which is consistent with the fact that DNA damages induce W/O switching in *C. albicans* (Fig. 5A). Furthermore, the switching frequency in the reverse direction was clearly reduced in *hat1*Δ/Δ cells (Fig. 5B). Thus, loss of Hat1 must stabilize the opaque state. Moreover, we performed quantitative mating assays with a *MTL*α/α tester strain to verify the mating-competent opaque form and the mating-incompetent white form. As expected, only opaque cells were able to mate with considerably higher efficiencies than mating-incompetent white cells (Fig. 5C). The slightly increased mating efficiency of white *hat1*Δ/Δ cells can be explained by increased switching to the opaque form that occurs during the assay. In summary, these data clearly indicate that loss of Hat1 induces W/O switching towards the opaque phase and therefore triggers mating in *C. albicans*.

The C. albicans *NuB4 complex binds and acetylates free histone H4 at lysine 5 and 12*

The efficient repair of DNA damages requires extensive chromatin remodelling and histone deposition, which has been linked to lysine acetylation (Costelloe *et al.*, 2006; Chen *et al.*, 2008; Ejlassi-Lassallette *et al.*, 2010). Thus, to investigate if Hat1 is involved in this process, we characterized its subcellular localization and if it catalyses the acetylation of histone H4 in a deposition-related pattern. Therefore, we constructed strains expressing fully functional GFP-tagged alleles of Hat1 and Hat2 (Fig. S4A). Interestingly, both proteins mainly localized to the nucleus (Fig. 6A). Furthermore, nuclear localization of Hat2 partially required a functional Hat1, since Hat2 lost its predominant nuclear localization in the absence of Hat1 but not vice versa. Importantly, genomic reintegration of *HAT1* fully restored the normal nuclear targeting of Hat2 (Fig. 6A). Furthermore, to confirm the physical interaction of Hat1 with Hat2 in *C. albicans*, we constructed strains expressing a functional myc-tagged allele of Hat1 (Fig. S4A), and performed co-immunoprecipitation experiments. Interestingly, we were able to co-immunoprecipitate a 43 kDa protein with Hat1-myc, which was identified as

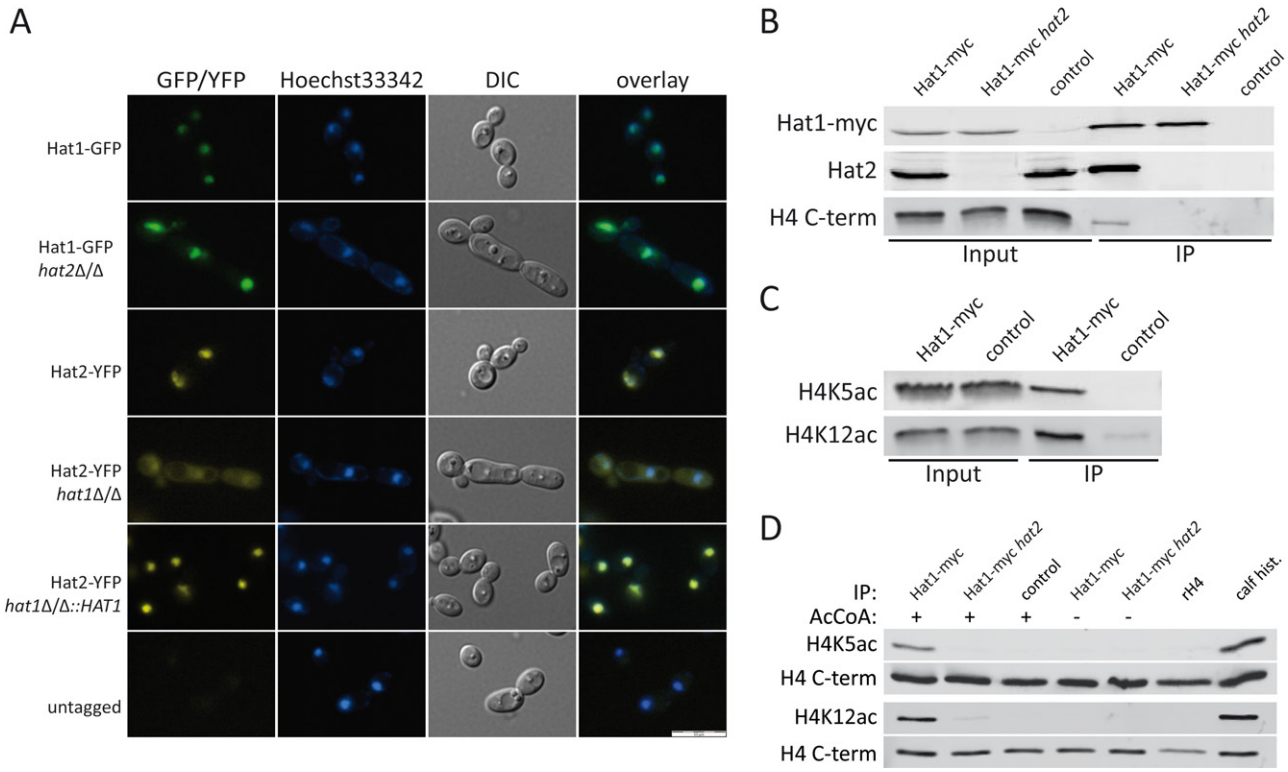


Fig. 6. The *C. albicans* Hat1/Hat2 complex localizes to the nucleus and acetylates free histone H4.
A. GFP-tagged Hat1 and YFP-tagged Hat2 show primarily nuclear localization (Hat1-GFP; Hat2-YFP). Deletion of *HAT2* does not change the localization of Hat1 (Hat1-GFP *hat2*Δ/Δ), but nuclear localization of Hat2 is partially Hat1-dependent (Hat2-YFP *hat1*Δ/Δ). Genomic reintegration of Hat1 restored the wild-type localization of Hat2 (Hat2-YFP *hat1*Δ/Δ*::HAT1*). Cells grown to the exponential growth phase in SC medium at 30°C were inspected. Nuclear localization was confirmed by Hoechst 33342 staining. A wild-type strain (untagged) was used as control. Scale bar corresponds to 10 μm.
B. Immunoprecipitation (IP) of myc-tagged Hat1 in a wild-type (Hat1-myc) and a *hat2*Δ/Δ background (Hat1-myc *hat2*) was performed with an anti-myc antibody. The IPs were subjected to SDS-PAGE analysis, followed by immunoblotting with specific antibodies for the myc-tag, Hat2 and the C-terminus of histone H4 respectively. An untagged wild-type strain was used as control. The amount of IP loaded corresponds to 40 OD_{600} units.
C. NuB4 incorporates H4K5ac and H4K12ac. Immunoprecipitation was performed as described in (B). Histone H4 acetylation was detected with acetylation-specific antibodies for K5 (H4K5ac) and K12 (H4K12ac). An untagged wild-type strain was used as control. The amount of IP loaded corresponds to 40 OD_{600} units.
D. Immunoprecipitation reactions were used for *in vitro* acetylation assays using recombinant unacetylated histone H4 and acetyl-CoA (AcCoA). H4K5ac and H4K12ac production by Hat1/Hat2 was detected by immunoblotting with antibodies specific for histone H4 acetyl-lysine 5 (H4K5ac) and acetyl-lysine 12 (H4K12ac). Detection of total histone H4 with an antibody recognizing the C-terminus (H4 C-term) served as loading control. Enzymatic activity of Hat1 was diminished in the absence of Hat2 (Hat1-myc *hat2*). No acetylation was detected with the IP from an untagged wild-type strain (control) or in the absence of acetyl-CoA. Recombinant histone H4 (rH4) and calf thymus histones (calf hist.) were controls.

Hat2 by immunoblotting using Hat2-specific rabbit polyclonal antibodies (Fig. 6B). Furthermore, we were also able to co-immunoprecipitate histone H4 acetylated at lysine 5 and lysine 12, both of which are diagnostic hallmarks for the deposition-related acetylation pattern of newly synthesized histone H4 (Fig. 6B and C) (Sobel *et al.*, 1995; Benson *et al.*, 2006). Interestingly, loss of Hat2 abrogated H4 binding by Hat1 (Fig. 6B). As expected, we failed to detect any changes in the acetylation status of total histone H4 in *hat1*Δ/Δ cells, because free histones acetylated by Hat1 represent only a minor fraction of the total histone H4 pool (Poveda *et al.*, 2004; Poveda and Sendra, 2008) (Fig. S4B). To confirm that Hat1 is indeed acetylating histone H4, we performed immunoprecipitation coupled with *in vitro* acetylation assays. Hat1 indeed acetyl-modified free histone H4 to yield H4K5ac and H4K12ac, and the presence of Hat2 was required for full enzymatic activity of Hat1 (Fig. 6D). Importantly, no signal was obtained in the absence of acetyl-CoA, excluding the possibility that the H4K5ac and H4K12ac signals in lane 1 are due to endogenous histone H4 in the IP. These data unequivocally demonstrate that Hat1 and Hat2 are part of the NuB4 complex in *C. albicans*, which binds and acetylates free histone H4 in a pattern characteristic for newly synthesized histones to be used for chromatin assembly.

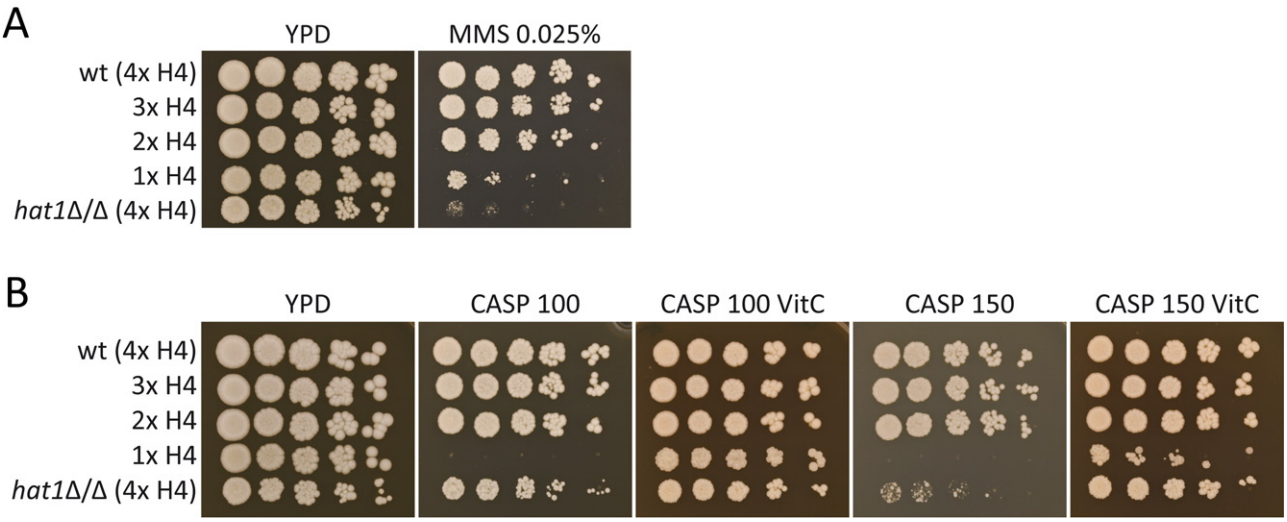


Fig. 7. Reduction of histone H4 gene dosage causes sensitivity to DNA damage and CASP.
A. Reduction to a single copy of histone H4 (1× H4) leads to MMS hypersensitivity. Strains carrying different copy numbers of histone H4 genes were spotted in fivefold serial dilutions onto YPD plates containing methylmethane sulphonate (MMS). Plates were incubated for 3 days at 30°C.
B. Single copy histone H4 cells (1× H4) show elevated sensitivity to CASP, which can be rescued by addition of vitamin C as an antioxidant. Experiment was performed as described in (A). Plates contained 100 or 150 ng ml^{-1} CASP and 25 mM VitC as indicated.

Reduction of histone H4 levels mimics inactivation of NuB4

The NuB4 complex is involved in the incorporation of histone H4-H3 dimers into nascent chromatin (Ai and Parthun, 2004). Interfering with this process by deleting NuB4 components debilitates DNA damage repair, which might be caused by defective histone H4 deposition during the final steps of the repair process. To determine if the observed phenotypes upon loss of Hat1 or Hat2 result from insufficient histone H4 incorporation, we aimed to mimic these phenotypes by reducing the gene dosage of histone H4 in *C. albicans*, since reduction to a single histone H4 gene impairs growth and leads to a constitutively pseudohyphal morphology (Zacchi *et al.*, 2010). Therefore, we sequentially deleted histone H4 genes in *C. albicans*, revealing a sharply increased MMS sensitivity of the single copy strain, which phenocopied the sensitivities of *hat1*Δ/Δ and *hat2*Δ/Δ cells (Fig. 7A). Strikingly, cells containing only one copy of histone H4 also showed markedly increased CASP sensitivity, which was again rescued by vitamin C (Fig. 7B). These data clearly show that interfering with the histone supply or deposition in *C. albicans* impairs DNA repair and leads to ROS-mediated CASP sensitivity, reminiscent of the phenotype observed for a lack of NuB4 components.

Discussion

In this study, we analyse the functions of the *C. albicans* NuB4 histone acetyltransferase complex in DNA damage repair and morphogenesis, as well as antifungal drug resistance. We show that *C. albicans* requires a fully functional NuB4 complex for efficient repair of DNA damages, normal cell proliferation and maintenance of the yeast morphology. Importantly, our data support the notion that interfering or blocking the function or assembly of the NuB4 complex may have therapeutic relevance for fungal diseases.

C. albicans *NuB4 complex is required for repair of various types of DNA damages*

The genetic removal of *HAT1* or *HAT2* impairs the repair of various types of DNA lesions (Fig. 2A and C). MMS and EMS lead to base alkylation, which is primarily repaired by base excision repair (Drablos *et al.*, 2004; Gocke *et al.*, 2009). Furthermore, when the DNA polymerase encounters MMS-induced DNA lesions, it converts them to double-strand breaks (Drablos *et al.*, 2004), whose repair requires distinct but overlapping pathways (Chapman *et al.*, 2012). In contrast, 4-NQO produces a kind of DNA damage, which is mainly repaired by nucleotide excision repair (Ikenaga *et al.*, 1975; Williams *et al.*, 2010). In addition, *hat1*Δ/Δ cells are also hypersensitive to UV irradiation, resulting in pyrimidine dimers, which are removed by nucleotide excision repair (Franklin *et al.*, 1985; Hoeijmakers, 2001). Thus, we demonstrate here that the NuB4 complex is involved in various DNA damage repair processes. This is consistent with a putative role in the restoration of a functional chromatin structure in the final steps of the repair process, because chromatin remodelling

must occur during the repair of different types of DNA damages (Costelloe *et al*., 2006).

Accumulating spontaneous DNA damage in the absence of Hat1 is followed by filamentation

Treatment of *C. albicans* with DNA-damaging agents triggers cellular elongation and the development of pseudohyphae. Interestingly, deletion of different components involved in the repair of DNA damage also leads to constitutive pseudohyphal growth (Andaluz *et al*., 2006; Shi *et al*., 2007). Obviously, the repair of spontaneous DNA damages appearing during the cell cycle in *C. albicans* requires a functional repair machinery. If this is not the case, activation of the DNA damage checkpoint and cell cycle arrest ultimately trigger cell elongation (Shi *et al*., 2007). We show here that inactivation of NuB4 complex components causes accumulation of endogenous DNA damages in the absence of any genotoxic stress (Fig. 2E and F). Furthermore, lack of Hat1 leads to inefficient repair of MMS-induced double-strand breaks (Fig. 3B and D). Thus, spontaneous DNA damages that arise during cell cycle cannot be repaired efficiently and accumulate in the absence of Hat1. In addition, cell elongation in the absence of Hat1 is clearly preceded by the accumulation of DNA lesions, indicating that DNA damage is the trigger for filamentation in the absence of Hat1 or Hat2 (Fig. 2G). We would like to emphasize that our results cannot rule out the possibility that DNA damage arises also more frequently in the absence of Hat1. In fact, there is evidence from *S. cerevisiae* indicating that the Hat1 orthologue could play a role in histone deposition during DNA replication, because it physically interacts with the origin recognition complex (ORC) (Suter *et al*., 2007). Thus, an inactive NuB4 complex could destabilize the replication fork, which would promote a fork collapse and subsequent accumulation of DNA damages. The clarification of this possibility will require further experiments. Interestingly, in our experimental set-up, we observed that elongated wild-type cells appearing after transient MMS treatment did not revert back to the yeast morphology within 300 min after removing MMS, even though Rad52-GFP foci had declined to about 10% (Fig. 3B and C). Obviously, the trigger for elongation must last longer than the presence of the foci. The completion of DNA repair, including the restoration of a functional chromatin structure after Rad52 removal might be required for a reversion to the yeast form. In fact, restoration of functional chromatin may signal the completion of DNA repair and enable checkpoint release (Chen *et al*., 2008). Therefore, completion of the repair process might be required to neutralize the elongation trigger, which may take longer than 300 min in our experimental set-up. However, 24 h after MMS removal, wild-type cells almost fully reverted to the yeast morphology (data not shown).

Lack of Hat1 increases CASP sensitivity and white-to-opaque switching

We also demonstrate that the genetic removal of *HAT1* or *HAT2* renders cells hypersusceptible to the commonly used antifungal drug CASP (Fig. 4C). Treatment with this drug induces oxidative stress, and antioxidants can suppress CASP sensitivity (Kelly *et al*., 2009; Wurtele *et al*., 2010). Interestingly, we show that CASP treatment induces ROS in *C. albicans* (Figs 4A and S2A), and cells lacking NuB4 components or DNA damage repair mutants are also hypersensitive to CASP in a ROS-dependent manner. This links DNA damage repair to antifungal susceptibility in this important fungal pathogen. Notably, a defective NuB4 complex does not affect susceptibility to other cell wall-damaging drugs or agents known to cause cell wall stress, including SDS or Calcofluor White, excluding the possibility that a cell wall defect might be responsible for the observed CASP sensitivity (data not shown). Thus, ROS-mediated DNA damage might be a secondary and indirect antifungal effect of CASP, which is most likely independent of the β-(1,3)-D-glucan synthase inhibition. Hence, interfering with the function of the NuB4 complex activity or other components implicated in the histone H4-H3 deposition pathway could be of interest in order to increase efficiency of CASP treatment, especially in resistant strains with mutations in Fks1, which stands out as the main mechanism of CASP resistance discovered to date (Balashov *et al*., 2006). Notably, efflux-based resistance mediated by ectopic overexpression of the Cdr2 ABC transporter in laboratory strains as well as in clinical isolates can also increase CASP tolerance (Schuetzer-Muehlbauer *et al*., 2003).

The inactivation of the NuB4 complex has also profound consequences on the phenotypic switching process. Genotoxic agents as well as several other environmental stimuli, which cause growth inhibition, increase W/O switching frequencies (Alby and Bennett, 2009). Interestingly, even slowing down the normal cell cycle is sufficient to induce this phenotype due to accumulation of opaque-specific factors that elicit the switch to the opaque state (Alby and Bennett, 2009). Hence, the slow growth phenotype of *hat1*Δ/Δ cells is most likely the cause for the increased switching frequency.

The C. albicans *NuB4 complex acetylates histone H4 in a deposition-related pattern*

We demonstrate that the *C. albicans* NuB4 complex binds and acetylates free histone H4 at K5 and K12 (Fig. 6B and D). Importantly, acetylation of H4K8 was undetectable

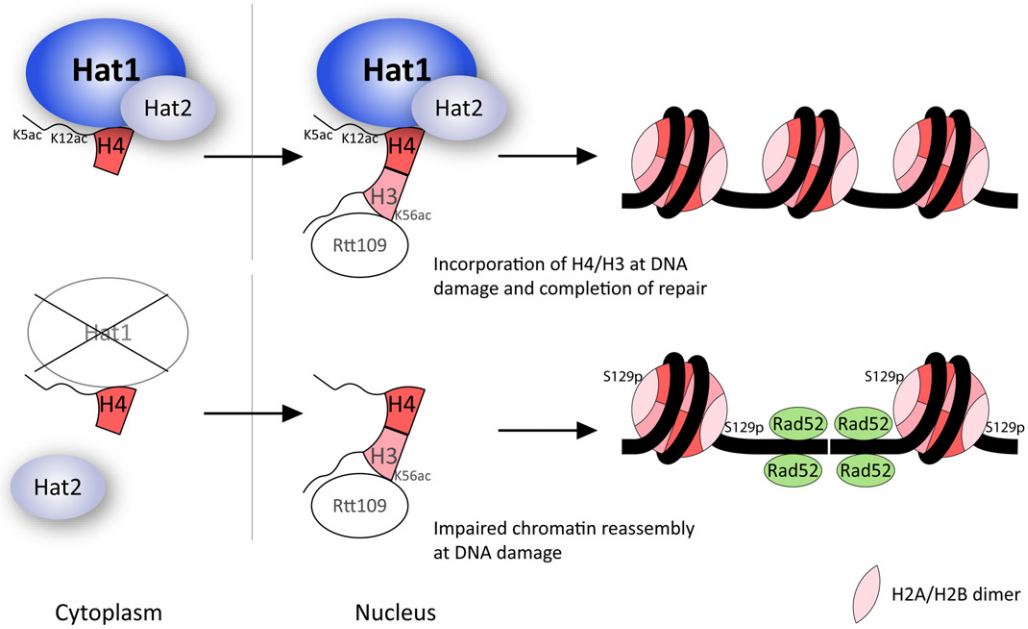


Fig. 8. Model for NuB4 function in DNA damage repair in *Candida albicans*. Hat1/Hat2 acetylate newly synthesized histone H4 in a deposition-related pattern to produce cytoplasmic H4K5ac and H4K12ac. The complex is transferred into the nucleus and histone H3, which is acetylated by Rtt109 to H3K56ac (Lopes da Rosa *et al*., 2010), becomes associated. Subsequently, modified histone H4-H3 are incorporated into chromatin at sites of DNA damage to restore chromatin structure. In the absence of Hat1, newly synthesized histone H4 remains unacetylated. Furthermore, incorporation of histone H4-H3 into chromatin at sites of DNA damage is impaired leading to accumulation of endogenous DNA lesions. This in turn causes a cell cycle delay, which results in cell elongation, pseudohyphal growth and increased white-to-opaque switching.

under these conditions, suggesting a strict specificity of Hat1 for H4K5 and H4K12 (Fig. S4C). This is a highly conserved hallmark acetylation pattern for newly synthesized histone H4 (Sobel *et al*., 1995; Benson *et al*., 2006; Campos *et al*., 2010). Furthermore, the observation that a reduction of histone H4 gene dosage in *C. albicans* phenocopies the genetic removal of Hat1 strongly suggests that the H4 pool available for chromatin deposition is affected upon inactivation of the NuB4 complex (Fig. 7). Thus, we propose a model whereby Hat1 primarily modifies newly synthesized histones, prior to their incorporation into chromatin at sites of DNA lesions (Fig. 8). Although acetylation of H4K5 and H4K12 is closely correlated with histone deposition, its importance in chromatin assembly varies among different organisms and processes analysed. For example, mutation of H4K5 and H4K12 in *S. cerevisiae* has no effect on histone deposition onto a 2 μ plasmid or in an *in vitro* nucleosome assembly assay (Ma *et al*., 1998; Zhang *et al*., 1998). In contrast, it was recently shown in *Physarum polycephalum* that histone H4K5ac and H4K12ac promote chromatin assembly (Ejlassi-Lassallette *et al*., 2010). Furthermore, deletion of *HAT1* as well as mutation of its target lysine residues impairs histone turnover in *S. cerevisiae* (Verzijlbergen *et al*., 2011). Therefore, whether H4K5 and H4K12 acetylation is required directly for efficient histone H4 incorporation or whether the essential function of *C. albicans* NuB4 in this process is restricted to binding and delivery of histones to histone chaperones for subsequent chromatin incorporation remains to be determined. Hat2 seems to be required for both scenarios, since it facilitates binding of Hat1 to histone H4 (Fig. 6B) and it is therefore also necessary for H4 acetylation (Fig. 6D). This role is obviously conserved, because *S. cerevisiae* Hat1 also requires Hat2 for binding of histone H4 (Parthun *et al*., 1996). Accordingly, deletion of Hat2 phenocopies the lack of Hat1 in *S. cerevisiae* (Kelly *et al*., 2000; Ge *et al*., 2011) as well as in *C. albicans* (Figs 1 and 2A).

Although, Hat1 was originally purified from cytoplasmic extracts (Parthun *et al*., 1996), subsequent work indicates that Hat1 is a predominantly nuclear enzyme (Ai and Parthun, 2004; Poveda *et al*., 2004). This is consistent with our data, although NuB4 components required for nuclear localization differ between *S. cerevisiae* and *C. albicans*. Interestingly, nuclear localization of Hat2 in *C. albicans* partially requires Hat1 but not vice versa (Fig. 6A). In contrast, *S. cerevisiae* Hat1 requires Hat2 for proper localization (Poveda *et al*., 2004), indicating substantial differences in HAT nuclear targeting between these two species. Obviously, Hat1 only transiently localizes to the cytoplasm to bind and acetylate histone H4, which is followed by nuclear translocation. Interestingly, Hat1 is indeed found in complexes with proteins involved in nuclear import of histone H4 (Mosammaparast *et al*., 2002; Campos *et al*.,

2010). In addition to chromatin assembly, acetylation of H4K5 and H4K12 is also involved in the nuclear targeting of histone H4 in *S. cerevisiae*, suggesting an additional function of Hat1 in nuclear histone H4 translocation (Glowczewski *et al*., 2004; Blackwell *et al*., 2007). If this is also the case in *C. albicans* remains to be determined.

In conclusion, we show here that the NuB4 complex plays important roles in facilitating DNA damage repair and modulating fungal morphogenesis and antifungal drug tolerance in *C. albicans*. This has come as a surprise, since Hat1 seems dispensable for normal growth in other organisms as demonstrated by deletion of *HAT1* in *Schizosaccaromyces pombe* or chicken cells (Barman *et al*., 2006; Benson *et al*., 2007). In *S. cerevisiae*, deletion of *HAT1* or *HAT2* alone does not show any sensitivity or growth phenotypes, due to a functional redundancy with histone H3 acetylation (Parthun *et al*., 1996; Qin and Parthun, 2002). Obviously, expressing a functional NuB4 complex is more important for *C. albicans* when compared with other organisms. Thus, *C. albicans* is to the best of our knowledge the first eukaryotic organism, which depends on histone H4 processing by a type B HAT for repair of endogenous DNA damage, normal cell growth and morphogenesis. Interestingly, slower growth rates and morphological defects due to deletion of DNA damage repair proteins have also been associated with a reduction in virulence (Hao *et al*., 2009; Lopes da Rosa *et al*., 2010). While we are certainly interested to identify possible virulence phenotypes using *in vivo* models, the slow growth rate of *hat1*Δ/Δ mutants makes it difficult to reliably assess a possible virulence defect due to reduced fitness when compared with wild-type cells (Chauhan *et al*., 2005). Nevertheless, preliminary virulence experiments in mouse models *in vivo* indicate attenuated virulence of cells lacking Hat1 (M. Tscherner *et al*., unpubl. data). The growth defect observed *in vitro* may lead to fitness defects *in vivo*, explaining reduced virulence. In contrast, *in vitro* assays with phagocytes indicate increased resistance to killing by mouse neutrophils, which could be a consequence of altered recognition due to the pseudohyphal morphology of cells lacking Hat1 (M. Tscherner *et al*., unpubl. data).

Nevertheless, our data suggest that the pharmacological inhibition of Hat1 might be a promising strategy to reduce the pathogenicity of *C. albicans* or render this pathogen more susceptible to echinocandins. Indeed, there is rising evidence that histone-modification inhibitory drugs (HiMoIDs) could provide a promising approach to treat fungal infections. For example, treatment with HDAC inhibitors can influence the interaction of *C. albicans* with immune cells (Simonetti *et al*., 2007) and alter antifungal drug sensitivity (Smith and Edlind, 2002). Furthermore, blocking the Hos2 histone deacetylase (HDAC) complex leads to reduced virulence (Hnisz *et al*., 2010). Interestingly, a specific Hos2 inhibitor termed MGCD290 (MethylGene, Montreal, Canada) has entered clinical trials as potential antifungal drug (Pfaller *et al*., 2009). We would like to point out that Hos2 shows even higher conservation between *C. albicans* and humans, with a Protein BLAST *E*-value of 7×10^{-174}, while Hat1 yields a lower albeit still significant *E*-value of 3×10^{-36}. Therefore, Hat1 can be considered as a potential antifungal target, despite the fact that the primary sequence is conserved and the target lysine residues are identical in higher eukaryotes. In addition, genetic or chemical inhibition of the HAT Rtt109 and the HDAC Hst3, which target mainly H3K56, reduces *C. albicans* virulence in mouse infection models (Lopes da Rosa *et al*., 2010; Wurtele *et al*., 2011). Since Rtt109 acts in a parallel pathway with Hat1 by acetylating newly synthesized histone H3, we propose that the targeted pharmacological inhibition of the NuB4 complex or other components of the same pathway could be a promising future strategy to combat fungal infections.

Experimental procedures

Media, growth conditions and growth inhibition assays

Rich medium (YPD) and synthetic complete medium (SC) were prepared as previously described (Kaiser *et al*., 1994). Cultures were grown at 30°C overnight, diluted to an OD_{600} of 0.1 the next morning, grown until cells reached the logarithmic growth phase and used for the experiments unless otherwise indicated. To assay sensitivity phenotypes cultures were diluted to 1×10^6 cells per millilitre and fivefold serial dilutions were prepared in distilled water. Identical volumes were spotted on agar plates containing the indicated substances. Plates were incubated at 30°C for 3 days and pictures were taken using a SPImager (S&P Robotics, Toronto, ON). MMS, EMS and 4-NQO were obtained from Sigma-Aldrich (Vienna, Austria). Stock solutions of 4-NQO were prepared in acetone. Caspofungin (Merck, Whitehouse Station, NJ) and ascorbic acid (Sigma-Aldrich, Vienna, Austria) were prepared as stock solutions in sterile water. To determine sensitivity to UV irradiation, 200 cells were plated on YPD plates and irradiated with 4 mJ in a Stratalinker 2400 (Stratagene, La Jolla, CA). Colonies were counted after incubation at 30°C for 3 days and survival rate was determined relative to plates without irradiation. For cfu determination after transient MMS treatment, logarithmically growing cells were incubated at 30°C in medium containing the indicated MMS concentration. Before MMS addition (0 h), and at the indicated time points after MMS addition, cultures were diluted and plated onto YPD plates. Colonies were counted after incubation at 30°C for 3 days and viability was determined relative to the zero time point.

Plasmid and strain construction

The complete list of *C. albicans* strains, plasmids and primers used in this study are listed in Tables S1, S2 and S3 respectively. All strains heterozygous for the mating type were derived from the clinical isolate SC5314 (Gillum *et al*., 1984).

Gene deletion mutants in this background were generated using the recyclable *SAT1*-flipping cassette (Reuss *et al*., 2004). For deletion of *RAD51*, *RAD52*, *RAD53*, *RTT109* and histone genes, we used a modified plasmid with an additional BglII site in which the *SAT1* marker was replaced by the *NAT1* marker derived from pJK863 (Shen *et al*., 2005). The *RAD53* deletion cassette was constructed using a fusion-PCR strategy (Noble and Johnson, 2005) with a fragment containing the *NAT1* marker and the FLP recombinase fused to the upstream and downstream region. Gene complementation constructs were created by cloning corresponding genes including upstream and downstream regions required for the genomic targeting into pSFS2A. All *MTL***a**/**a** strains were derived from DHCA202, which is a *MTL***a/a** derivative of SN152 (Hnisz *et al*., 2009). Gene deletion mutants in this background were done using the fusion-PCR strategy with the *Candida maltosa LEU2* and *C. dubliniensis HIS1* markers (Noble and Johnson, 2005). The *MTL*α/α mating tester strain has been previously described (Hnisz *et al*., 2009). For C-terminal fluorescent tagging, pFA6a-GFP-NAT1 and pFA6a-YFP-NAT1 were created by cloning GFP and YFP derived from pGFP-HIS1 and pYFP-HIS1 (Gerami-Nejad *et al*., 2001), respectively, into pFA6a-3HA-NAT1. The pFA6a-3HA-NAT1 vector containing the dominant *NAT1* marker was created by exchanging the marker in pFA6a-3HA-kanMX6 (Longtine *et al*., 1998) by the *NAT1* marker, including *TEF1* promoter and terminator derived from pJK863 (Shen *et al*., 2005). For construction of the *HAT1* and *HAT2* fluorescent protein tagging cassettes, the *3′* part of the coding sequence and the terminator region were fused with a fragment containing the fluorescent protein and the *NAT1* marker using fusion-PCR (Noble and Johnson, 2005). For tagging of *HAT2* with a recyclable marker, YFP derived from pYFP-HIS1 (Gerami-Nejad *et al*., 2001) was cloned into pSFS3b to yield pSFS3b-YFP and the fusion-PCR strategy was applied. For C-terminal myc-tagging pFA6a-9myc-NAT1 was constructed by replacing the 3HA tag in pFA6a-3HA-NAT1 with a 9-myc tag. The tagging cassette was constructed as described for fluorescent protein tagging. Transformation of *C. albicans* was done via electroporation as described previously (Reuss *et al*., 2004).

Colony morphology analysis, microscopy and live cell imaging

Colony morphology and microscopic analysis were performed as described previously (Hnisz *et al*., 2010). For fluorescence microscopy cells were grown in SC medium, washed once in distilled water and used for microscopy. Nuclear staining was done by adding 2 μg ml^{-1} Hoechst 33342 dye (bis-Benzimide H 33342 trihydrochloride, Sigma, Vienna, Austria) directly to the medium for 15 min at 30°C. For live cell imaging the ONIX Microfluidic Perfusion Platform (CellASIC, Hayward, CA) with Y04C Microfluidic plates was used. Cells were grown in SC medium to exponential phase and loaded into the culture chambers of the Y04C plate. The media flow rate was set to 1 psi and cells were grown at 30°C for 2 h. Then, the medium was switched to SC containing 0.02% MMS for 90 min. After that, cells were grown again in SC medium till the end of the experiment. Pictures were taken every 10 min during the whole experiment. For Rad52-GFP foci determination five pictures were obtained for each field of cells at 1 μm intervals along the *z*-axis and analysed.

ROS measurements

To determine intracellular ROS levels, overnight cultures were diluted to an OD_{600} of 0.25 in SC medium with 10% glucose to avoid CASP-induced flocculation (Gregori *et al*., 2011), and cultured for 1 h at 30°C. After loading with 20 μM DHE (Invitrogen GmbH, Vienna, Austria) or 20 μM DHR (Invitrogen) for 1 h, cells were treated with 250 ng ml^{-1} CASP for 2.5 h. Cells were washed once with distilled water followed by FACS analysis with FL1-H (DHR) or FL3-H (DHE) on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Microbroth dilution assays

To determine the drug sensitivity to CASP in liquid assays, a modified protocol of the microbroth dilution assay was used (Sanglard *et al*., 1995). Briefly, overnight cultures were diluted to an OD_{600} of 0.1, grown at 30°C in YPD until cells reached an OD_{600} of about 1, and an inoculum of 2.5×10^4 cells per millilitre was prepared. Aliquots of 100 μl of twofold concentrated CASP solutions were distributed in duplicates in a flat bottom microtitre plate. After adding a 100 μl inoculum, plates were incubated at 30°C for 48 h in a humid environment to avoid evaporation. OD_{600} was determined in a Victor^2 plate reader (PerkinElmer, Waltham, MA).

RNA isolation and RT-qPCR

RNA isolation was done as previously described with some modifications (Hnisz *et al*., 2010). Briefly, logarithmically growing cells were harvested at 1500 *g* for 3 min at 4°C and washed once in ice-cold distilled water. Cells were resuspended in 1 ml of TRI Reagent (Sigma-Aldrich, Vienna, Austria). Afterwards, 200 μl glass beads (425–600 μm, Sigma-Aldrich) were added and cells were broken at 6 m s^{-1} for 45 s on a FastPrep instrument (MP Biomedicals, Illkirch, France). After centrifugation at 14 000 *g* for 15 min at 4°C, the supernatant was transferred into a fresh tube and 200 μl of chloroform was added. After another centrifugation step, the supernatant was transferred to a fresh tube and RNA was precipitated by addition of 500 μl of isopropanol for 20 min on ice, washed once with 70% ethanol and resuspended in distilled water. Afterwards, 5 μg of total RNA was treated with DNase I (Fermentas, St. Leon-Rot, Germany). After PCI extraction and ethanol precipitation, 1 μg of total RNA was used for reverse transcription using the RevertAid Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany). cDNA amplification was monitored quantitatively by SYBR Green incorporation in a Realplex Mastercycler (Eppendorf, Vienna, Austria) using the KAPA SYBR FAST Master Mix Universal (Peqlab, Erlangen, Germany). Amplification curves were analysed using the Realplex Software (Eppendorf). Relative quantification of mRNAs was performed using the efficiency corrected ΔΔCt method (Pfaffl, 2001). *RIP1* was used as housekeeping gene (Hnisz *et al*., 2010). Quantification and statistics analysis (Student's *t*-test) was performed in Excel (Microsoft).

Preparation of whole-cell extracts and immunoblotting

Whole-cell extracts for immunoblotting were prepared by the TCA (trichloroacetic acid) method exactly as described previously (Mamnun *et al.*, 2004). For immunoblot analysis cell lysates equivalent to 0.5 OD_{600} units were separated by SDS-PAGE and transferred to nitrocellulose membranes (Protran BA79, Millipore, Billerica, MA). For separation of histones, 20% SDS-PAGE gels were used while all other proteins were separated in 12% gels. For detection of total histone H4 irrespective of the acetylation status we used an antibody against its C-terminus (ab10158, Abcam, Cambridge, UK). For detection of acetylated histone H4, we used antibodies recognizing H4K5ac (ab51997, Abcam), H4K8ac (39172, Active Motif, La Hulpe, Belgium) or H4K12ac (07-959, Millipore). Histone H2A phosphorylation was detected with an antibody specific for phosphorylated serine 129 (39271, Acitve Motif, La Hulpe, Belgium) and as loading control an antibody against total histone H2A was used (39236, Active Motif). Tagged proteins were visualized using antibodies against GFP (11814460001, Roche Diagnostics GmbH, Vienna, Austria) and c-Myc (ab32, Abcam) respectively. Calf histones were obtained from Sigma-Aldrich (H9250, Vienna, Austria) and chicken core histones from Millipore (13-107, Billerica, MA).

Rabbit polyclonal anti-Hat2 antibodies and purification

Polyclonal anti-Hat2 antibodies were raised in rabbits against a peptide corresponding to amino acid 363–382 of *C. albicans* Hat2. The peptide was expressed in *E. coli* as a glutathione *S*-transferase fusion protein and purified via glutathione sepharose beads (GE Healthcare, Vienna, Austria). About 1 mg of purified antigen was used for immunization of one New Zealand White Rabbit (Charles River, Sulzfeld, Germany). The antiserum was used at a dilution of 1:1000 and specificity was tested using appropriate cell extracts of wild-type *C. albicans* and mutants lacking Hat2 as a control.

Immunoprecipitation

Cells were grown in 100 ml of YPD to an OD_{600} of 2.0, harvested, washed and resuspended in 1 ml of lysis buffer [50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, complete protease inhibitor cocktail (Roche Diagnostics GmbH, Vienna, Austria)]. Afterwards, an equal volume of glass beads (425–600 µm, Sigma-Aldrich, Vienna, Austria) was added and cells were broken by shaking eight times at 6 m s^{-1} for 30 s on a FastPrep instrument (MP Biomedicals, Illkirch, France). Extracts were cleared by centrifugation twice at 20 000 *g*. For immunoprecipitation of myc-tagged protein 30 µl of EZview Red anti-c-Myc Affinity Gel (Sigma-Aldrich) was washed six times in 700 µl of lysis buffer, added to the lysates and incubated overnight. Beads were pelleted at 1000 *g*, washed three times with lysis buffer and resuspended in Lämmli loading buffer for SDS-PAGE analysis. Aliquots of IPs corresponding to 40 OD_{600} units were loaded.

In vitro *histone acetylation assay*

Immunoprecipitation reactions used for *in vitro* acetylation assays were prepared as described above with modifications. Buffer A [50 mM HEPES pH 8.0, 400 mM $(NH_4)_2SO_4$, 5% glycerol, 0.5 mM EDTA, complete protease inhibitor cocktail (Roche Diagnostics GmbH, Vienna, Austria)] was used as lysis buffer and beads were resuspended in the end in 20 µl of PBS containing a protease inhibitor cocktail. Aliquots of 3 µl of the immunoprecipitation reactions were used for acetylation assays. Reactions contained 1 µg of recombinant histone H4 (Sigma-Aldrich, Vienna, Austria), 40 mM acetyl coenzyme A, 50 mM NaH_2PO_4 pH 7.4, 15 mM β-mercaptoethanol, 10% glycerol and complete protease inhibitor cocktail (Roche Diagnostics GmbH) in a total volume of 20 µl and were incubated for 15 min at 37°C. To stop the reaction, 20 µl of 2× Lämmli buffer was added and samples were incubated at 70°C for 10 min. Aliquots of 5 µl were used for SDS-PAGE and immunoblot analysis.

White-opaque switching and mating assays

White-opaque switching assays and quantitative mating assays were performed precisely as described previously (Hnisz *et al.*, 2009).

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Supporting information

Additional supporting information may be found in the online version of this article.

Supporting Information

The histone acetyltransferase Hat1 facilitates DNA damage repair and morphogenesis in *Candida albicans*

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Figure S1

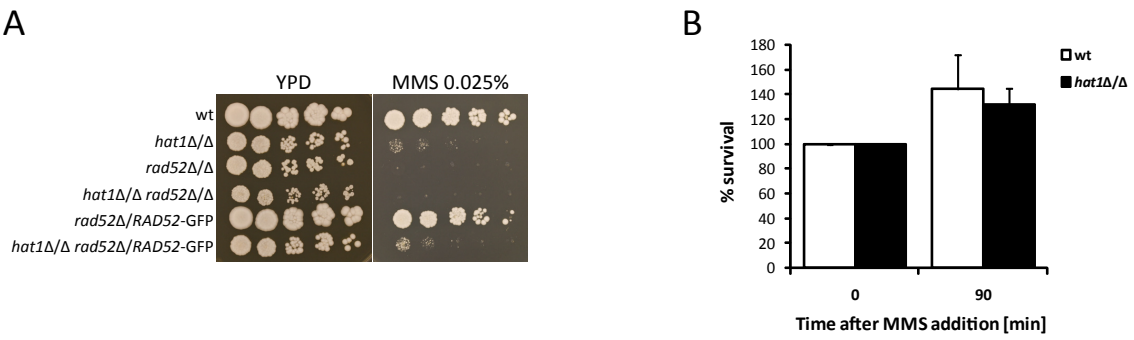

Fig. S1. C-terminally GFP-tagged Rad52 is functional and *hat1*Δ/Δ cells survive live cell imaging conditions.
A. Functional Rad52-GFP alleles confer resistance to MMS. Five-fold serial dilutions of indicated strains were spotted onto YPD plates containing the indicated compound. Plates were incubated at 30°C for 3 days.
B. Viability of cells treated with 0.02% MMS for 90 minutes. Exponentially growing cells were incubated in MMS-containing SC medium at 30°C. To determine CFUs, cells were diluted and plated onto YPD plates. Colonies were counted after 3 days incubation at 30°C. Survival relative to the zero minute control was calculated. Data are shown as mean + SD from two independent experiments.

Figure S2

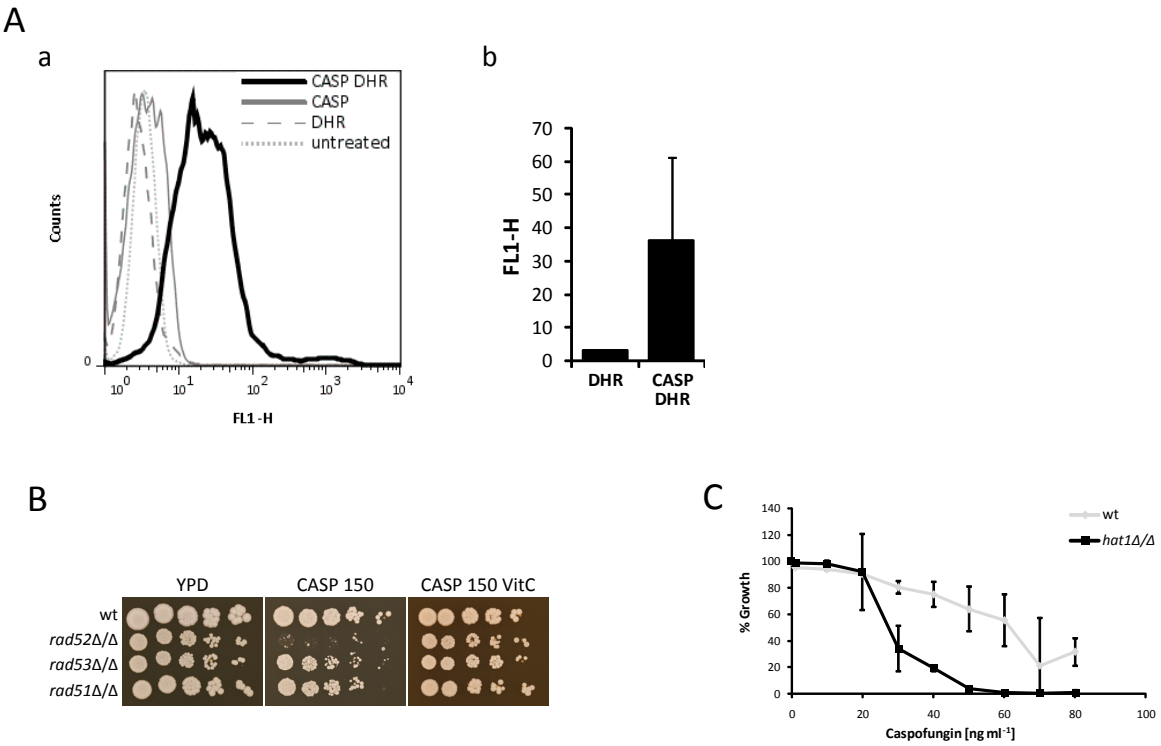

Fig. S2. CASP treatment leads to accumulation of ROS and lack of DNA repair proteins or Hat1 results in hypersensitivity to CASP.
A. Intracellular ROS production after CASP treatment was measured by FACS using dihydrorhodamine 123 (DHR). Log-phase cells were loaded with 20 µM DHR for 1 hour, treated with 250 ng ml^{-1} CASP for 2.5 hours and analyzed by FACS (a). The mean fluorescent intensity of each sample was determined for ROS quantification. Data are shown as mean + SD from two independent experiments (b).
B. Deletion of DNA damage repair genes encoding Rad52, Rad53 and Rad51 renders cells sensitive to CASP, which can be rescued by addition of vitamin C (VitC) as antioxidant. Five-fold serial dilutions of indicated strains were spotted onto YPD plates containing 150 ng ml^{-1} CASP and 25 mM VitC. Plates were incubated at 30°C for 3 days.
C. Microbroth dilution assay to determine CASP sensitivity of cells lacking Hat1 was performed as described in Experimental Procedures. OD_{600} was measured after 48h incubation at 30°C. Data are shown as mean ± SD from three independent experiments.

Figure S3

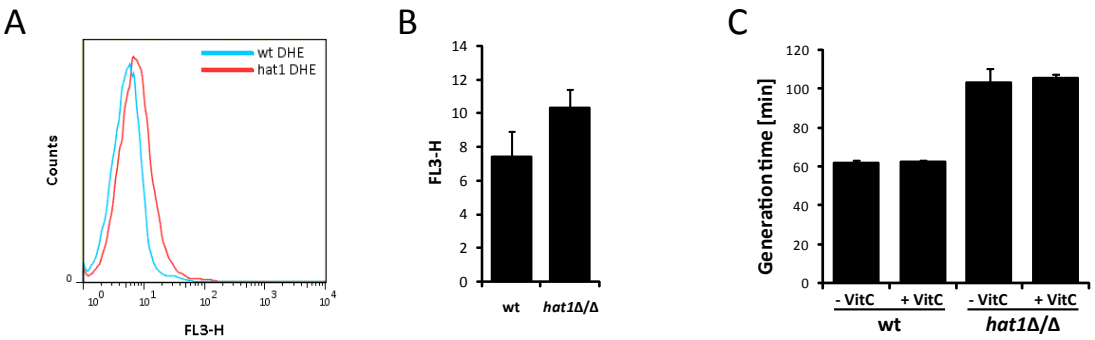

Fig. S3. Constitutively elevated ROS levels are not causing the slow growth phenotype of *hat1*Δ/Δ cells.
A. Intracellular ROS levels in logarithmically growing wild type and *hat1*Δ/Δ cells were quantified with dihydroethidium (DHE) as described in the Experimental Procedures section.
B. Quantification of cellular ROS levels shown in panel A. The mean fluorescent intensity of each sample was determined. Data are shown as mean + SD from two independent experiments.
C. Generation times of wild type and *hat1*Δ/Δ cells were determined in the presence and absence of vitamin C. Cells were grown in YPD medium at 30°C with or without 25 mM vitamin C and OD_{600} values were measured every two hours. The generation times were calculated by fitting an exponential function on the exponential parts of the growth curves. Data are shown as mean + SD from two independent experiments.

Figure S4

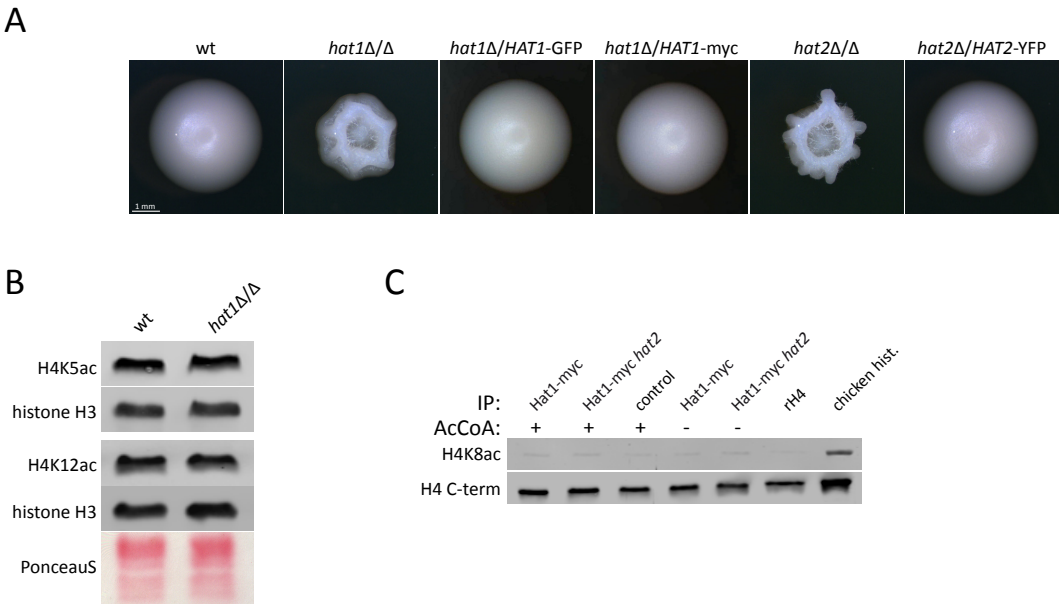

Fig. S4. C-terminally tagged alleles of Hat1 and Hat2 are fully functional. Deletion of *HAT1* does not alter total histone H4 acetylation status and Hat1 does not acetylate histone H4K8.

A. Cells carrying C-terminally tagged alleles of Hat1 or Hat2 show wild type colony morphology. Images were taken after 3 days of incubation on YPD plates at 30°C. Scale bar corresponds to 1 mm.

B. Total histone H4K5 and H4K12 acetylation is unchanged in *hat1*Δ/Δ cells. Immunoblotting of acetylated histone H4K5 and H4K12 from whole cell extracts from strains grown to the logarithmic growth phase in YPD at 30°C. Reprobing for total histone H3 visualized cellular histone levels. The Ponceau S stained membrane was used as a loading control.

C. Hat1 has no activity on histone H4K8. Hat1-myc IPs were prepared as described in the Experimental Procedures section and used for *in vitro* acetylation assays as described in Fig. 6. H4K8ac was detected by immunoblotting with an antibody specific for histone H4 acetyl-lysine 8 (H4K8ac). Detection of total histone H4 with an antibody recognizing the C-terminus (H4 C-term) served as loading control. The IP from an untagged wild type strain was included (control). Recombinant histone H4 (rH4) and chicken core histones (chicken hist.) were controls.

Table S1. *C. albicans* strains used in this study

Description	Name	Parent	Genotype	Reference	Figure
wt	SC5314	Clinical isolate		Gillum *et al* , 1984	Figures 1, 2, 3, 4, 6, 7
HAT1 /*hat1* Δ	CA-MT003	SC5314	*HAT1* /*hat1* Δ::SAT1-FLP	This study	
HAT1 /*hat1* Δ	CA-MT007	CA-MT003	*HAT1* /*hat1* Δ::FRT	This study	Figures 1, 2, 4
hat1 Δ/Δ	CA-MT011	CA-MT007	*hat1* Δ::FRT/*hat1* Δ::SAT1-FLP	This study	
hat1 Δ/Δ	CA-MT014	CA-MT011	*hat1* Δ::FRT/*hat1* Δ::FRT	This study	Figures 1, 2, 4, 7
hat1 Δ/Δ::*HAT1*	CA-MT049	CA-MT014	*hat1* Δ::FRT/*hat1* Δ::*HAT1* -SAT1-FLP	This study	
hat1 Δ/Δ::*HAT1*	CA-MT050	CA-MT049	*hat1* Δ::FRT/*hat1* Δ::*HAT1* -FRT	This study	Figures 1, 2, 4
HAT2 /*hat2* Δ	CA-MT027	SC5314	*HAT2* /*hat2* Δ::SAT1-FLP	This study	
HAT2 /*hat2* Δ	CA-MT031	CA-MT027	*HAT2* /*hat2* Δ::FRT	This study	Figures 1, 2, 4
hat2 Δ/Δ	CA-MT035	CA-MT031	*hat2* Δ::FRT/*hat2* Δ::SAT1-FLP	This study	
hat2 Δ/Δ	CA-MT039	CA-MT035	*hat2* Δ::FRT/*hat2* Δ::FRT	This study	Figures 1, 2, 4
hat2 Δ/Δ::*HAT2*	CA-MT098	CA-MT039	*hat2* Δ::FRT/*hat2* Δ::*HAT2* -SAT1-FLP	This study	
hat2 Δ/Δ::*HAT2*	CA-MT101	CA-MT098	*hat2* Δ::FRT/*hat2* Δ::*HAT2* -FRT	This study	Figures 1, 2, 4
hat1 Δ/Δ *HAT2* /*hat2*Δ	CA-MT083	CA-MT014	*hat1* Δ/Δ *HAT2* /*hat2* Δ::SAT1-FLP	This study	
hat1 Δ/Δ *HAT2* /*hat2*Δ	CA-MT088	CA-MT083	*hat1* Δ/Δ *HAT2* /*hat2* Δ::FRT	This study	
hat1 Δ/Δ *hat2* Δ/Δ	CA-MT155	CA-MT088	*hat1* Δ/Δ *hat2* Δ::FRT/*hat2* Δ::SAT1-FLP	This study	
hat1 Δ/Δ *hat2* Δ/Δ	CA-MT161	CA-MT155	*hat1* Δ/Δ *hat2* Δ::FRT/*hat2* Δ::FRT	This study	Figures 1, 2, 4
RAD52 /*rad52* Δ	CA-MT195	SC5314	*RAD52* /*rad52* Δ::NAT1-FLP	This study	
RAD52 /*rad52* Δ	CA-MT198	CA-MT195	*RAD52* /*rad52* Δ::FRT	This study	
RAD52 /*rad52* Δ	CA-MT196	CA-MT014	*hat1* Δ/Δ *RAD52* /*rad52* Δ::NAT1-FLP	This study	
RAD52 /*rad52* Δ	CA-MT199	CA-MT196	*hat1* Δ/Δ *RAD52* /*rad52* Δ::FRT	This study	
Rad52-GFP	CA-MT230	CA-MT198	*rad52* Δ::FRT/*RAD52* -GFP-NAT1	This study	Figures 2, 3
Rad52-GFP *hat1* Δ/Δ	CA-MT232	CA-MT199	*hat1* Δ/Δ *rad52* Δ::FRT/*RAD52* -GFP-NAT1	This study	Figures 2, 3
rad52Δ/Δ	CA-MT205	CA-MT198	*rad52* Δ::FRT/*rad52* Δ::NAT1-FLP	This study	
rad52Δ/Δ	CA-MT207	CA-MT205	*rad52* Δ::FRT/*rad52* Δ::FRT	This study	Figure 4
RAD53 /*rad53* Δ	CA-MT091	SC5314	*RAD53* /*rad53* Δ::NAT1-FLP	This study	
RAD53 /*rad53* Δ	CA-MT095	CA-MT091	*RAD53* /*rad53* Δ::FRT	This study	
rad53Δ/Δ	CA-MT169	CA-MT095	*rad53* Δ::FRT/*rad53* Δ::NAT1-FLP	This study	
rad53Δ/Δ	CA-MT170	CA-MT169	*rad53* Δ::FRT/*rad53* Δ::FRT	This study	Figure 4
RAD51 /*rad51* Δ	CA-MT209	SC5314	*RAD51* /*rad51* Δ::NAT1-FLP	This study	
RAD51 /*rad51* Δ	CA-MT211	CA-MT209	*RAD51* /*rad51* Δ::FRT	This study	

rad51Δ/Δ	CA-MT221	CA-MT211	*rad51* Δ::FRT/*rad51* Δ::NAT1-FLP	This study	
rad51Δ/Δ	CA-MT227	CA-MT221	*rad51* Δ::FRT/*rad51* Δ::FRT	This study	Figure 4
RTT109 /*rtt109* Δ	CA-ES003	SC5314	*RTT109* /*rtt109* Δ::NAT1-FLP	This study	
RTT109 /*rtt109* Δ	CA-ES004	CA-ES003	*RTT109* /*rtt109* Δ::FRT	This study	
rtt109Δ/Δ	CA-ES007	CA-ES004	*rtt109* Δ::FRT/*rtt109* Δ::NAT1-FLP	This study	
rtt109Δ/Δ	CA-ES008	CA-ES007	*rtt109* Δ::FRT/*rtt109* Δ::FRT	This study	Figure 4
rtt109 Δ/Δ *HAT1* /*hat1* Δ	CA-ES061	CA-ES008	*rtt109* Δ/Δ *HAT1* /*hat1* Δ::SAT1-FLP	This study	
rtt109 Δ/Δ *HAT1* /*hat1* Δ	CA-ES070	CA-ES061	*rtt109* Δ/Δ *HAT1* /*hat1* Δ::FRT	This study	
rtt109 Δ/Δ *hat1* Δ/Δ	CA-ES074	CA-ES070	*rtt109* Δ/Δ *hat1Δ* ::FRT/*hat1Δ* ::SAT1-FLP	This study	
rtt109 Δ/Δ *hat1* Δ/Δ	CA-ES076	CA-ES074	*rtt109* Δ/Δ *hat1Δ* ::FRT/*hat1Δ* ::FRT	This study	Figure 4
wt	DHCA202	SN152	*arg4* Δ/*arg4* Δ *his1* Δ/*his1* Δ *leu2* Δ/*leu2* Δ *URA3* /*ura3* Δ::λimm434 *IRO1* /*iro1* Δ::λimm434	Hnisz *et al* ., 2009	Figure 5
HAT1 /*hat1* Δ	DHCA361	DHCA202	*HAT1* /*hat1* Δ::*CdHIS1*	This study	
hat1 Δ/Δ	DHCA362	DHCA361	*hat1* Δ::*CdHIS1* /*hat1* Δ::*CmLEU2*	This study	Figure 5
hat1 Δ/Δ::*HAT1*	DHCA363	DHCA362	*hat1* Δ::*CmLEU2* /*hat1* Δ::*HAT1* -SAT1	This study	Figure 5
Mating tester strain (α/α)	DHCA210	SC5314	*ade2* Δ::FRT/*ade2* Δ::FRT	Hnisz *et al* ., 2009	
Hat1-GFP	CA-MT086	CA-MT007	*hat1* Δ::FRT/*HAT1* -GFP-NAT1	This study	Figure 6
hat2 Δ/Δ *HAT1* /*hat1* Δ	CA-MT097	CA-MT039	*hat2* Δ/Δ *HAT1* /*hat1* Δ::SAT1-FLP	This study	
hat2 Δ/Δ *HAT1* /*hat1* Δ	CA-MT100	CA-MT097	*hat2* Δ/Δ *HAT1* /*hat1* Δ::FRT	This study	
Hat1-GFP *hat2* Δ/Δ	CA-MT131	CA-MT100	*hat2* Δ/Δ *hat1* Δ::FRT/*HAT1* -GFP-NAT1	This study	Figure 6
Hat2-YFP	CA-ES005	CA-MT031	*hat2* Δ::FRT/*HAT2* -YFP-NAT1	This study	Figure 6
Hat2-YFP *hat1* Δ/Δ	CA-ES027	CA-MT088	*hat1* Δ/Δ *hat2* Δ::FRT/*HAT2* -YFP-NAT1-FLP	This study	
Hat2-YFP *hat1* Δ/Δ	CA-ES031	CA-ES027	*hat1* Δ/Δ *hat2* Δ::FRT/*HAT2* -YFP-FRT	This study	Figure 6
Hat2-YFP *hat1* Δ/Δ::*HAT1*	CA-ES034	CA-ES031	*hat1* Δ/Δ::*HAT1* -SAT1-FLP *hat2* Δ::FRT/*HAT2* -YFP-FRT	This study	
Hat2-YFP *hat1* Δ/Δ::*HAT1*	CA-ES037	CA-ES034	*hat1* Δ/Δ::*HAT1* -FRT *hat2* Δ::FRT/*HAT2* -YFP-FRT	This study	Figure 6
Hat1-myc	CA-MT251	CA-MT007	*hat1* Δ::FRT/*HAT1* -9myc-NAT1	This study	Figure 6
Hat1-myc *hat2*	CA-MT253	CA-MT100	*hat2* Δ/Δ *hat1* Δ::FRT/*HAT1* -9myc-NAT1	This study	Figure 6
3x H4	CA-ES011	SC5314	*HHF1* /*hhf1* Δ::NAT1-FLP	This study	
3x H4	CA-ES016	CA-ES011	*HHF1* /*hhf1* Δ::FRT	This study	Figure 7
2x H4	CA-ES019	CA-ES016	*hhf1* Δ::FRT/*hhf1* Δ::NAT1-FLP	This study	
2x H4	CA-ES020	CA-ES019	*hhf1* Δ::FRT/*hhf1* Δ::FRT	This study	Figure 7
1x H4	CA-ES028	CA-ES020	*hhf1* Δ/Δ *HHF22* /*hhf22* Δ::NAT1-FLP	This study	
1x H4	CA-ES030	CA-ES028	*hhf1* Δ/Δ *HHF22* /*hhf22* Δ::FRT	This study	Figure 7

Table S2. Plasmids used in this study

Plasmids	Parent	Relevant inserts and cloning sites	Reference
pSFS2a			Reuss *et al* ., 2004
pSFS2a-*HAT1* urdr	pSFS2a	*PvuI* 5'*HAT1XhoI* -SAT1-FLP-*SacII* 3'*HAT1SacI*	This study
pSFS2a-*HAT1* int	pSFS2a	*PvuIHAT1PvuI* -SAT1-FLP	This study
pSFS2a-*HAT2* urdr	pSFS2a	*ApaI* 5'*HAT1XhoI* -SAT1-FLP-*SacII* 3'*HAT1SacI*	This study
pSFS2a-*HAT2* int	pSFS2a	*ApaIHAT2XhoI* -SAT1-FLP	This study
pSFS2a-7185urdr	pSFS2a	*ApaI* 5'orf19.7185*XhoI* -SAT1-FLP-*SacII* 3'orf19.7185*SacI*	This study
pSFS2a-6843urdr	pSFS2a	*ApaI* 5'orf19.6843*XhoI* -SAT1-FLP-*SacII* 3'orf19.6843*SacI*	This study
pSFS3b	pSFS2a	FRT-FLP-*PaeI* NAT1*NheI* -FRT-*BglII*	This study
pSFS3b-*RAD52* urdr	pSFS3b	*KpnI* 5'*RAD52ApaI* -NAT1-FLP-*BglII* 3'*RAD52NotI*	This study
pSFS3b-*RAD51* urdr	pSFS3b	*KpnI* 5'*RAD51ApaI* -NAT1-FLP-*BglII* 3'*RAD51NotI*	This study
pSFS3b-*RTT109* urdr	pSFS3b	*KpnI* 5'*RTT109ApaI* -NAT1-FLP-*BglII* 3'*RTT109NotI*	This study
pSFS3b-*HHF1* urdr	pSFS3b	*KpnI* 5'*HHF1ApaI* -NAT1-FLP-*BglII* 3'*HHF1NotI*	This study
pSFS3b-*HHF22* urdr	pSFS3b	*KpnI* 5'*HHF22ApaI* -NAT1-FLP-*BglII* 3'*HHF22NotI*	This study
pGFP-*HIS1*			Gerami-Nejad *et al* ., 2001
pYFP-*HIS1*			Gerami-Nejad *et al* ., 2001
pFA6a-3HA-kanMX6			Longtine *et al.* , 1998
pFA6a-3HA-NAT1	pFA6a-3HA-kanMX6	3HA-*ApaI* NAT1*SacI*	This study
pFA6a-GFP-NAT1	pFA6a-3HA-NAT1	*SmaI* GFP*AscI* -NAT1	This study
pFA6a-YFP-NAT1	pFA6a-3HA-NAT1	*SmaI* YFP*AscI* -NAT1	This study
pSFS3b-YFP	pSFS3b	*KpnI* YFP*ApaI* -FLP-NAT1	This study
pFA6a-9myc-NAT1	pFA6a-3HA-NAT1	*XmaI* 9myc*AscI* -NAT1	This study

Table S3. Oligonucleotide primers used in this study

Name	Sequence (5' - 3')*
Gene deletion constructs based on the SAT1-flipping strategy (Reuss *et al.* , 2004)	
5C_CaHAT1	CTGGGAACTAAAGTTCAAGATGACG
55_CaHAT1	gctagggccctagccgatcgAGTTACTGCTCATCAGCCATTG
53_CaHAT1	gtcgctcgagcgacCATTGATTAAGTCTTGATGTGATTG
35_CaHAT1	gctaccgcggtagcTAAGTATATTTGTATTATTAGCTAGAAAACTAGTG
33_CaHAT1	gtcggagctccgaccgatcgTAATATGACAATAACCGCCTCTG
3C_2 CaHAT1	AAGTTCTTCTTCTTCTGCCAGTGG
5C_CaHAT2	TTACATTCACCAACTGCAGTGACC
55_CaHAT2	acgtgggcccgatcgGAAACGAAGCCGAGACATAGGC
53_CaHAT2	atcgctcgagGGCTGGTGTTGAAAAGAAAAAGTGAC
35_CaHAT2	acgtccgcggTAAAACTGGCCCTAGCTAGAGAC
33_CaHAT2	atcggagctctcgagcgatcgCCTAGTCTAAATGTTTAGCTGTGTGG
3C_CaHAT2	GTTTTGGCTGTAAGTGACAAGTGC
5C_Ca7185	GTCTTTCAGTAATTCTATCGGTTGGG
55_Ca7185	acgtgggcccgatcgTATTCGCAGTGGCACATTTGC
53_Ca7185	atcgctcgagCATTGGATCGGTATATTTGCCTTTAC
35_Ca7185	acgtccgcggGTAAAGAGTTGAATAGTTTTAATTGTGTG
33_Ca7185	atcggagctctcgagcgatcgCCTTCACCTTAAGTCTGTCACC
3C_Ca7185	TAGTGAAGGGTGCGAGATTCG
5C_Ca6843	CAACAATAGAAGAATTGGGAGTTCG
55_Ca6843	acgtggtacCTAATTCATGACCTTTGGATCG
53_Ca6843	acgtgggcccTATTGATGCGGGTTTTATCAG
35_Ca6843	actgcggccgcATAGTAAGCAGTTGCTTTTGTAG
33_Ca6843	actgggcccgagctcATAGAGCACAAAACTCCGAG
3C_Ca6843	AAATATCATGTGAACAACTCAATCC
5C_CaRAD52	GCGAATCAATTGTCAACCCAG
55_CaRAD52	atcggtaccAAGTTGGGCATTGTATTGCAG
53_CaRAD52	atcgggcccCATATTACAAGGTTTATAACTGTG
35_CaRAD52	gctgctgcaggtcgaccggtgtcggagatctGAGGAGGGTTATAGGTATCTAG
33_CaRAD52	atcgggcccgcggccgcGATGATATATCTCTAACGTTGTC
3C_CaRAD52	CTTCAGAAAATTCATTATATGGTCC
5C_CaRAD51	GTTAGAATTATAGTTGGGTTCTTAATC
55_CaRAD51	atcggtaccAAGTACCAATAACACCTTGAAGC
53_CaRAD51	atcgggcccTGTGTGGGGGTTGTCAGTATTTG
35_CaRAD51	gctgctgcaggtcgaccggtgtcgggcggccgcGTTTGTTTGTACTATCCCAAAGTTG
33_CaRAD51	atcgggcccTTCATCACAGGAGAGCTCTGATC
3C_CaRAD51	TTGCTCTGTCCCAACTTTGC
5C_CaRTT109	TTTAAGTTTATAGGTGGTGTATTTGG
55_CaRTT109	catggtaccATCCCAGTTAATTGTTTACCATCTG
53_CaRTT109	tcagggcCCTAAAACAACAAGATCTTGATGTAG
35_CaRTT109	agtagatctATCGACGATAACCTCACAC
33_CaRTT109	atcgggcccgcggccgCGTTATCTTGCAGCATGATAG
3C_CaRTT109	CTAGAAGTTGTAGTCTCTTGTGATG
5C_CaHHF1	GTATTGTTTTGTTTGTGCCTGG
55_CaHHF1	atccgatcggtaccCTCTTTTATTCTTCCCCATC
53_CaHHF1	atcgggcccGATTAATTGATTTATTTGTAGATG
35_CaHHF1	atcagatctACTGGGTTGTATTTCTAATTGTC

33_CaHHF1	atcgggcccgcggccgcAAACTCAGTGAGCTGTTACG
3C_CaHHF1	CCTCCATCAATTGCTTCAAACC
5C_CaHHF22	CGTCTTTCTCTCTCTTGTGTAC
55_CaHHF22	atcggtaccCCAAGATGAGAATAGATGGAAC
53_CaHHF22	atcgggcccAAAGGAGAATATTAAAGCTTCAATG
35_CaHHF22	atcagatctCTTCTTTTCTTTATGTTTTTCGATTC
33_CaHHF22	atcgcggccgcTTTTCAGGCTACTTCAAAGG
3C_CaHHF22	CTCAAAGTCATACAGTCAAGCTAAC
5C_CaRAD53	TGTGTTTCATTGCTGGTTGC
55_CaRAD53	actgggcccGTTTCTTCATTAACTCCGTCACG
53_CaRAD53	gcccggtacccaattcgcccATGTAGTTTGGTAAATTAAGGGTG
35_CaRAD53	gcgcgcttggcgtaatcatggGCATATATACAAGCATTTCCTATAG
33_CaRAD53	agtctcgagCACTCAATCAAAACCTAGACGAG
3C_CaRAD53	ACCCAACATCCTTCCTCG
SATflipp_oe_fwd	GGGCGAATTGGGTACCGG
SATflipp_oe_rev	CCATGATTACGCCAAGCGC
SATflipp_5C	TTTGGAACTAACGATGCATACGAC
SATflipp_3C	CCTAACATATGTGAAGTGTGAAGGG
heukan3	CATCATCTGCCCAGATGCGAAG
Gene deletion constructs based on the fusion-PCR strategy (Noble & Johnson, 2005)	
55_CA779	AGTTACTGCTCATCAGCCATTG
53_CA779	cacggcgcgcctagcagcggCATTGATTAAGTCTTGATGTGATTG
35_CA779	gtcagcggccgcatccctgcTAAGTATATTTGTATTATTAGCTAGAAAACTAGTG
33_CA779	TAATATGACAATAACCGCCTCTG
Construction of pSFS3b and plasmids for C-terminal tagging	
pSFS BglIImut fwd	GGAACTTCAGATCTACTAGTTCTAGAGC
pSFS BglIImut rev	GCTCTAGAACTAGTAGATCTGAAGTTCC
CaACT-T_PaeI_rev	atggcatgcTTTATGATGGAATGAATGGGATG
pSFS2_NheI_fwd	atggctagcCAATCAAAGGTGGTCCTGC
AgTEF-P_PaeI_fwd	atggcatgcAGCTTGCCTCGTCCCCGC
AgTEF-T_NheI_rev	atggctagcCTGGATGGCGGCGTTAGTATCG
ADH1T_ApaI_rev	tcgagggcccATATTACCCTGTTATCCCTAGCG
pFA6a_backb_SacI_fwd	acgtgagctcCGGTCTCCCTATAGTGAGTCG
FP_KpnISmaI_fwd	atgcccgggtaccATGTCTAAAGGTGAAGAATTATTCAC
FP_AscI_rev	atcggcgcgccTTATTTGTACAATTCATCCATACCATG
9myc_XmaI_fwd	agtcccgggTCCGGTTCTGCTGCTAGTGG
9myc_AscI_rev	agtggcgcgCCACTTTTTAGCTAGTGGATCCG
Construction of tagging cassettes	
FPoe_fwd	gccgcaggtTCTAAAGGTGAAGAATTATTCAC
pFA6a-backb_rev	ccgacaccggtcgacctgcagcaGCAGGTTAACCTGGCTTATCG
35_2_CaHAT1	gctgctgcaggtcgaccggtgtcggCGGTTGATTAAGGTAGTAAACCTG
33_2 CaHAT1	CAATAACCGCCTCTGAAATATCTCC
RT5_CaHAT1	TTAGTCTTATTGTTCATTGAGGCAGG
CaHAT1-FPTag_rev	cttcacctttagaacctgcggcCACTTTTTGCTTTTTGGAAACG
INT5_CaHAT1	CTAGCTAGCTAGCCTGTTACTGAAGAAGAAGTTGATGC
5C_CaHAT2_FPTag	GACACTGAAACCAGGTACTCAC
55_2_CaHAT2_FPTag	AATACTATGCCGAGTCCTCC

53_CaHAT2-FPTag	cttcacctttagaacctgcggcCTCTAAAATCGACACATCTACTTC
35_CaHAT2-pFA	gctgctgcaggtcgaccggtgtcggAACTGGCCCTAGCTAGAG
35_CaHAT2-pSFS	gcgcttggcgtaatcatggAACTGGCCCTAGCTAGAG
33_2_CaHAT2	CCAATACCAAACCACAAAGCC
CaHAT1-9myc_oe_rev	ccactagcagcagaaccggaCACTTTTTGCTTTTTGGAAACG
9myc_CaHAT1_oe_fwd	cgtttccaaaaagcaaaaagtgTCCGGTTCTGCTGCTAGTGG
55Tag_2_RAD52	AAACGAGTCTATGGCAGTTG
53Tag_CaRAD52	gtgaataattcttcacctttagaacctgcggcTTGGTTAACAGTCGTATTAGC
33_2_RAD52	CAATGGTTTGGGTTATAGGATG
RT-qPCR (Reference)	
RT5_CaECE1	CTGTTGTTGCTGGTATCATTGCTG
RT3_CaECE1	CAACGTCATCATTAGCTCCATCTC
RT5_CaHWP1	GAAACCTCACCAATTGCTCCAG
RT3_CaHWP1	GTAGAGACGACAGCACTAGATTCC
RT5_CaSAP5	TGACAAGGCCAAGTACAGTGG
RT3_CaSAP5	GAGCACCTATGGCATAGAGAATG
RT5_CaRIP1	TGCTGACAGAGTCAAGAAACC (Hnisz *et al.* , 2010)
RT3_CaRIP1	GAACCAACCACCGAAATCAC (Hnisz *et al.* , 2010)
RT5_CaRAD6	ACGTATGCAACAAGATCCAC
RT3_CaRAD6	CAAATGGTGTATCTGAAGGTCC
RT5_CaMEC1	GACTGTTCGAGGTTCAGATGG
RT3_CaMEC1	TTCTTGCCTCTGTACTGGTG
RT5_CaRAD17	GACGAGTTTATGATTGGGTGG
RT3_CaRAD17	TTGACAACTACGCTCGACTG
RT5_CaRFX2	GTAACCCTTCAGTTCCACCC
RT3_CaRFX2	AATTTCGCAGTTTCTTTCGGAG

* Lower case and upper case letters denote exogenous and endogenous sequences, respectively.

5.2 Results: The Candida albicans *histone acetyltransferase 1 regulates stress resistance and virulence via distinct chromatin assembly pathways*

5.2.1 Introduction

Eukaryotic chromatin is densely packed with the nucleosome being its basic repeating unit (Luger *et al.*, 1997). This structure represents a barrier for enzymes reading or modifying genomic DNA. Thus, disassembly and reassembly of histones, the components of nucleosomes, within chromatin is essential for various biological processes including transcription, replication and DNA repair (Chen *et al.*, 2008, Franco *et al.*, 2005, Tsukuda *et al.*, 2005, Schwabish & Struhl, 2004, Kaplan *et al.*, 2003, Saunders *et al.*, 2003). Under acute environmental stress conditions, expression of stress genes changes rapidly requiring massive chromatin reorganization at promoters and coding sequences (Klopf *et al.*, 2009, Zhao *et al.*, 2005). Interestingly, several studies found connections between chromatin-remodelling factors and repression of stress-induced as well as stress-repressed loci (Stevenson & Liu, 2013, Zunder & Rine, 2012, Klopf *et al.*, 2009, Shivaswamy & Iyer, 2008, Zabaronick & Tyler, 2005). Incorporation of histones into chromatin is essential for efficient gene repression. Increasing the histone density can hamper binding of activators and inhibit RNA polymerase progression (Mellor, 2006, Kristjuhan & Svejstrup, 2004, Schwabish & Struhl, 2004).

Two key players in the deposition of histones are type A histone acetyltransferases (HATs) and histone chaperones. Type A HATs specifically acetylate free histones immediately after synthesis and show at least partial cytoplasmic localisation. Hat1 was the first type A HAT identified and was found to be conserved throughout the eukaryotic kingdom. (Parthun, 2012, Parthun *et al.*, 1996). Together with a subunit called Hat2 in *Saccharomyces cerevisiae* (RbAp46/48 in higher eukaryotes) this enzyme is able to acetylate histone H4 at lysine 5 and 12 (Parthun *et al.*, 1996). After binding of an additional subunit in the nucleus the so-called NuB4 complex is formed which is responsible for histone deposition at sites of DNA damage (Parthun, 2007, Poveda *et al.*, 2004). Rtt109 is another fungal-specific HAT involved in DNA damage repair associated histone deposition by acetylating free histone H3 at lysine 56 (Chen *et al.*, 2008, Driscoll *et al.*, 2007). Interestingly, telomeric silencing is also defective in *S. cerevisiae and Schizosaccharomyces pombe hat1*Δ mutants (Tong *et al.*, 2012, Kelly *et al.*, 2000). Thus, Hat1 is involved in the generation or maintenance of repressive chromatin structures.

Hat1 functions together with various histone chaperones (Yang *et al.*, 2013, Campos *et al.*, 2010, Barman *et al.*, 2008). This class of proteins is able to bind histones thereby avoiding unspecific binding to DNA and facilitating correct incorporation into nucleosomes (Das *et al.*, 2010). Two main chromatin assembly pathways include the hallmark histone chaperone complexes CAF-1 and HIR. While CAF-1 is well known to function in replication-coupled chromatin assembly recent reports

indicate also a role in transcription regulation (Yu *et al.*, 2013, Huang *et al.*, 2007, Shibahara & Stillman, 1999). In contrast, HIR is involved in replication-independent chromatin assembly and is a repressor of histone genes outside of S-phase (Spector *et al.*, 1997). Hat1 was found in complexes together with CAF-1 as well as HIR suggesting a central role in the delivery of newly synthesized histones for incorporation via distinct pathways (Yang *et al.*, 2013, Barman *et al.*, 2008).

In the fungal pathogen *Candida albicans* Hat1 is required for efficient repair of endogenous as well as exogenous DNA damages (Tscherner *et al.*, 2012). Reactive oxygen species (ROS) produced by immune cells to kill the pathogen can cause DNA damage (Salmon *et al.*, 2004). Furthermore, lack of DNA damage repair proteins as well as the histone acetyltransferase Rtt109 in *C. albicans* can reduce resistance to killing by immune cells as well as clearance by the host immune system (Lopes da Rosa *et al.*, 2010, Hao *et al.*, 2009, Chauhan *et al.*, 2005). Thus, in this work we aimed to determine the role of Hat1 in oxidative stress resistance and virulence in *C. albicans*. Surprisingly, we could show that loss of Hat1 or Hat2 leads to increased oxidative stress resistance. Furthermore, reduced susceptibility of the *hat1*Δ/Δ strain to H_2O_2 is accompanied by hyperinduction of oxidative stress genes. Deletion of *CAC2*, a component of the CAF-1 complex mimics this phenotype suggesting a negative role in transcriptional regulation for Hat1 and CAF-1. Furthermore, genome-wide transcriptional profiling revealed that cells lacking Hat1 or Cac2 are indeed defective in efficient repression of specific groups of genes in the absence and presence of H_2O_2. As expected due to the increased oxidative stress resistance *hat1*Δ/Δ cells are also more resistant to killing by murine neutrophils. Furthermore, cells lacking Hat1 are not cleared from infected mice. However, *hat1*Δ/Δ cells are severely attenuated in virulence. Interestingly, we could also identify Hat1 and the HIR complex as negative regulators of azole tolerance in *C. albicans*. Thus, our data strongly suggest that Hat1 has a central and novel role in transcriptional regulation of stress genes via distinct chromatin assembly pathways which seems to be specific for *C. albicans*.

5.2.2 Deletion of NuB4 components increases H_2O_2 resistance and azole tolerance

In a previous study, we identified the *C. albicans* NuB4 complex being essential for efficient repair of exogenous as well as endogenous DNA damage (Tscherner *et al.*, 2012). *C. albicans* DNA damage repair mutants show increased susceptibility to ROS produced by immune cells to kill the pathogen. Therefore, we asked if inactivation of the NuB4 complex would render this pathogen more susceptible to H_2O_2. However, deletion of *HAT1* or *HAT2* increased the resistance to H_2O_2 determined by spot dilution assays (Fig. 5-1A). Importantly, reintegration of *HAT1* or *HAT2* at its endogenous locus fully restored the wild-type phenotype (Fig. 5-1A).

Due to this unexpected resistance phenotype we performed a phenotypic screening and applied a set of different stress conditions including cell wall stress (CFW, CR), osmotic stress (NaCl), oxidative stress (tBOOH), heavy metal stress ($CdCl_2$) as well as antifungals (Voric., Itrac., AmB) to the *hat1*Δ/Δ mutant (Fig. 5-1). For most conditions we did not observe any difference between the wild-type and the *hat1*Δ/Δ strain (Fig. 5-1B). However, lack of Hat1 increased the resistance to the oxidizing agent tert-butyl hydroperoxide (tBOOH) indicating a specific role for Hat1 in the regulation of oxidative stress resistance (Fig. 5-1C). Interestingly, we also observed that deletion of *HAT1* increased the tolerance to different azoles, a widely used class of antifungals targeting the lanosterol 14-α-demethylase thereby blocking the ergosterol biosynthesis (Fig. 5-1D). Furthermore, deletion of *HAT2* or *HAT1* and *HAT2* mimicked deletion of *HAT1* and reintegration of both genes at their endogenous loci fully restored the wild-type phenotype (Fig. 5-1D). To confirm that the observed resistance phenotypes are independent of NuB4s function in DNA damage repair we determined sensitivities of different DNA damage repair mutants to H_2O_2 and voriconazole. Importantly, neither deletion of the gene encoding the histone H3 specific acetyltransferase *RTT109* nor the absence of the repair protein Rad52 yielded in comparable oxidative stress or voriconazole resistance (Fig. 5-1C and E). Therefore, we conclude that these phenotypes are independent of NuB4s role in DNA damage repair. These data suggest that Hat1 has an additional and novel function in *C. albicans* in the regulation of stress resistance.

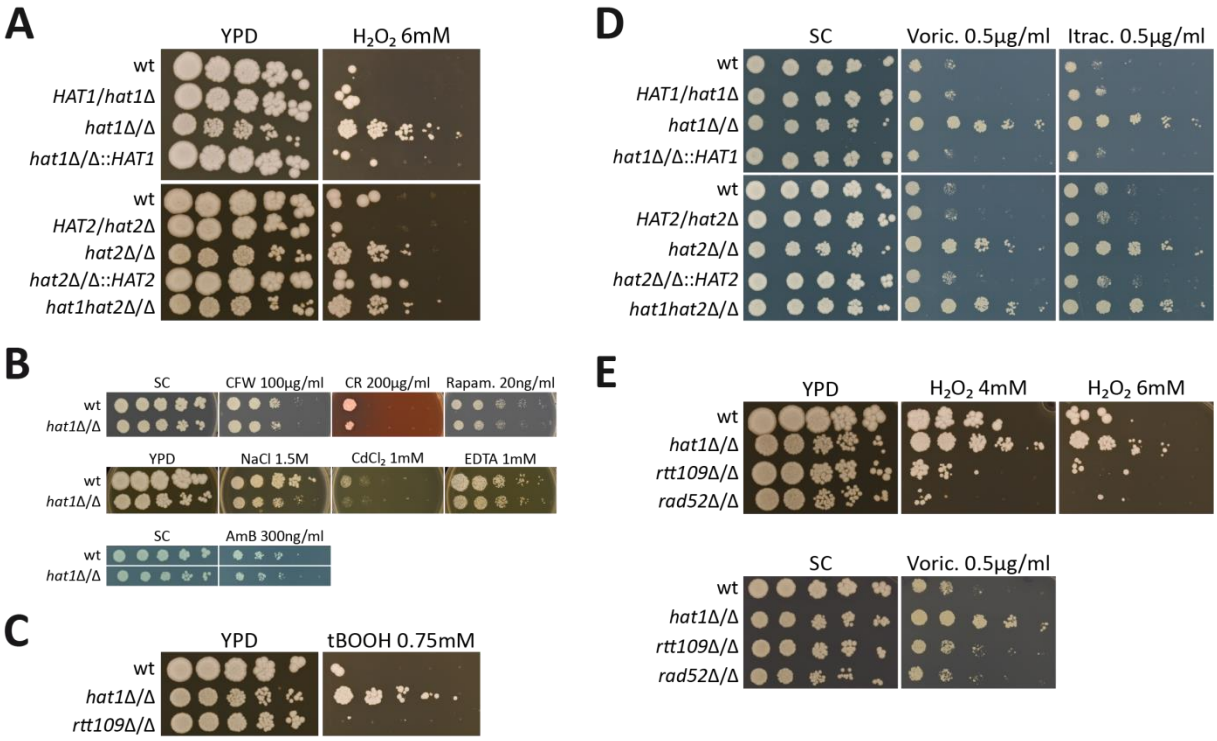

Figure 5-1: Deletion of *HAT1* and *HAT2* increases oxidative stress resistance and azole tolerance.
A. Cells lacking Hat1 or Hat2 show increased resistance to H_2O_2. Lack of both genes mimics the corresponding single deletion strains.
B. Resistance to other stress conditions is unchanged in *hat1*Δ/Δ cells. CFW: Calcofluor White; CR: Congo Red; Rapam.: Rapamycin; AmB: Amphotericin B;
C. Deletion of *HAT1* increases resistance to tert-butyl hydroperoxide (tBOOH). Lack of Rtt109 does not affect tBOOH sensitivity.
D. Loss of Hat1 causes reduced susceptibility to Voriconazole (Voric.) and Itraconazole (Itrac.). Deletion of *HAT2* or *HAT1* and *HAT2* mimics loss of Hat1.
E. Deletion of *RTT109* or *RAD52* does not increase H_2O_2 resistance or azole tolerance.
A-E. Fivefold serial dilutions were spotted on agar plates containing the indicated substances and pictures were taken after incubation at 30°C for 3 days.

5.2.3 Inactivation of chromatin assembly pathways mimics lack of Hat1

Hat1 is involved in the deposition of histone H3-H4 dimers at sites of DNA damage as well as in heterochromatic regions in different species. Different histone chaperones are responsible for the incorporation of histones into nucleosomes via distinct chromatin assembly pathways. We speculated that a defect in chromatin assembly is causing the observed resistance phenotypes. Therefore, we aimed to create deletion mutants of a set of genes encoding histone chaperones or histone chaperone complex components known to interact or copurify with Hat1 or to regulate gene expression in other species (Table 5-1). Genes were deleted in a wild-type as well as a *hat1*Δ/Δ background and the mutant's sensitivities to H_2O_2 and voriconazole were examined.

Table 5-1: Selected histone chaperone genes for deletion in *C. albicans*.
Histone chaperone genes chosen for deletion are listed. +/- indicates successful deletion.

Gene Name	ORF number	Complex	Deleted	Reference
CAC2	orf19.6670	CAF-1	+	(Zhang *et al.*, 2012, Barman *et al.*, 2008)
HIR1	orf19.2099	HIR/HIRA	+	(Yang *et al.*, 2013)
RTT106	orf19.1177		+	(Silva *et al.*, 2012, Zunder & Rine, 2012)
ASF1	orf19.3715		-	(Campos *et al.*, 2010, Barman *et al.*, 2008)
SPT16	orf19.2884	FACT	-	(Nair *et al.*, 2011)
SPT6	orf19.7136			(Klopf *et al.*, 2009, Adkins & Tyler, 2006)

Strikingly, deletion of *CAC2*, a subunit of the CAF-1 complex, also increased the resistance to H_2O_2 and tBOOH (Fig. 5-2A). Furthermore, quantification of the H_2O_2 resistance by determination of the survival rate in liquid culture confirmed this result (Fig. 5-2B). However, deletion of *CAC2* had only a minor effect on the azole susceptibility determined by spot dilution assays (Fig. 5-2C). In contrast, deletion of *HIR1*, a component of the HIR complex, dramatically increased the tolerance to voriconazole, but did not alter the H_2O_2 susceptibility (Fig. 5-2A a and C). Importantly, both genes belong to the same epistasis group since the double deletion strain did not show increased azole resistance compared to the corresponding single deletions determined by spot dilution assays and growth inhibition in liquid culture (Fig. 5-2C and D).

Deletion of *RTT106* did not alter susceptibility to H_2O_2 and voriconazole (Fig 5-2A and C). Unfortunately, we and others could not obtain homozygous deletion mutants for Asf1 and Spt16 indicating that they might be essential (Buurman *et al.*, 2002, Davis *et al.*, 2002). Successful deletion of the gene encoding the histone chaperone Spt6 in *C. albicans* was already described (Al-Rawi *et al.*, 2010). We included this strain in our analysis and found out, that deletion of *SPT6* increased H_2O_2 resistance. Reliable determination of the azole sensitivity of the homozygous *spt6* mutant was not possible due to its extremely slow growth rate. However, deletion of one *SPT6* allele also decreased the susceptibility to voriconazole (Fig. 5-2E). In a previous study, we showed that a strain with reduced histone H4 gene dosage mimics deletion of *HAT1*. Interestingly, the strain carrying only a single copy of the histone H4 gene also showed increased H_2O_2 resistance and elevated tolerance to voriconazole (Fig. 5-2F). These data indicate that defects in chromatin assembly can lead to increased resistance to H_2O_2 and voriconazole. Furthermore, distinct histone deposition pathways regulate the resistance phenotypes observed upon deletion of *HAT1*.

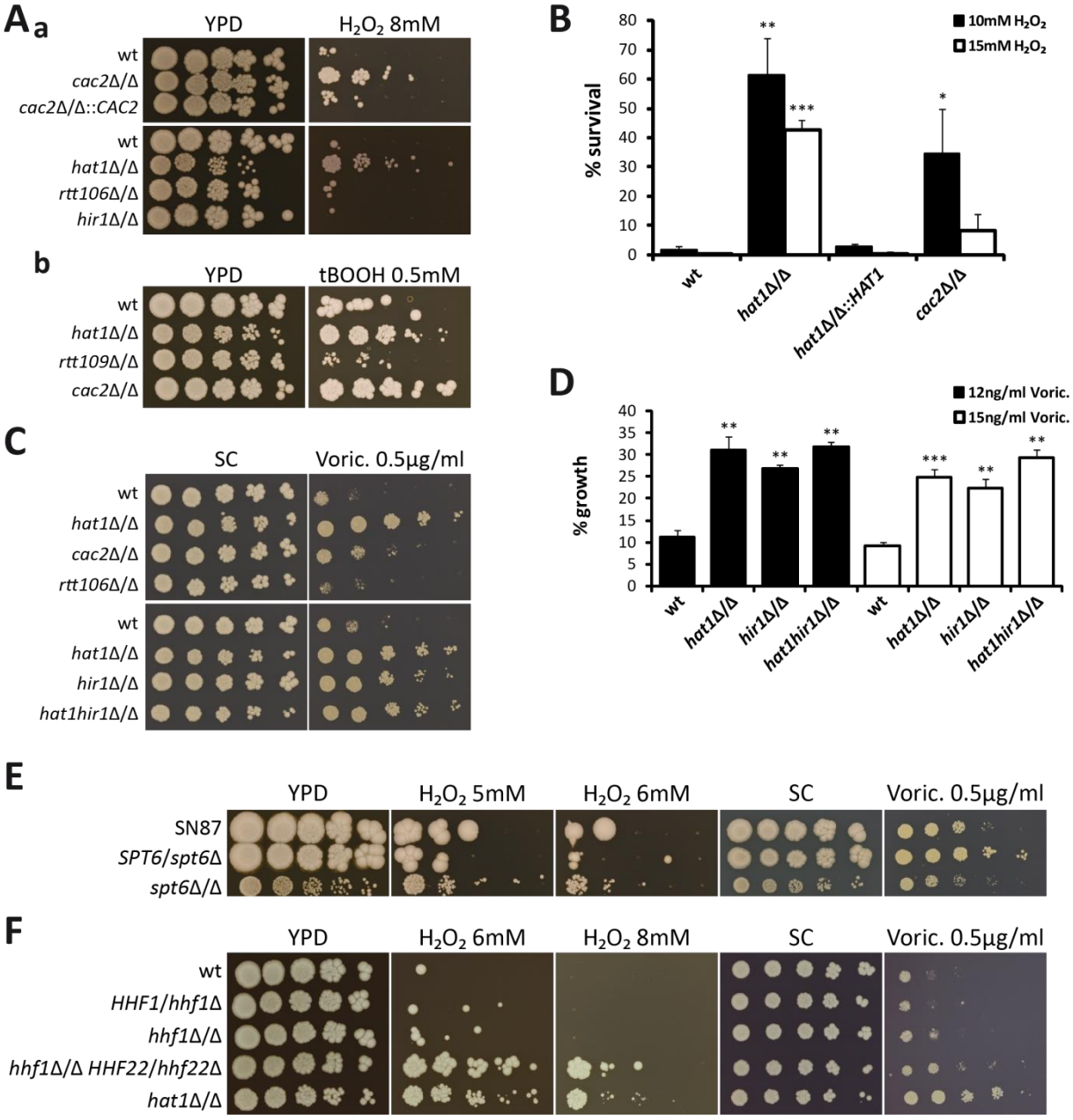

Figure 5-2: Lack of histone chaperones mimics deletion of *HAT1*.

A. Loss of Cac2 increases H_2O_2 resistance. Deletion of *RTT106* or *HIR1* does not affect susceptibility to hydrogen peroxide (a). Cells lacking Cac2 show increased resistance to tert-butyl hydroperoxide (tBOOH). Loss of Rtt109 does not affect tBOOH resistance (b). Fivefold serial dilutions were spotted on agar plates containing the indicated substances and pictures were taken after incubation at 30°C for 3 days.

B. Deletion of *HAT1* or *CAC2* increases survival to transient hydrogen peroxide treatment. Exponentially growing cells were treated with the indicated concentrations of H_2O_2 for 2 hours. Cells were plated and colonies counted after 3 days of incubation on YPD plates at 30°C to determine viability. Data are shown as mean + SD from three independent experiments.

C. Deletion of *HIR1* reduces voriconazole (Voric.) susceptibility. The *hat1hir1*Δ/Δ double deletion strain mimics lack of Hat1. Loss of Cac2 has only a minor effect and deletion of *RTT106* does not alter azole susceptibility. Experiment was performed as described in A.

D. Increased azole tolerance of *hat1*Δ/Δ, *hir1*Δ/Δ and *hat1hir1*Δ/Δ was confirmed using a liquid growth inhibition assay. Logarithmically growing cells were diluted into medium containing the indicated concentrations of Voriconazole (Voric.) and incubated at 30°C for 18 hours. OD_{600} was determined and growth inhibition relative to untreated samples was calculated. Data are shown as mean + SD from three independent experiments.

E. Deletion of *SPT6* increases H_2O_2 resistance and azole tolerance. Fivefold serial dilutions were spotted on agar plates containing the indicated substances and pictures were taken after incubation at 30°C for 5 days.

F. Reduction of histone gene dosage decreases H_2O_2 and azole susceptibility. Experiment was performed as described in A.

B and D. **P*<0.05, ***P*<0.01 and ****P*<0.001 relative to wild-type (Student's t-test).

5.2.4 Resistance phenotypes upon loss of Hat1 are specific to *C. albicans*

Next we wanted to determine if the role of Hat1 in regulating oxidative stress resistance and azole tolerance is conserved in other species. Therefore, we analysed the effect of *HAT1* deletion on H_2O_2 and voriconazole resistance in *Saccharomyces cerevisiae*. However, loss of Hat1 did not lead to increased resistance to H_2O_2 or voriconazole (Fig. 5-3A). Furthermore, deletion of *CAC2* or *HIR1* did not lower the sensitivity to H_2O_2 and voriconazole in *S. cerevisiae*. Only loss of Rtt109 and Asf1 had a minor, if any, effect on H_2O_2 sensitivity, but did not increase azole tolerance (Fig. 5-3A). Next we assessed the effect of *HAT1* deletion in another fungal pathogen, *Candida glabrata*. As for *S. cerevisiae,* loss of Hat1 did not increase resistance to H_2O_2 or voriconazole (Fig. 5-3B). Furthermore, no phenotype was observed upon deletion of *HAT2* in *C. glabrata*. Finally, lack of Hat1 in *Schizosaccharomyces pombe* did not lead to a comparable resistance phenotype as observed in *C. albicans* either (Fig. 5-3C). Thus, the role of Hat1 in regulating oxidative stress resistance and azole tolerance is not widely conserved and seems to be specific for *C. albicans* and perhaps more closely related *Candida* species.

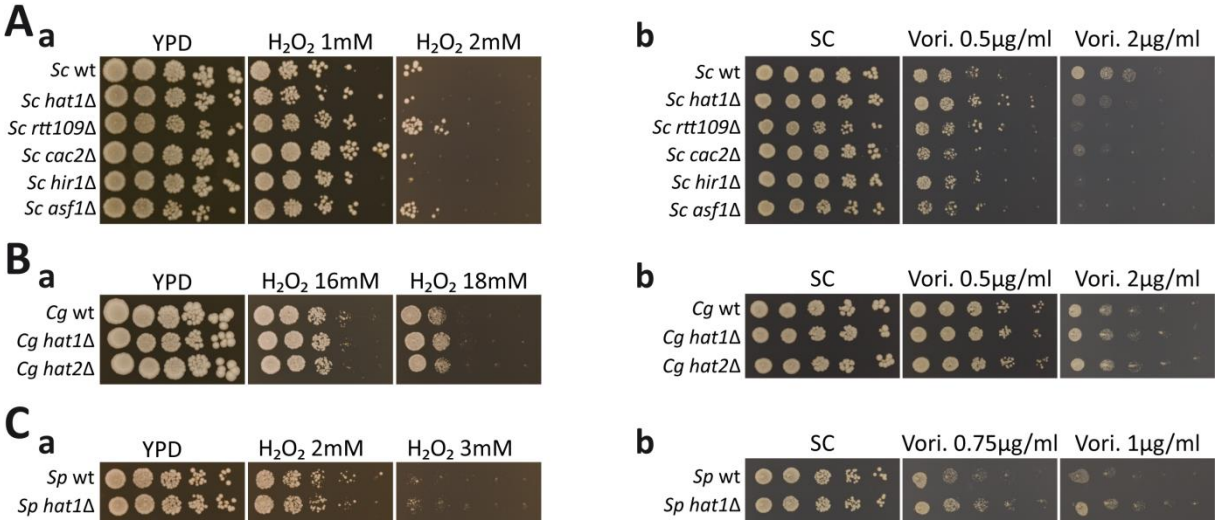

Figure 5-3: Resistance phenotypes caused by loss of Hat1 are specific for *C. albicans*.
A. Deletion of *HAT1* or histone chaperones in *S. cerevisiae* has no effect on H_2O_2 resistance (a). Lack of *S. cerevisiae* Hat1 or histone chaperones does not increase azole tolerance (b). Fivefold serial dilutions were spotted on agar plates containing the indicated substances and pictures were taken after incubation at 30°C for 3 days.
B. Deletion of *HAT1* or *HAT2* in *C. glabrata* has no effect on H_2O_2 resistance (a) or azole tolerance (b). Experiment was performed as described in A.
C. Loss of Hat1 in *S. pombe* does not increase H_2O_2 resistance (a) and has only a minor effect on azole susceptibility (b). Experiment was performed as described in A.

5.2.5 Deletion of *HAT1* and *CAC2* causes hyperinduction of oxidative stress genes

We speculated that the increased H_2O_2 resistance upon deletion of *HAT1* as well as *CAC2* might be due to derepression or elevated induction levels of oxidative stress genes. Therefore, we examined transcription levels of the *CAT1* gene encoding the catalase, which is the main enzyme responsible for detoxification of H_2O_2.

We detected derepression of *CAT1* upon deletion of *HAT1* as well as *CAC2* under non-inducing conditions (Fig. 5-4A). However, upregulation of *CAT1* under non-inducing conditions was also detected for a *rtt109*Δ/Δ control strain, which does not show increased resistance to H_2O_2. Thus, this moderate derepression of *CAT1* is most likely due to a general stress response in these deletion mutants. Furthermore, to assess the induction levels we added 1.6 mM H_2O_2 to the cultures and examined *CAT1* expression over time. Importantly, no loss of viability was observed even after 1 hour treatment with this concentration (Fig. 5-4B). In wild-type cells the maximum induction level was reached after 45 min exposure and started to decline afterwards. Interestingly, lack of Hat1 as well as Cac2 resulted in faster induction as well as increased maximum expression levels indicating that these two proteins negatively regulate the induction kinetics of *CAT1* (Fig. 5-4C). However, loss of Hat1 had a more profound effect on *CAT1* expression than lack of Cac2.

In addition, we also determined expression levels of *OYE22*, *PST3* and *OYE23*, three known oxidative stress induced genes (Enjalbert *et al.*, 2006, Wang *et al.*, 2006). These genes encode two NADPH oxidoreductases and a flavodoxin, respectively. Similar to the results obtained for *CAT1*, we observed hyperinduction of *OYE22*, *PST3* and *OYE23* in the *hat1*Δ/Δ mutant (Fig. 5-4D). Again, the *cac2*Δ/Δ strain showed a less pronounced phenotype. This result correlates with reduced H_2O_2 resistance of the *cac2*Δ/Δ strain compared to the *hat1*Δ/Δ mutant (Fig. 5-2B). Importantly, we could not detect any difference in the *CAT1* induction level between the wild-type and the *rtt109*Δ/Δ control strain (Fig. 5-4E). Furthermore, we also wanted to know, if the observed increase in *CAT1* transcription also leads to elevated catalase activity in the mutants, which could explain the resistance to H_2O_2. Therefore, we prepared whole cell extracts from cells before and after 60 min exposure to H_2O_2 and determined catalase activity spectrophotometrically. We could detect elevated enzyme activity for both mutants already under non-inducing conditions, although as expected at a low level (Fig. 5-4F). Furthermore, upon H_2O_2 induction *hat1*Δ/Δ as well as *cac2*Δ/Δ cells showed increased catalase activity when compared to the wild-type indicating that these two mutants are more efficient in the degradation of hydrogen peroxide (Fig. 5-4F).

In agreement with the assumption that increased induction levels of oxidative stress genes cause the observed resistance phenotype, *hat1*Δ/Δ cells are still more resistant to H_2O_2 even after pretreatment with a low dose of hydrogen peroxide (Fig. 5-4G). Furthermore, to determine if catalase hyperinduction is the main reason for the increased resistance of the *hat1*Δ/Δ mutant we

deleted the *CAT1* gene in a wild-type and a *hat1*Δ/Δ background and assayed the H_2O_2 resistance of these mutants. As expected, the *cat1*Δ/Δ strain showed increased sensitivity to H_2O_2 (Fig. 5-4H). However, the double deletion strain still showed increased resistance to H_2O_2 when compared to the *cat1*Δ/Δ single mutant indicating that increased catalase expression is not the only reason for the hydrogen peroxide resistance of the *hat1*Δ/Δ strain (Fig. 5-4H). In summary, our results indicate that the NuB4 and the CAF-1 complexes negatively regulate oxidative stress gene expression in *C. albicans*. Furthermore, loss of Hat1 as well as Cac2 leads to increased H_2O_2 detoxification and resistance to hydrogen peroxide.

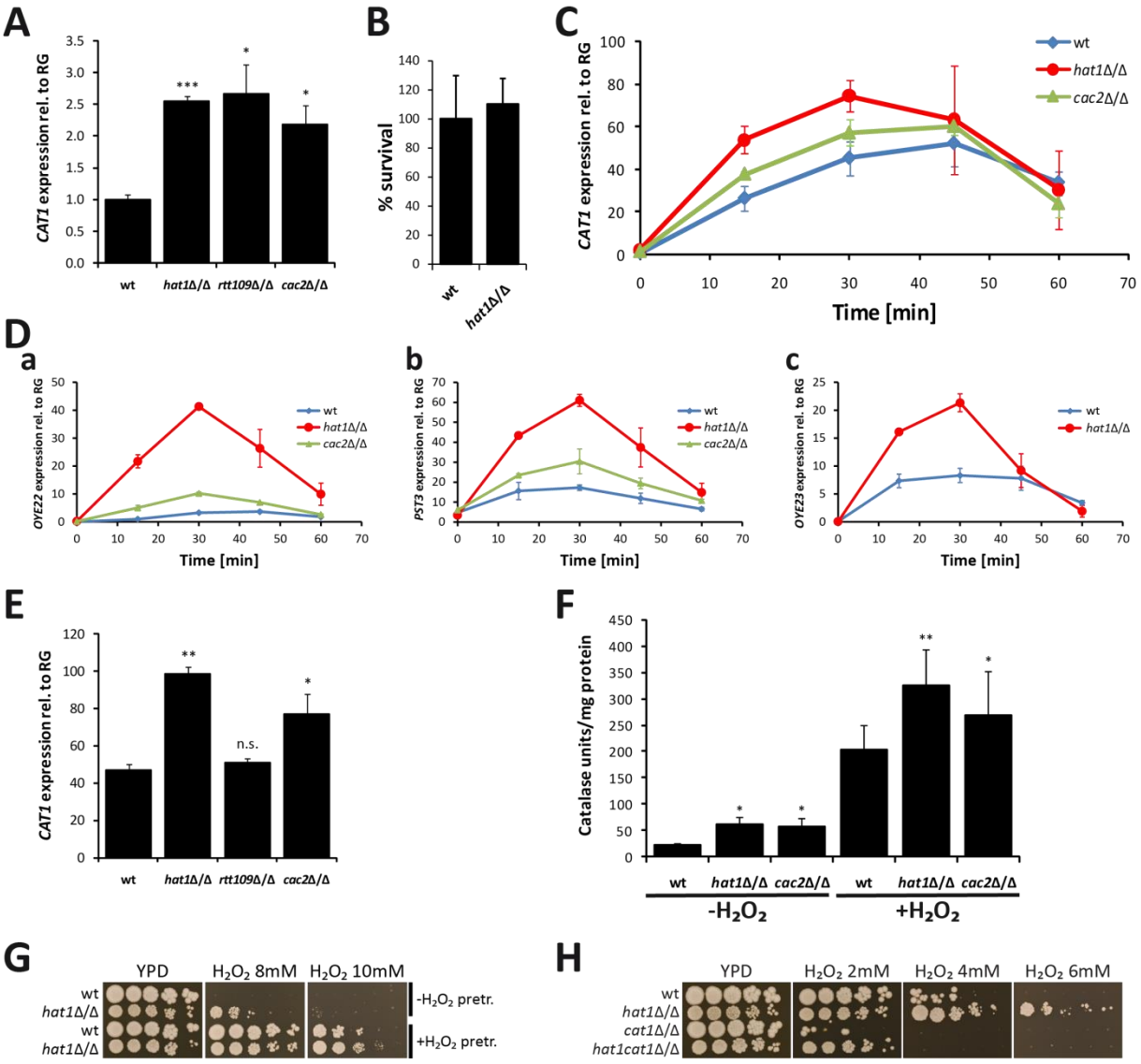

Figure 5-4: Hat1 and Cac2 negatively regulate oxidative stress genes and H_2O_2 degradation.

A. Derepression of *CAT1* in logarithmically growing cells was detected by RT-qPCR. Transcript levels were normalized to the expression level of *PAT1*. Data are shown as mean + SD from 3 independent experiments.

B. *C. albicans* does not loose viability upon treatment with 1.6 mM H_2O_2 for 60 min. Exponentially growing cells were treated. Cultures were diluted and plated onto YPD plates. Colonies were counted after 3 days of incubation at 30°C to determine viability relative to the 0 time point. Data are shown as mean + SD from three independent experiments.

C. Catalase is hyperinduced in *hat1*Δ/Δ and *cac2*Δ/Δ cells. *CAT1* expression levels were measured by RT-qPCR upon induction with 1.6 mM H_2O_2 over 60 minutes. Transcript levels were normalized to the expression level of *PAT1*. Data are shown as mean + SD from 3 independent experiments.

D. Oxidative stress genes *OYE22* (a), *PST3* (b) and *OYE23* (c) are hyperinduced in *hat1*Δ/Δ and *cac2*Δ/Δ cells. Experiment was performed as described in C. Data are shown as mean + SD from two independent experiments.

E. Increased *CAT1* induction levels were only observed for *hat1*Δ/Δ and *cac2*Δ/Δ cells, but not for cells lacking Rtt109. Cells were treated with 1.6 mM H_2O_2 for 30 min. Transcript levels were normalized to the expression level of *PAT1*. Data are shown as mean + SD from 3 independent experiments.

F. *CAT1* hyperinduction leads to increased catalase activity in *hat1*Δ/Δ and *cac2*Δ/Δ cells. Catalase activity was determined in whole cell extracts isolated from cells before and after 60 minutes treatment with 1.6 mM H_2O_2. Data are shown as mean + SD from three independent experiments.

G. Loss of Hat1 leads to increased H_2O_2 resistance even in pretreated cells. Logarithmically growing cells with or without pretreatment with 0.4 mM H_2O_2 for 60 min were spotted in fivefold serial dilutions on plates containing the indicated concentrations of hydrogen peroxide. Pictures were taken after incubation at 30°C for 3 days.

H. Loss of Hat1 causes H_2O_2 resistance even in the absence of Cat1. Cells lacking Hat1, Cat1 or both were spotted on plates containing the indicated concentrations of H_2O_2. Pictures were taken after incubation at 30°C for 3 days.

A-F. *P<0.05, **P<0.01 and ***P<0.001 relative to the corresponding wild-type (Student's t-test).

5.2.6 Expression of specific gene groups is affected in the *hat1*Δ/Δ mutant

Our data suggest that Hat1 as well as Cac2 are involved in the regulation of gene expression in *C. albicans*. To identify the complete set of genes affected by deletion of *HAT1* and *CAC2* during logarithmic growth as well as oxidative stress we determined the transcriptional profiles of *hat1*Δ/Δ and *cac2*Δ/Δ cells on a genome-wide scale. Therefore, we performed RNA-Seq analysis with cDNA prepared from cells before and after exposure to 1.6 mM H_2O_2 for 30 minutes. Furthermore, we also included the *rtt109*Δ/Δ strain as a control which mimics lack of Hat1 in terms of morphology and accumulation of DNA damage, yet it is not resistant to H_2O_2.

Without treatment the vast majority of differentially regulated genes in all of the three mutants showed increased expression when compared to the wild-type (Fig. 5-5A a). Interestingly, the *hat1*Δ/Δ strain showed the highest number of differentially regulated genes among the three mutants consistent with Hat1 being involved in multiple processes in *C. albicans*. For all of the three mutants the upregulated genes are predominantly genes with low expression levels in the wild-type (Fig. 5-5B a). Upon treatment with H_2O_2 a large set of 278 genes showed increased expression in all of the three mutants when compared to the wild-type. Interestingly, most of the upregulated genes in the mutants were repressed in the wild-type upon H_2O_2 treatment (Fig. 5-5A c and 5-5B b). Surprisingly, we found only 15 H_2O_2 induced genes with more than two-fold increased expression in the *hat1*Δ/Δ and the *cac2*Δ/Δ strains compared to the wild-type (Fig. 5-5C). Furthermore, genes with reduced expression levels in the mutants compared to the wild-type were enriched for genes induced upon H_2O_2 treatment (Fig. 5-5B b and 5-5C).

In addition to protein-coding genes we also analysed the expression of non-coding features including tRNAs and small RNAs. Among the 188 non-coding RNAs analysed 73 were repressed more than two-fold in the wild-type upon H_2O_2 treatment. Interestingly, in all of the three mutants repression of non-coding RNAs upon H_2O_2 treatment was severely affected resulting in increased transcript levels when compared to the wild-type upon treatment (Fig. 5-5D).

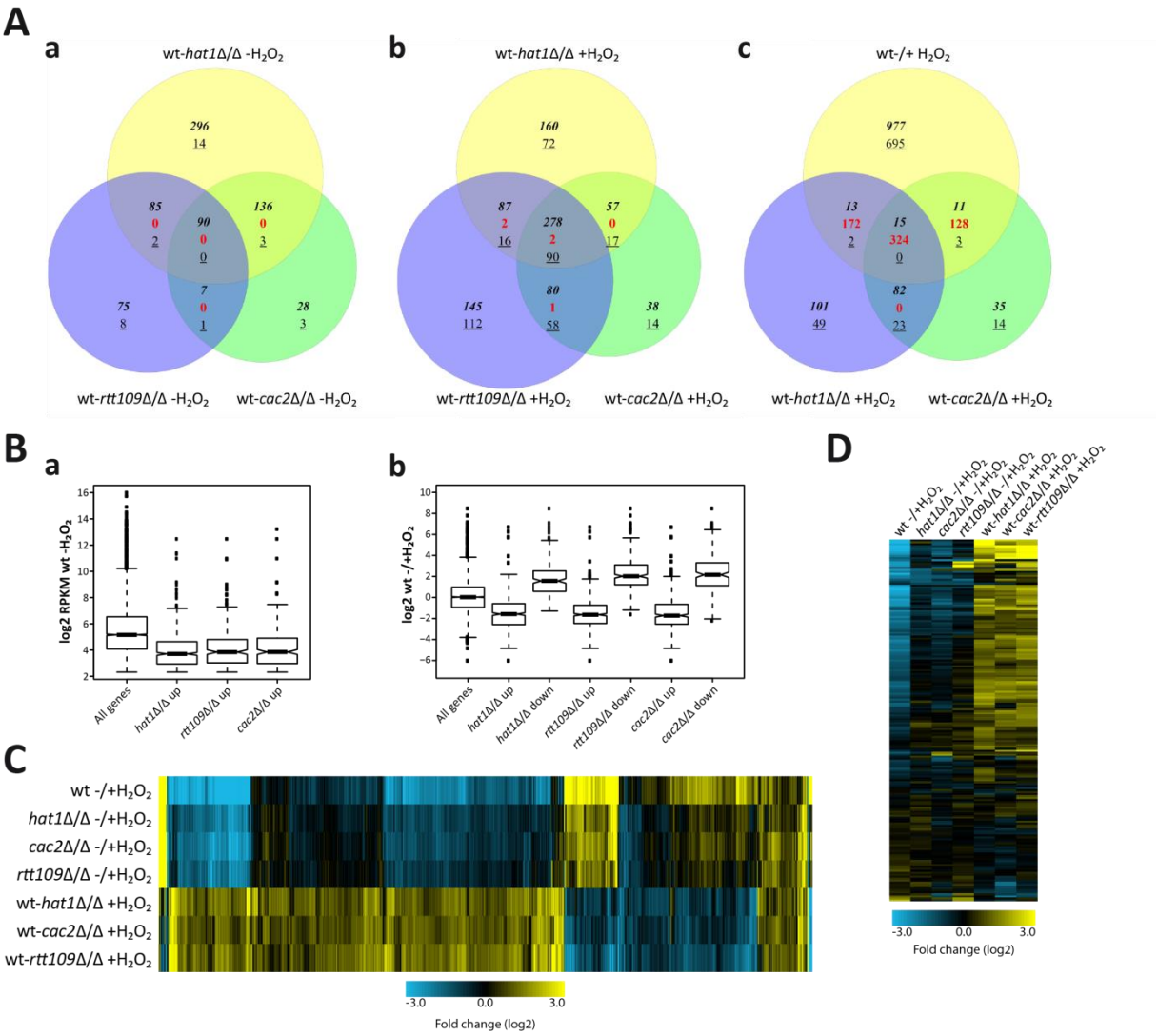

Figure 5-5: Deletion of *HAT1* leads to defects in gene repression upon oxidative stress.
A. Venn diagrams showing the overlaps of differentially regulated genes determined by RNA-Seq in the *hat1*Δ/Δ, *cac2*Δ/Δ and *rtt109*Δ/Δ mutants in the absence (a) or presence (b) of H_2O_2. The overlaps between genes regulated in the wild-type upon H_2O_2 exposure and the *hat1*Δ/Δ as well as the *cac2*Δ/Δ mutants versus the wild-type are depicted in (c). Bold numbers indicate upregulated genes and underlined numbers downregulated genes. Numbers in red indicate contraregulated genes i.e. genes upregulated in at least one condition included in the diagram and downregulated in the other.
B. Genes with increased expression in the *hat1*Δ/Δ, *cac2*Δ/Δ and *rtt109*Δ/Δ mutants are lowly expressed in the wild-type. Wild-type RPKM values of all genes or genes upregulated in the indicated mutants without treatment are shown in (a). The distribution of RNA fold changes in the wild-type upon H_2O_2 treatment of genes up- and downregulated in the indicated mutants, respectively, is shown (b).
C. Deletion of *HAT1*, *CAC2* and *RTT109* leads to defects in the repression of genes upon H_2O_2 treatment. Genes differentially regulated in at least one mutant versus the wild-type upon H_2O_2 exposure were selected for hierarchical clustering.
E. Loss of *HAT1*, *CAC2* and *RTT109* causes defects in the repression of non-coding RNAs. All non-coding RNAs (including tRNAs) were selected for hierarchical clustering.
A-D. Differentially regulated genes or non-coding RNAs were defined by a fold change >=2 and P-value <0.05.

To further characterize differentially expressed genes we performed GO term enrichment analysis. Without H_2O_2 treatment upregulated genes in the *hat1*Δ/Δ mutant were enriched for catabolic genes and oxidative stress response genes (Fig. 5-6A a). However, we could not detect enrichment for DNA damage response genes most likely due to the fact that Hat1 is involved in

different processes in *C. albicans*. This leads to a high number of differentially expressed genes in the mutant and could hamper detection of enriched GO groups. Therefore, we analysed subsets of differentially expressed genes based on their expression in the three different mutants. As expected due to their DNA damage phenotype, genes upregulated in the *hat1*Δ/Δ and the *rtt109*Δ/Δ mutants were strongly enriched for DNA damage repair genes (Fig. 5-6A b). In addition, monocarboxylic acid and lipid catabolic genes were also changed. Among the genes upregulated in the *hat1*Δ/Δ as well as the *cac2*Δ/Δ mutant were predominantly oxidative stress response genes and again catabolic genes comparable to the groups enriched in the *hat1*Δ/Δ *rtt109*Δ/Δ set (Fig. 5-6A c). Interestingly, arginine biosynthesis genes were specifically enriched in the *hat1*Δ/Δ *cac2*Δ/Δ set. As expected, genes upregulated in all of the three mutants contained the same groups of catabolic genes, that were already enriched in the *hat1*Δ/Δ *rtt109*Δ/Δ and the *hat1*Δ/Δ *cac2*Δ/Δ sets, respectively (Fig. 5-6A d). Interestingly, glyoxylate cycle genes were specifically affected in all of the three mutants. When we analysed the 296 genes upregulated only in the *hat1*Δ/Δ mutant we found that genes involved in mitochondrion degradation were still enriched as in the total *hat1*Δ/Δ set (Fig. 5-6A e).

Next, we analysed the genes with differential expression levels in the mutants relative to the wild-type upon H_2O_2 treatment. No specific GO term enrichment for the *hat1*Δ/Δ strain alone or in combination with another mutant was found for genes with reduced expression compared to the wild-type. However, in all of the three mutants especially glycolysis genes were highly enriched (data not shown). Strikingly, when we analysed genes with higher expression than the wild-type we detected strong enrichment for genes involved in non-coding RNA (ncRNA) processing as well as ribosome biogenesis for the *hat1*Δ/Δ mutant (Fig. 5-6B a). Interestingly, these genes were not enriched in the other mutants but specifically in the *hat1*Δ/Δ strain (Fig. 5-6B b and c). Groups enriched in all of the mutants contained genes involved in transport processes (Fig. 5-6B b).

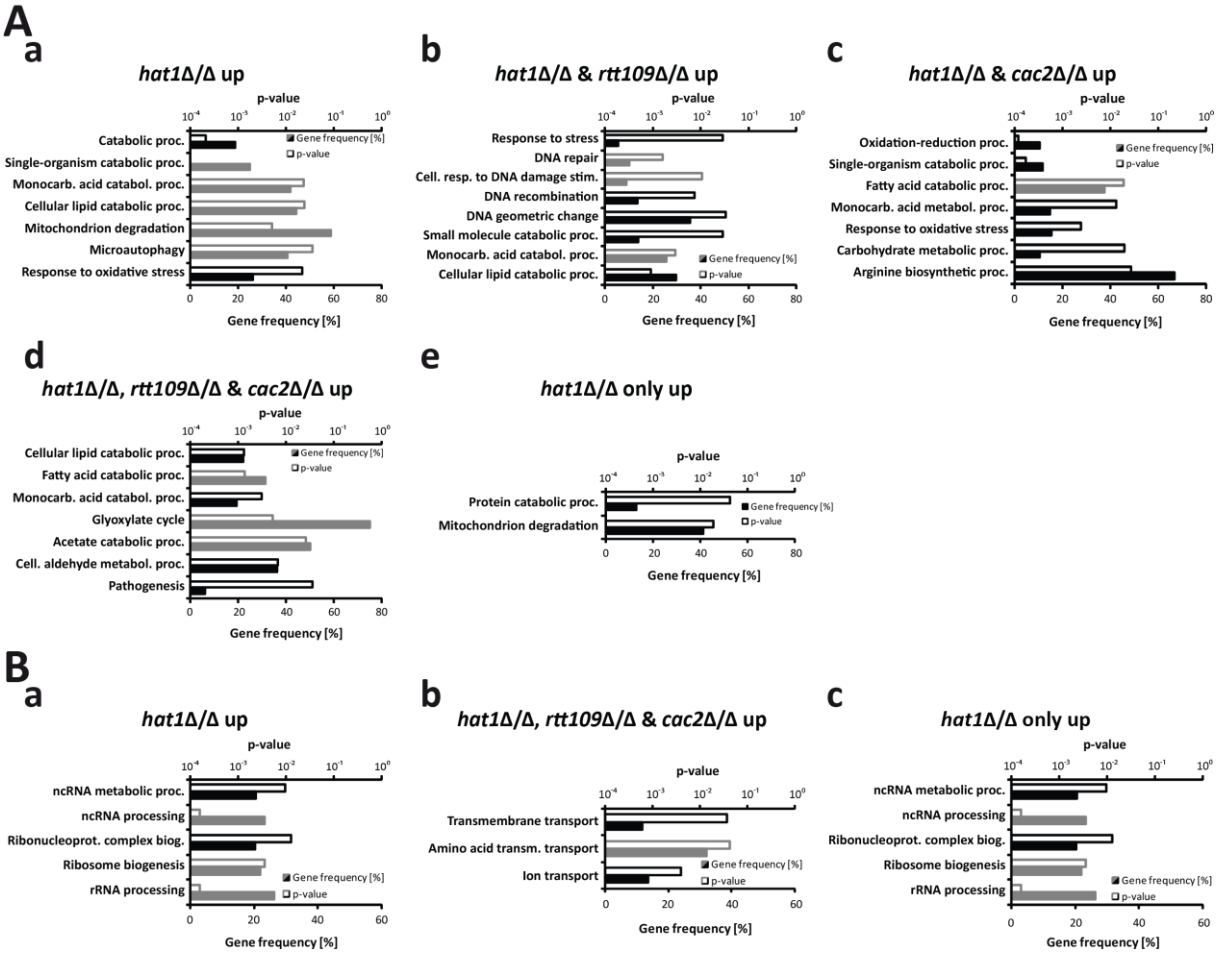

Figure 5-6: Specific functional gene groups are upregulated in cells lacking Hat1.

A. Specific GO terms are enriched in the absence of H_2O_2 within genes upregulated in the *hat1*Δ/Δ strain alone or in the overlap with the *cac2*Δ/Δ and *rtt109*Δ/Δ strain, respectively. Enriched GO terms without H_2O_2 treatment within all genes upregulated in the *hat1*Δ/Δ mutant (a), within genes commonly upregulated in two or all three of the mutants (b-d) as well as within genes upregulated only in the *hat1*Δ/Δ mutant (e) and not in the other strains are presented.

B. GO terms enriched upon H_2O_2 treatment within all genes upregulated in the *hat1*Δ/Δ mutant (a), within genes upregulated in all of the three mutants (b) and within genes upregulated only in the *hat1*Δ/Δ strain (c) are shown. The corresponding *P*-values (empty bars) and the percentage of genes changed within the GO group (filled bars) are presented. Black colour indicates main GO groups and grey colour indicates subgroups of the main group above.

A-B. Differentially regulated genes were defined by a fold change >=2 and P-value <0.05. proc.: process; monocarb.: monocarboxylic; catabol.: catabolic; cell.: cellular; resp.: response; stim.: stimulus; metabol.: metabolic; ribonucleoprot.: ribonucleoprotein; biog.: biogenesis; transm.: transmembrane;

In summary, loss of Hat1 mainly leads to increased expression of genes belonging to different functional groups, which is in accordance with Hat1 being involved in different processes in *C. albicans*. Furthermore, distinct functional groups affected by the loss of Hat1 are also upregulated in either the *rtt109*Δ/Δ or the *cac2*Δ/Δ strain indicating that Hat1 might function in specific processes together with Rtt109 or Cac2.

5.2.7 Hat1 regulates ROS detoxification and neutrophil survival

The absence of Hat1 and Cac2 leads to upregulation of oxidative stress genes and resistance to hydrogen peroxide. ROS is produced by immune cells to kill *C. albicans* during the infection process (Frohner *et al.*, 2009). The fungus counteracts this attack by upregulating ROS detoxifying enzymes of which the superoxide dismutases Sod5 and Sod4 were shown be essential for survival of *C. albicans* upon phagocytosis (Frohner *et al.*, 2009). Interestingly, our RNA-Seq data revealed upregulation of both Sods in cells lacking Hat1 or Cac2. Induction of *SOD4* and *SOD5* in the *hat1*Δ/Δ strain was also confirmed by qPCR analysis (Fig. 5-7A). Therefore, we asked if the deletion of *HAT1* or *CAC2* influences the ability to detoxify and survive ROS produced by immune cells.

ROS production during interaction of *C. albicans* with bone-marrow derived murine macrophages was determined by measuring luminol dependent chemiluminescence (Frohner *et al.*, 2009). Interestingly, ROS production was significantly reduced upon deletion of *HAT1* or *CAC2* compared to the wild-type (Fig. 5-7B and C). Furthermore, deletion of *SOD5* encoding the enzyme mainly responsible for superoxide detoxification in a *hat1*Δ/Δ background dramatically increased the ROS levels in the interaction. However, ROS levels upon interaction with the *hat1sod5*Δ/Δ double mutant did not reach the levels of the *sod5*Δ/Δ mutant (Fig. 5-7C). Thus, it might be possible that less ROS is produced by the macrophages upon interaction with the *hat1*Δ/Δ mutant. However, the observed upregulation of Sod4 could also account for the difference in ROS accumulation between *sod5*Δ/Δ and *hat1sod5*Δ/Δ strains. Furthermore, increased expression of the glutathione reductase Grx2 in *hat1*Δ/Δ cells could also be responsible for the reduced ROS levels (data not shown) (Miramon *et al.*, 2012).

Phagocytosis is required for ROS production by the NADPH oxidase (Frohner *et al.*, 2009). To exclude that a difference in phagocytosis of the pseudohyphal *hat1*Δ/Δ cells accounts for the observed reduction in ROS accumulation, we performed a phagocytosis assay. We could not detect any differences in the rate of phagocytosis upon infection with the *hat1*Δ/Δ strain or the wild-type strain (Fig. 5-7D). Furthermore, we analysed NADPH oxidase activation in macrophages upon interaction of wild-type and mutant cells by western blot analysis. However, we observed no difference in the phosphorylation levels of the NADPH oxidase subunit p40phox upon interaction with *hat1*Δ/Δ cells or wild-type cells (Fig. 5-7E). Therefore, reduced ROS accumulation upon interaction with *hat1*Δ/Δ cells most likely resembles increased ROS detoxification by the mutant. Interestingly, reduction of histone levels mimicked the phenotype observed for the *hat1*Δ/Δ and *cac2*Δ/Δ mutants demonstrated by a reduced ROS accumulation upon interaction with a single copy histone H4 strain (Fig. 5-7B). In addition, interaction of macrophages with a *rtt109*Δ/Δ mutant did not lead to significant changes in the ROS levels compared to the wild-type (Fig. 5-7B). Importantly, this mutant grows in a constitutively pseudohyphal morphology due to accumulating DNA damages

similar to the *hat1*Δ/Δ strain (Lopes da Rosa *et al.*, 2010). These data and the fact that the *cac2*Δ/Δ mutant does not show any morphology defects strongly argue against the possibility that the observed reduction in ROS accumulation is due to an indirect effect caused by altered cell morphology.

To determine the resistance to killing by immune cells we used murine bone-marrow neutrophils, because our wild-type *C. albicans* strain is not efficiently killed by ROS produced by macrophages in our experimental setup (Frohner *et al.*, 2009). Furthermore, we observed the same reduction in ROS accumulation upon interaction with neutrophils (Fig. 5-7F). Interestingly, while only about 35% of the wild-type cells were able to survive a 1 hour interaction with neutrophils, about 55% of the *hat1*Δ/Δ cells were still alive at the end of the experiment (Fig. 5-7G). Furthermore, reintegration of *HAT1* fully restored the wild-type phenotype. These data strongly suggest that *HAT1* deletion causes increased neutrophil survival due to reduced ROS accumulation in the interaction.

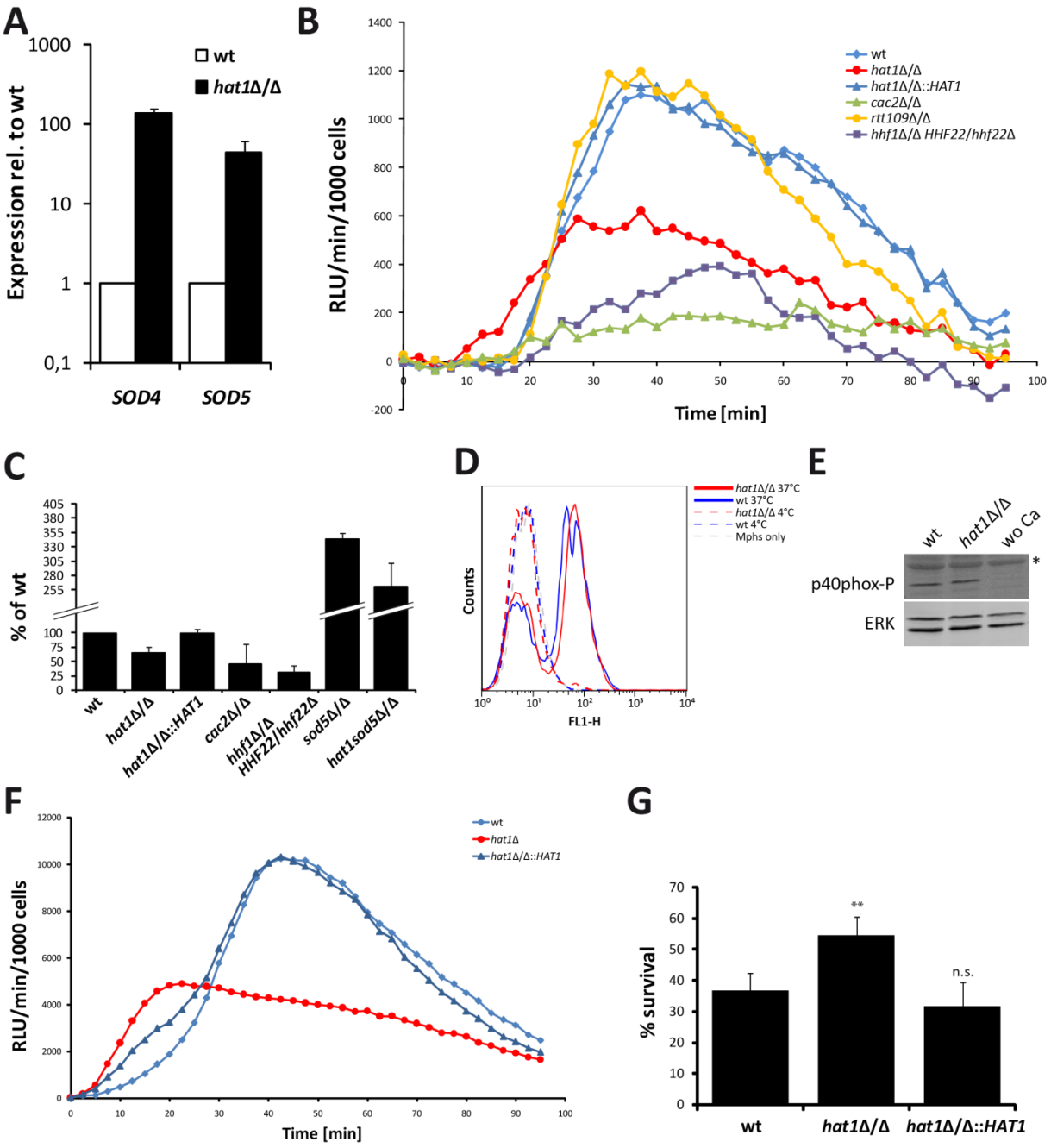

Figure 5-7: Higher ROS detoxification capacity of *hat1*Δ/Δ cells causes resistance to neutrophil killing.

A. Superoxide dismutases Sod4 and Sod5 are induced in *hat1*Δ/Δ cells. Expression levels of *SOD4* and *SOD5* in logarithmically growing cells were detected by RT-qPCR. Transcript levels are shown as fold change to wild-type. Data are shown as mean + SD from 3 independent experiments.

B. Infection of macrophages with *hat1*Δ/Δ or *cac2*Δ/Δ cells causes reduced ROS accumulation. ROS levels were determined by measuring luminol-dependent chemiluminescence [relative luciferase units (RLU) min^{-1} per 1000 immune cells] in 2.5 min intervals during interaction of the indicated *C. albicans* strains with bone marrow-derived murine macrophages (BMDMs). One representative experiment is shown. Data were reproduced in three independent experiments.

C. Quantification of total ROS release upon interaction with BMDMs. Experiment was performed as described in B. The area under the curve within 90 min of interaction was calculated. Data are shown as mean + SD from three independent experiments.

D. Cells lacking Hat1 are phagocytosed at the same rate as the wild-type. Phagocytosis was quantified by measuring the fraction of BMDMs containing labelled *C. albicans* cells upon 45 min interaction at 37°C (5% CO_2) by FACS. Control reactions were kept at 4°C. BMDMs without *C. albicans* were also included (Mphs only). One representative experiment is shown. Data were reproduced in two independent experiments.

Figure 5-7 (continued): E. NADPH oxidase is activated to the same extent upon infection with wild-type or *hat1*Δ/Δ cells. Activation of the NADPH oxidase in BMDMs upon 30 min interaction was determined by detection of the phosphorylated p40phox subunit. ERK levels served as loading control. An uninfected control was included (wo Ca). The asterisk marks a cross reaction. One representative experiment is shown. Data were reproduced in two independent experiments.
F. Infection of neutrophils with *hat1*Δ/Δ cells causes reduced ROS accumulation. ROS levels were determined by measuring luminol-dependent chemiluminescence [relative luciferase units (RLU) min^{-1} per 1000 immune cells] in 2.5 min intervals during interaction of the indicated *C. albicans* strains with murine bone marrow neutrophils. One representative experiment is shown. Data were reproduced in two independent experiments.
G. Cells lacking Hat1 show increased survival to neutrophil killing. Survival of *C. albicans* cells upon one hour interaction with murine bone marrow neutrophils was determined by plating and CFU counting. Data are shown as mean + SD from three independent experiments. $^{**}P<0.01$ relative to the wild-type (Student's *t*-test).

5.2.8 Cells lacking Hat1 show reduced virulence but persist in mouse kidneys

Deletion of *HAT1* causes a reduced growth rate with morphological defects (Fig. 5-8A) (Tscherner *et al.*, 2012), which has been shown to reduce virulence of several mutants (Lopes da Rosa *et al.*, 2010, Chauhan *et al.*, 2005, Hwang *et al.*, 2003b). However, cells lacking Hat1 are also more resistant to killing by immune cells (Fig. 5-7G). Therefore, we asked if deletion of *HAT1* influences the virulence of *C. albicans*.

To answer this question we used a mouse model of systemic candidiasis. Infection was performed via the tail vein and fungal burdens in kidneys were followed, because this is the main organ targeted by *C. albicans* (MacCallum & Odds, 2005). Interestingly, after 24 hours mice infected with the *hat1*Δ/Δ mutant showed significantly reduced CFUs in the kidney compared to the wild-type or the reintegrant (Fig. 5-8B). However, the fungal burden of the mutant increased until day 7 after infection to reach almost the same levels as the wild-type and the reintegrant. Thus, cells lacking Hat1 are not cleared from infected mice and are able to compensate the *in vitro* growth defect *in vivo*. To further investigate the virulence properties of the *hat1*Δ/Δ strain, we determined the survival rate of infected mice. Interestingly, 15 days post infection 83% of wild-type infected mice had died, whereas 100% of the mice infected with the *hat1*Δ/Δ strain were still alive (Fig. 5-8C). Even after 32 days only one mouse infected with the *hat1*Δ/Δ mutant had died. Furthermore, mice infected with the reintegrant showed an intermediate survival rate, which was however not significant when compared to the wild-type strain (Fig. 5-8C). Although the majority of mice survived the infection with cells lacking Hat1, the mutant was not cleared from the kidneys in 4 out of 5 individuals (Fig. 5-8D). Instead the fungal burden stayed high until the end of the experiment. Furthermore, two mice survived infection with the reintegrant and for both *Candida* was not cleared (Fig. 5-8D). Thus, again the revertant strain showed an intermediate phenotype most likely due to haploinsufficiency.

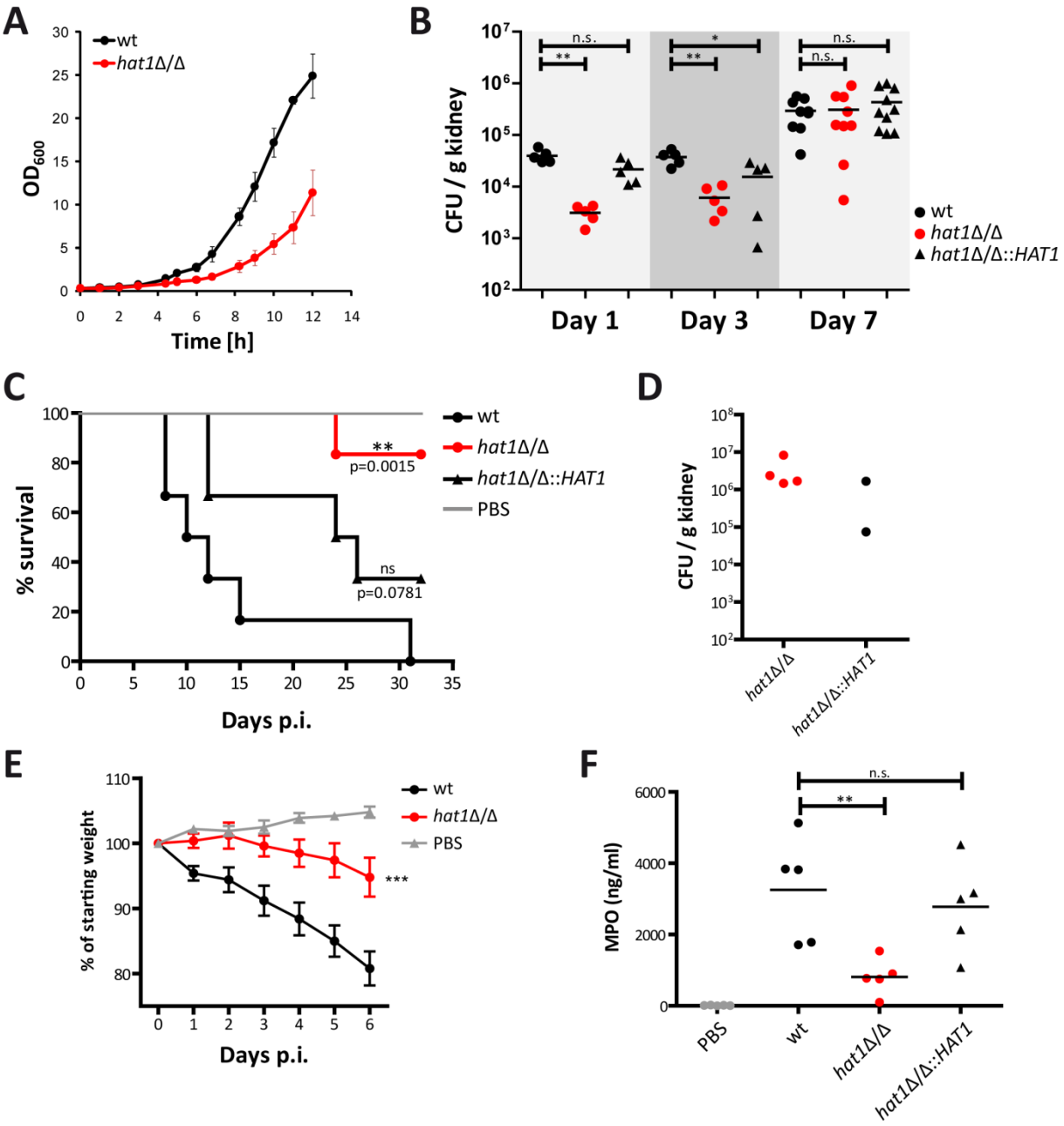

Figure 5-8: Cells lacking Hat1 show reduced virulence but persist in mouse kidneys.
A. Reduced growth rate of the *hat1*Δ/Δ strain was determined by measuring OD_{600} of cells growing in YPD at 30°C.
B. Cells lacking Hat1 are not cleared from kidneys. At the indicated time points fungal burdens in kidneys of mice infected with *C. albicans* strains were determined and expressed as CFUs per gram kidney. Groups of 5-10 mice were analysed at each time point and statistical significance was determined using the non-parametric Mann-Whitney-test. **P*<0.05 and ***P*<0.01 relative to the corresponding wild-type.
C. *hat1*Δ/Δ cells are defective in killing the host. Survival of mice infected with the indicated strains was monitored over 32 days post infection (p.i.). The data are presented as Kaplan-Meier survival curves. Groups of 6 mice were infected per *C. albicans* strain. Statistical significance was determined using the Log-rank test.
D. Fungal burdens in kidneys of surviving mice from panel C were determined and expressed as CFUs per gram organ. One mouse infected with the *hat1*Δ/Δ strain was able to clear *Candida*.
E. Body weight of mice infected with the indicated *Candida* strains was determined daily until day 6 post infection (p.i.). Significance was determined relative to wild-type infected mice using Two-way ANOVA. ****P*<0.001.
F. Infection with *hat1*Δ/Δ cells leads to reduced neutrophil activation. Myeloperoxidase (MPO) levels in kidneys of infected mice were determined 7 days post infection. Statistical significance was determined by Student's *t*-test. ***P*<0.01 relative to the wild-type.

In addition, body weight measurement of infected mice revealed a striking difference between the wild-type and the *hat1*Δ/Δ mutant. As expected, mice infected with the wild-type rapidly lost body weight after infection. However, upon infection with the *hat1*Δ/Δ strain weight loss was dramatically reduced (Fig. 5-8E). Thus, deletion of *HAT1* strongly attenuates virulence in a mouse model of systemic candidiasis. Fungal burdens are comparable between the *hat1*Δ/Δ mutant and the wild-type but the mutant is strongly attenuated in killing the host. Therefore, we further investigated the host response and determined neutrophil activity in kidneys of infected mice. We did this by measuring myeloperoxidase (MPO) levels in mouse kidneys 7 days post infection. Interestingly, mice infected with the *hat1*Δ/Δ mutant showed dramatically reduced MPO levels compared to the wild-type and the reintegrant (Fig. 5-8F). Thus, either there are less neutrophils infiltrating the organ or they are not activated to the same extent as for the wild-type. Both scenarios in addition to the mutant's increased capacity to detoxify ROS would support persistence of the mutant in the host. In summary, our data suggest that the *hat1*Δ/Δ strain is able to compensate its *in vitro* growth defect *in vivo* and persist in the host. However, cells lacking Hat1 are severely compromised in killing the host.

6 Conclusions and Discussion

In the course of this thesis the role of the histone acetyltransferase Hat1 in DNA damage repair, oxidative stress resistance, azole tolerance and virulence in the fungal pathogen *C. albicans* was analysed. Besides a conserved role in the repair of exogenous DNA lesions our data indicate that *C. albicans* Hat1 is also indispensable for efficient processing of intracellular DNA damages. This is in concert with other studies showing that the removal of components of the DNA damage repair machinery leads to accumulation of DNA lesions and cell cycle arrest (Lopes da Rosa *et al.*, 2010, Shi *et al.*, 2007). Thus, in contrast to other organisms *C. albicans* seems to be extremely sensitive to perturbations of the DNA damage repair machinery. During the course of this work an essential role for Hat1 in mammalian development and genome stability was identified (Nagarajan *et al.*, 2013). Mouse embryonic fibroblasts have proliferation defects and accumulate DNA damages in the absence of Hat1, which is reminiscent of the phenotype observed for *C. albicans*. These data indicate that *C. albicans* might be suitable model system to study different aspects of chromatin assembly.

In addition to its role in the repair of DNA lesions our data provide strong evidence for a novel function of Hat1 in regulating the response to oxidative stress and azole treatment, which seems to be specific to *C. albicans* and has not been described in other organisms so far. Furthermore, resistance phenotypes as well as misregulated genes upon loss of Hat1 are shared by different chromatin modifiers involved in independent chromatin assembly pathways. Thus, our data suggest that Hat1 has a central function in the flux of histones used for nucleosome formation in *C. albicans* thereby affecting various cellular processes.

6.1 Regulation of catalase expression via chromatin assembly

Interestingly, our data suggest that there are substantial differences in the functions of chromatin assembly factors in *C. albicans* compared to other species. Neither the NuB4 complex nor the CAF-1 complex were shown to be involved in the regulation of oxidative stress in other organisms so far. Interestingly, in *S. cerevisiae* Rtt109 negatively regulates catalase expression together with Asf1 via histone deposition (Klopf *et al.*, 2009). Furthermore, the *S. cerevisiae rtt109*Δ mutant showed slightly increased H_2O_2 resistance (Fig. 5-3A a). However, in *C. albicans RTT109* deletion had no effect on catalase expression or peroxide resistance (Fig. 5-1E and 5-4E). Thus, it's tempting to speculate that Hat1 and Rtt109 switched functions during evolution. If Hat1 regulates oxidative stress gene expression in *C. albicans* also via Asf1 is still unclear. Unfortunately, deletion of Asf1 in *C. albicans* was not possible indicating that it might be essential. Interestingly, Asf1 is also essential for survival in *S. pombe* and *Drosophila melanogaster* (Schulz & Tyler, 2006, Umehara *et al.*, 2002). Thus,

there might be more redundancy among histone chaperone functions in *S. cerevisiae* than in *C. albicans* and other species.

Interestingly, Spt6 negatively regulates catalase expression in *S. cerevisiae* (Klopf *et al.*, 2009). The observed H_2O_2 resistance phenotype of the *C. albicans spt6*Δ/Δ mutant might indicate conservation of this function in both species. Further experiments need to be done to determine expression levels of oxidative stress genes in cells lacking Spt6. Interestingly, the ATP-dependent chromatin remodeller Ino80 functions together with Spt6 and in parallel with Asf1/Rtt109 in the regulation of catalase expression in *S. cerevisiae* (Klopf *et al.*, 2009). Additional studies are needed to clarify if this function of Ino80 is conserved in *C. albicans* or if there are also differences in the roles of ATP-dependent chromatin remodeller in this organism. We could show that deletion of *HAT1* as well as *CAC2* leads to hyperinduction of oxidative stress genes including *CAT1*, the gene encoding for the catalase. However, deletion of the catalase in a *hat1*Δ/Δ background did not lower the H_2O_2 sensitivity to the level of the *cat1*Δ/Δ single knock-out (Fig. 5-4H). Therefore, catalase-independent mechanisms must also contribute to the H_2O_2 resistance of the *hat1*Δ/Δ deletion strain.

We could also detect hyperinduction of other oxidative stress genes in cells lacking Hat1, which could cause reduced H_2O_2 sensitivity. Overexpression of *OYE2* encoding a NADPH oxidoreductase in *S. cerevisiae* increases resistance to H_2O_2-induced cell death (Odat *et al.*, 2007). Interestingly, homologues of this enzyme are indeed hyperinduced in a *hat1*Δ/Δ deletion strain (Fig. 5-4D a and c). We also observed higher induction levels for *PST3* encoding a putative flavodoxin upon loss of Hat1 (Fig. 5-4D b). Notably, overexpression of a flavodoxin has been shown to increase resistance to H_2O_2 in *Pseudomonas aeruginosa* (Moyano *et al.*, 2014). However, if the above-mentioned genes indeed contribute to the observed resistance phenotype of the *hat1*Δ/Δ and the *cac2*Δ/Δ mutants still has to be proven.

6.2 Repression of genes via distinct chromatin assembly pathways

To our knowledge there are no data available suggesting a role of Hat1 together with the HIR complex in regulating azole resistance in any other organism. However, novel functions for the *C. albicans* CAF-1 and the HIR complexes in the regulation of white-opaque switching were discovered recently (Stevenson & Liu, 2013). These two complexes are required for histone deposition via distinct pathways. While the HIR complex functions in replication-independent chromatin assembly, CAF-1 is involved in replication-coupled histone deposition (Green *et al.*, 2005, Shibahara & Stillman, 1999, Stillman, 1986). The HIR complex was initially identified as negative regulator of histone genes and later a role in the repression of cryptic and antisense transcription was discovered (Anderson *et al.*, 2009, Xu *et al.*, 1992, Osley & Lycan, 1987). Thus, it's tempting to speculate that in *C. albicans*

Hat1 and Hir1 function together in a chromatin assembly pathway which is responsible for negative regulation of genes conferring azole resistance. In line with this hypothesis *HAT1* is indeed repressed in *C. albicans* cells treated with itraconazole (De Backer *et al.*, 2001).

Although, the CAF-1 complex can assemble histones in a replication-coupled manner, it was also found to influence the rate of replication-independent histone incorporation and transcription (Yu *et al.*, 2013, Ray-Gallet *et al.*, 2011, Kim *et al.*, 2009). Therefore, NuB4 and CAF-1 might act together to regulate specific target genes such as oxidative stress genes in *C. albicans* by facilitating histone incorporation during transcription thereby increasing the histone density. Consistently, we saw a large overlap in the differentially expressed genes in *hat1*Δ/Δ and the *cac2*Δ/Δ mutants (Fig. 5-5A). Unfortunately, we were not able to obtain a *hat1cac2*Δ/Δ double deletion strain to be able to perform epistasis analysis. Thus, we cannot rule out the possibility that Hat1 and Cac2 regulate H_2O_2 resistance independently.

Genome-wide transcriptional profiling of logarithmically growing *hat1*Δ/Δ, *cac2*Δ/Δ and *rtt109*Δ/Δ mutants yielded almost exclusively in upregulation of genes further indicating repressive functions of these three proteins. Notably, different GO terms were enriched in the *hat1*Δ/Δ mutant alone or in the overlaps with one of the other mutants indicating specific functions for Hat1 in different processes including DNA damage repair, arginine biosynthesis and mitochondrion degradation (Fig. 5-6). Interestingly, expression of arginine biosynthesis genes is inhibited in *S. cerevisiae* by Rtt109 together with Asf1 under repressing conditions (Lin & Schultz, 2011). However, in *C. albicans* this group of genes is derepressed in *hat1*Δ/Δ and *cac2*Δ/Δ, but not in *rtt109*Δ/Δ cells (Fig. 5-6A). Thus, Hat1/Cac2 might have taken over this function from Rtt109. Although hyperinduction of genes connected to H_2O_2 resistance was observed upon loss of Hat1 (Fig. 5-4), the majority of oxidative stress induced genes did not show increased induction levels in the *hat1*Δ/Δ or *cac2*Δ/Δ strain upon treatment (Fig. 5-5C). Genes involved in oxidative stress response were only enriched in both mutants without treatment (Fig. 5-6A c). Furthermore, among the genes which were induced in the wild-type upon treatment only 81 showed more than 1.5 fold increase in the *hat1*Δ/Δ mutant. However, even in this group was no GO enrichment for oxidative stress genes found. This could be in part explained by the fact, that other oxidative stress genes might have different induction kinetics and we could have missed their expression maximum. Furthermore, we also observed faster induction kinetics for *CAT1* in cells lacking Hat1 or Cac2 (Fig. 5-4C). This might also apply to other oxidative stress genes even if they reach similar maximum expression levels in the mutants and the wild-type. Nevertheless, our results indicate that hyperinduction as for the catalase is not the primary effect seen upon loss of Hat1 or Cac2 after H_2O_2 treatment. However, all three mutants had defects in efficiently repressing genes upon H_2O_2 stress. Interestingly, GO term enrichment suggests a specific role for Hat1 in the regulation of ncRNA processing upon H_2O_2 stress.

Nevertheless, further experiments are required to confirm the RNA-Seq data and to clarify the reason for the H_2O_2 resistance observed for the *hat1*Δ/Δ and the *cac2*Δ/Δ mutants.

Repressing functions for Hat1 and Cac2 have been reported, although mainly in the context of heterochromatin formation and telomeric silencing (Mersfelder & Parthun, 2008, Kaufman *et al.*, 1997, Monson *et al.*, 1997). Although we could detect some enrichment of upregulated genes near telomeres in the absence of H_2O_2, this does not seem to be the main cause for the observed defects in transcriptional repression (Table 6-1).

Table 6-1: Location and telomere enrichment of upregulated genes in *hat1*Δ/Δ, *rtt109*Δ/Δ and *cac2*Δ/Δ mutants.
Total genes: Total number of genes upregulated in the respective mutant. Telomere proximal genes: Number of genes upregulated in the respective mutant and located at least partially within 20 kb of the chromosome end. Differentially regulated genes were defined by a fold change >=2 and P-value <0.05. Statistical significance was determined using Fisher's exact test.

Condition	Strain	Total genes	Telomere proximal genes	p-value
w/o H_2O_2	*hat1*Δ/Δ	571	31	0.00176
	*rtt109*Δ/Δ	240	16	0.00324
	*cac2*Δ/Δ	243	16	0.00357
w H_2O_2	*hat1*Δ/Δ	583	22	0.30790
	*rtt109*Δ/Δ	587	26	0.05564
	*cac2*Δ/Δ	454	11	0.47640

6.3 Inactivation of Hat1 compromises virulence of C. albicans

Although infection of macrophages with the *hat1*Δ/Δ mutant caused less total ROS accumulation, we observed faster accumulation of ROS in the beginning of the interaction. This effect cannot be simply explained by the pseudohyphal morphology of cells lacking Hat1 since the *rtt109*Δ/Δ strain has the same morphological defect yet does not show faster ROS accumulation. Thus, this effect seems to be specific for the loss of Hat1 and might be caused by upregulated factors that trigger ROS production. Further studies are needed to identify these factors.

Interestingly, cells lacking Hat1 are severely impaired in killing infected mice although they show a similar increase in fungal burden between day 3 and day 7 post infection (Fig. 5-8). It's tempting to speculate that cells lacking Hat1 cause less damage to host organs and therefore promote mouse survival. In line with this hypothesis is the fact that *hat1*Δ/Δ cells degrade ROS more efficiently. This does not only protect the mutant from being cleared by the immune system but it might also cause reduced tissue damage, since reactive oxygen species produced during the innate immune response are not only harming the pathogen but also the host (MacCallum *et al.*, 2009, Sriskandan & Altmann, 2008). Furthermore, the reduced number of activated neutrophils in kidneys

infected with *hat1*Δ/Δ cells could also explain less organ damage. In fact, the reason for a possible reduction in neutrophil influx could be less damage itself, because this cell type is known to be attracted by damaged tissue (McDonald & Kubes, 2011). Furthermore, it has been shown recently that downmodulation of the immune system can be advantageous for the host in *Candida albicans* infections (Majer *et al.*, 2012). Finally, ROS is also known to function as a signalling molecule during infections and reduced levels upon challenge with the *hat1*Δ/Δ mutant might impact the immune response (Mantegazza *et al.*, 2008).

There is also strong evidence that the observed virulence phenotype of the *hat1*Δ/Δ mutant is not just due to the role of this protein in DNA damage repair. Deletion of *RAD52* as well as *RTT109* also leads to pseudohyphal cell morphology and strong virulence defects in mouse models of systemic candidiasis (Lopes da Rosa *et al.*, 2010, Andaluz *et al.*, 2006, Chauhan *et al.*, 2005). However, the *rtt109*Δ/Δ mutant is efficiently cleared from kidneys of infected mice 3 days post infection (Lopes da Rosa *et al.*, 2010). Furthermore, for the *rad52*Δ/Δ strain kidney fungal burdens decline over 3 days post infection even with an inoculum concentration ten times higher than in our setup. Only using a three times higher inoculum dose as for the wild-type enables the *rad52*Δ/Δ mutant to kill the host and increases fungal burdens in kidneys to wild-type levels (Chauhan *et al.*, 2005). In contrast, for the *hat1*Δ/Δ mutant fungal burdens in kidneys increase to reach wild-type levels seven days post infection. However, cells lacking Hat1 are unable to kill the host efficiently over 32 days. Thus, the ability to persist within the host without killing it seems to be specific for cells lacking Hat1. Nevertheless, further experiments are required to clarify the role of the NuB4 complex during the infection process.

6.4 Evolution of specialized functions for chromatin assembly pathways in C. albicans

The loss of Hat1, Cac2 and Hir1 can be beneficial for *C. albicans* in our experimental setup. However, the inability to efficiently repress genes under stress conditions might be detrimental in the long run. Furthermore, loss of Hat1 and Cac2 also leads to defects in DNA damage repair and genome instability (Stevenson & Liu, 2013, Tscherner *et al.*, 2012). Thus, local regulation of NuB4/CAF-1- or NuB4/HIR-mediated repression might be more beneficial than complete absence of this mechanism. Further experiments are required to identify additional factors functioning together with NuB4 in the regulation of oxidative stress resistance or azole tolerance. This might also lead to the discovery of potential antifungal targets, which could be used to render cells more sensitive to ROS or azoles.

Interestingly, we did not observe comparable resistance phenotypes upon deletion of NuB4, CAF-1 and HIR components in *S. cerevisiae*, *C. glabrata* and *S. pombe*. Thus, in *C. albicans* these

complexes might have gained some functions during coevolution with the human host. However, deletion of NuB4 components in *C. glabrata*, which is also a human pathogen, did not yield the same phenotype. Thus, this adaptation might have happened later during evolution and is specific for *C. albicans* and perhaps more closely related species. The regulation of virulence-associated traits by chromatin modification has developed in different pathogens (Lopez-Rubio *et al.*, 2007). Also in *C. albicans* chromatin modifiers are involved in the regulation of virulence factors and antifungal tolerance (Lopes da Rosa & Kaufman, 2013, Hnisz *et al.*, 2010, Wurtele *et al.*, 2010, Simonetti *et al.*, 2007, Smith & Edlind, 2002). Therefore, modulation of chromatin modifying pathways seems to be a common strategy during coevolution with the host.

In summary, our results clearly demonstrate that there can be large differences in the functions of even highly conserved proteins between *C. albicans* and other fungi. Therefore, results obtained from classical model systems (e.g. baker's yeast) often cannot simply be transferred to this important pathogen.

7 Materials and Methods

Media, chemicals and growth conditions

Rich medium (YPD) and synthetic complete medium (SC) were prepared as previously described (Kaiser *et al.*, 1994). Fungal strains were routinely grown on YPD plates at 30°C. Hydrogen peroxide, tert-butyl hydroperoxide, EDTA, Calcofluor White, Congo Red, cadmium, sodium chloride, luminol and HRP Type VI were obtained from Sigma Aldrich, St Louis, MO. Voriconazole, Itraconazole and Amphotericin B were purchased from Discovery Fine Chemicals Ltd., Wimborne, UK. Rapamycin was obtained from LC Laboratories, Woburn, US. DMEM was purchased from PAA, Vienna, Austria.

Plasmid and strain construction

A list of fungal strains, plasmids and primers used in this study is shown in Tables 7-1, 7-2 and 7-3, respectively. All strains constructed in this work were derived from the clinical isolate SC5314 (Gillum *et al.*, 1984). Deletion of *CAC2* and *HIR1* was done using a modified version of the SAT1 flipper method (Reuss *et al.*, 2004). Briefly, the marker cassette was amplified using the pSFS3b plasmid and primers containing some 80bp homologous region to replace the whole coding sequence of the corresponding gene (Tscherner *et al.*, 2012). For deletion of *RTT106* two primer pairs were used to add the homologous regions in two sequential PCR steps. For deletion of *CAT1,* Yep352-SAT1-*CAT1*urdr was constructed by *in vivo* cloning in *S. cerevisiae* exactly as described previously (Krauke & Sychrova, 2011). Deletion of *SOD5* was done using the plasmid pSFS2a-*SOD5* (Frohner *et al.*, 2009). Transformation of *C. albicans* was done via electroporation (Reuss *et al.*, 2004). Correct integration of the deletion cassette and loss of the corresponding gene were confirmed by colony.

Colony PCR

A colony was resuspended in 25 µl H_2O in a PCR tube and incubated at 95°C for 10 minutes. Cell debris was spun down briefly and 5 µl of the supernatant was used as template for the PCR, which was performed using the DreamTaq Green DNA Polymerase (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions.

Table 7-1: Fungal strains used in this study.

Description	Name	Parent	Genotype	Reference	Figure
***C. albicans* strains**					
wt	SC5314	Clinical isolate	wild-type	Gillum *et al.*, 1984	Figures 5-1, -2, -4, -5, -6, -7
*HAT1/hat1*Δ	CA-MT007	SC5314	*HAT1/hat1*Δ::FRT	Tscherner *et al*., 2012	Figures 5-1
*hat1*Δ/Δ	CA-MT014	CA-MT007	*hat1*Δ::FRT/*hat1*Δ::FRT	Tscherner *et al*., 2012	Figures 5-1, -2, -4, -5, -6, -7
*hat1*Δ/Δ::*HAT1*	CA-MT050	CA-MT014	*hat1*Δ::FRT/*hat1*Δ::*HAT1*-FRT	Tscherner *et al*., 2012	Figures 5-1, -2, -6, -7
*HAT2/hat2*Δ	CA-MT031	SC5314	*HAT2/hat2*Δ::FRT	Tscherner *et al*., 2012	Figures 5-1
*hat2*Δ/Δ	CA-MT039	CA-MT035	*hat2*Δ::FRT/*hat2*Δ::FRT	Tscherner *et al*., 2012	Figures 5-1
*hat2*Δ/Δ::*HAT2*	CA-MT101	CA-MT098	*hat2*Δ::FRT/*hat2*Δ::*HAT2*-FRT	Tscherner *et al*., 2012	Figures 5-1
*hat1*Δ/Δ *hat2*Δ/Δ	CA-MT161	CA-MT014	*hat1*Δ/Δ *hat2*Δ::FRT/*hat2*Δ::FRT	Tscherner *et al*., 2012	Figures 5-1
*CAC2/cac2*Δ	CA-MT347	SC5314	*CAC2/cac2*Δ::FRT	This study	
*cac2*Δ/Δ	CA-MT363	CA-MT347	*cac2*Δ::FRT/*cac2*Δ::FRT	This study	Figure 5-2, -4, -5, -6
*cac2*Δ/Δ::*CAC2*	CA-SJ015	CA-MT363	*cac2*Δ::FRT/*cac2*Δ::*CAC2*-FRT	This study	Figure 5-2
*HIR1/hir1*Δ	CA-MT353	SC5314	*HIR1/hir1*Δ::FRT	This study	
*hir1*Δ/Δ	CA-MT376	CA-MT353	*hir1*Δ::FRT/*hir1*Δ::FRT	This study	Figure 5-2
*hat1*Δ/Δ *HIR1/hir1*Δ	CA-MT354	CA-MT014	*hat1*Δ/Δ *HIR1/hir1*Δ::FRT	This study	
*hat1 hir1*Δ/Δ	CA-MT400	CA-MT354	*hat1*Δ/Δ *hir1*Δ::FRT/*hir1*Δ::FRT	This study	Figure 5-2
*RTT106/rtt106*Δ	CA-MT396	SC5314	*RTT106/rtt106*Δ::FRT	This study	
*rtt106*Δ/Δ	CA-MT408	CA-MT396	*rtt106*Δ::FRT/*rtt106*Δ::FRT	This study	Figure 5-2
*rad52*Δ/Δ	CA-MT207	CA-MT198	*rad52*Δ::FRT/*rad52*Δ::FRT	Tscherner *et al*., 2012	Figure 5-1
*rtt109*Δ/Δ	CA-ES008	CA-ES004	*rtt109*Δ::FRT/*rtt109*Δ::FRT	Tscherner *et al*., 2012	Figures 5-1, -2, -4, -5, -6
*HHF1/hhf1*Δ	CA-ES016	SC5314	*HHF1/hhf1*Δ::FRT	Tscherner *et al*., 2012	Figure 5-2
*hhf1*Δ/Δ	CA-ES020	CA-ES016	*hhf1*Δ::FRT/*hhf1*Δ::FRT	Tscherner *et al*., 2012	Figure 5-2
*hhf1*Δ/Δ *HHF22/hhf22*Δ	CA-ES030	CA-ES020	*hhf1*Δ/Δ *HHF22/hhf22*Δ::FRT	Tscherner *et al*., 2012	Figure 5-2, -6
SN87	SN87	RM1000#2	*leu2*Δ/*leu2*Δ *his1*Δ/*his1*Δ *URA3/ura3*Δ::imm^{434} *IRO1/iro1*Δ::imm^{434}	Noble *et al*., 2010	Figure 5-2
*SPT6/spt6*Δ	JMB220	SN87	*spt6*Δ::*C.d.HIS1/SPT6 leu2*Δ/*leu2*Δ *his1*Δ/*his1*Δ *URA3/ura3*Δ::imm^{434} *IRO1/iro1*Δ::imm^{434}	Al-Rawi *et al*., 2010	Figure 5-2
*spt6*Δ/Δ	JMB31	JMB220	*spt6*Δ::*C.d.HIS1/spt6*Δ::*C.m.LEU2 leu2*Δ/*leu2*Δ *his1*Δ/*his1*Δ *URA3/ura3*Δ::imm^{434} *IRO1/iro1*Δ::imm^{434}	Al-Rawi *et al*., 2010	Figure 5-2
*CAT1/cat1*Δ	CA-MT473	SC5314	*CAT1/cat1*Δ::FRT	This study	
*cat1*Δ/Δ	CA-MT505	CA-MT473	*cat1*Δ::FRT/*cat1*Δ::FRT	This study	Figure 5-4
*hat1*Δ/Δ *CAT1/cat1*Δ	CA-MT474	CA-MT014	*hat1*Δ/Δ *CAT1/cat1*Δ::FRT	This study	
*hat1cat1*Δ/Δ	CA-MT488	CA-MT474	*hat1*Δ/Δ *cat1*Δ::FRT/*cat1*Δ::FRT	This study	Figure 5-4
*sod5*Δ/Δ	CA-IF063	CA-IF057	*sod5*Δ::FRT/*sod5*Δ::FRT	Frohner *et al*., 2009	Figure 5-6
*hat1*Δ/Δ *SOD5/sod5*Δ	CA-MT369	CA-MT014	*hat1*Δ/Δ *SOD5/sod5*Δ::FRT	This study	
*hat1sod5*Δ/Δ	CA-MT385	CA-MT369	*hat1*Δ/Δ *sod5*Δ::FRT/*sod5*Δ::FRT	This study	Figure 5-6
***S. cerevisiae* strains**					
Sc wt	BY4741		MATa; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0	Brachmann *et al*., 1998	Figure 5-3
*Sc hat1*Δ		BY4741	MATa; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0; *hat1*Δ::kanMX	Euroscarf collection	Figure 5-3
*Sc rtt109*Δ		BY4741	MATa; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0; *rtt109*Δ::kanMX	Euroscarf collection	Figure 5-3
*Sc cac2*Δ		BY4741	MATa; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0; *cac2*Δ::kanMX	Euroscarf collection	Figure 5-3
*Sc hir1*Δ		BY4741	MATa; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0; *hir1*Δ::kanMX	Euroscarf collection	Figure 5-3
*Sc asf1*Δ		BY4741	MATa; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0; *asf1*Δ::kanMX	Euroscarf collection	Figure 5-3
***C. glabrata* strains**					
Cg wt	his-	ATCC2001	*his3*Δ	Kitada *et al*., 1995	Figure 5-3
*Cg hat1*Δ	BM145	his-	*his3*Δ *hat1*Δ::NAT1	Schwarzmüller *et al*., *in revision*	Figure 5-3
*Cg hat2*Δ	BM14	his-	*his3*Δ *hat2*Δ::NAT1	Schwarzmüller *et al*., *in revision*	Figure 5-3
***S. pombe* strains**					
Sp wt	975		*h*$^+$	Leupold, 1950	Figure 5-3
*Sp hat1*Δ	LBP6	975	*h*$^+$ *hat1*Δ::kan	Benson *et al*., 2007	Figure 5-3

Table 7-2: Plasmids used in this study.

Plasmids	Parent	Relevant inserts and cloning sites	Reference
pSFS3b			Tscherner *et al*., 2012
YEp352-SAT1			Krauke *et al*., 2010
YEp352-SAT1-*CAT1*urdr	YEp352-SAT1	5'*HAT1*-FRT-SAT1-FLP-FRT-3'*HAT1*	This study
pSFS2a-*SOD5*	pSFS2a	*SacI*5'*SOD5NotI*-SAT1-FLP-*ApaI*3'*SOD5KpnI*	Frohner *et al*., 2009

Table 7-3: Primers used in this study.
Lower case and upper case letters denote exogenous and endogenous sequences, respectively.

Name	Sequence (5' - 3')*
Gene deletion constructs based on the SAT1-flipping strategy (Reuss *et al.*, 2004)	
CAC2del_fwd	atctttttacttagtaaaacaaatcttcaaatcgttcatttatccacatttccttaaagctagaacaacataagttattaCTCACTATAGGGCGAATTGG
CAC2del_rev	ccaacttcaattttgcattttatagcatcgcttgtatgtacataaatgtatataaacacaaattgttttactagttacTGAGCGGATAACAATTTCACAC
HIR1del_fwd	catcaacagaaaattacaccaaaaagaaaaaataatcaaaacaagacacttccaaacctaatcaactatataacaagccCTCACTATAGGGCGAATTGG
HIR1del_rev	gtttgcaaagagggtttcaaagccagttagagatacaaaggttgaatagttttgttaaaactagtatatgcattaacTGAGCGGATAACAATTTCACAC
RTT106del_fwd	tcgttcatggttgttgttgttgcggttacgcgacctggtCTCACTATAGGGCGAATTGG
RTT106del_rev	cttcttgataaatttggtgctaaatatcaataaaacaatTGAGCGGATAACAATTTCAC
RTT106del_fwd2	GGGCACCAAGTGCAAGATCGTTGCATTAGCATCAAACAATTCGTTCATGGTTGTTGTTG
RTT106del_rev2	AGTTACAAAACAACATCCATATAAAAGAGGTGCATTCACCCAGCTTCTTGATAAATTTG
55ic_CAT1	agctatgaccatgattacgaattcgagctcggtacccgggACCACAATGTAGCCAAGCAG
53ic_CAT1	ttctctagaaagtataggaacttcctcgaggggggcccggAATTCGTAAATGTTGGAGC
35ic_CAT1	atccactagttctagagcggccgccaccgcggtggagctcTAAGTGTGAACGAAACCAGG
33ic_CAT1	cgacgttgtaaaacgacggccagtgccaagcttgcatgcTGTTGTGCGAAAGTTGGTGG
Primers for integration check	
5C_CAC2	GTATTTGAAGGTGTTGGGACTGAG
3C_CAC2	GATGGTGTCCCCAATACCAC
5C_HIR1	ACCCTTGATGCTTCCAAACATC
3C_HIR1	GCTTGTAGCAAAACAATTGGTGG
5C_RTT106	GTACATCTCTCTTGGCAACGG
3C_RTT106	GGCCCTTGAAGAAGTTTATCATTGAG
5C_CAT1	GTAGAAGAAGTTATACCGAGGAAG
3C_CAT1	GATGATGGTTATAATTAGTGTTGGG
Primers for loss of gene PCR	
55Tag_CAC2	TACATATACTCACGTGCGAGTC
53Tag_CAC2	ccactagcagcagaaccggaCAGTATTAGTGTTGGCGTTATTCTC
55Tag_HIR1	TTATGTGTTGTGCTCGTTCCATG
53Tag_HIR1	ccactagcagcagaaccggaTATCAAATCAAGTAGTTCTTGACCTC
55Tag_RTT106	GTTATGGTAAACTGCCACAAGG
53Tag_RTT106	ccactagcagcagaaccggaTTCGATTTCAATACCTCTTTCAAACAC
RT-qPCR (Reference)	
RT5_CaRIP1	TGCTGACAGAGTCAAGAAACC (Hnisz *et al.*, 2010)
RT3_CaRIP1	GAACCAACCACCGAAATCAC (Hnisz *et al.*, 2010)
RT5_2 PAT1	CAGCAACTGATTTATCGGAATGG
RT3_2 PAT1	ACATCTTCAGGGTTAGGTGG
RT_SOD4_417s	GACAGTAAAGCTTACATTGGTGGG
RT_SOD4_529as	GCACTTGCAGTATCGTCACC
RT_SOD5_464s	CAATGGTACCAGATTGAACTGTGC
RT_SOD5_582as	AAGAAGTGTTGACTGCACTTTGAG
RT5_2 CaCAT1	GAGACCCATCTAAATTCCCAC
RT3_2 CaCAT1	CTTCATTGCTAGTCAAGTAATCCC

Spot dilution and liquid survival/growth inhibition assays

Spot dilution assays were performed as described previously (Tscherner *et al.*, 2012). Hydrogen peroxide pretreatment of cultures prior to spotting was achieved by incubation with 0.4 mM H_2O_2 for 60 min. For determination of H_2O_2 survival cells were grown overnight to an OD_{600} of 1 and treated with the indicated concentrations of hydrogen peroxide for 2 hours at 30°C. Before and after treatment cells were diluted and plated on YPD plates. Colonies were counted after 3 days incubation at 30°C and viability was determined relative to the samples plated before H_2O_2 addition. To quantify growth inhibition by azole treatment cells were grown to logarithmic phase in SC medium at 30°C. Cultures were diluted to an OD_{600} of 0.01 in SC medium with or without voriconazole at the indicated concentrations. OD_{600} was determined after growth at 30°C for 18 hours. Growth inhibition was calculated relative to untreated controls.

RNA isolation and RT-qPCR analysis

Cells were grown overnight to an OD_{600} of 1 at 30°C. For hydrogen peroxide treatment 1.6 mM H_2O_2 was added to the culture for the indicated period of time. RNA isolation and qPCR analysis was done as described previously (Tscherner *et al.*, 2012). For RT-qPCRs shown in Fig. 5-6, *RIP1* was used as reference gene (Hnisz *et al.*, 2010). Other RT-qPCRs were normalized to *PAT1* (Zordan *et al.*, 2006) due to changing transcript levels of *RIP1* upon H_2O_2 treatment (data not shown).

RNA-Sequencing (RNA-Seq) and analysis

After RNA isolation 10 µg total RNA were treated with DNase I (Fermentas, St. Leon-Rot, Germany) and purified using the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). 5 µg DNase treated RNA were used for rRNA depletion with the RiboMinus™ Eukaryote System v2 (Life Technologies, Carlsbad, CA). rRNA depleted samples were fragmented using the NEBNext Magnesium RNA Fragmentation Module (New England Biolabs, Frankfurt am Main, Germany) and purified with the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). SuperScript III reverse transcriptase (Life Technologies, Carlsbad, CA) was used for first strand synthesis. Priming was done with 3 µg random hexamers (Life Technologies, Carlsbad, CA). Samples were purified using Mini Quick Spin Columns (Roche, Basel, Switzerland) and second strand synthesis was done with the NEBNext mRNA Second Strand Synthesis Module (New England Biolabs, Frankfurt am Main, Germany). Final purification of double stranded cDNA was done with the MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Samples were further processed and sequenced on a HiSeq 2000 instrument (Illumina, San Diego, CA) at the Next Generation Sequencing Facility (CSF NGS unit, http://www.csf.ac.at) of the Campus Vienna Biocenter. For both conditions five biological replicates for the wild-type as well as the *hat1*Δ/Δ strain and three biological replicates for the *cac2*Δ/Δ as well

as the *rtt109*Δ/Δ mutants were sequenced. Reads were mapped onto the Assembly 21 of the *C. albicans* genome (http://www.candidagenome.org) using TopHat with a minimal intron length of 11 as well as a maximum intron length of 1338 (Trapnell *et al.*, 2009). Only uniquely mapping reads were allowed. Reads per kilobase in a million mapped reads (RPKM) values were calculated with Cufflinks (Trapnell *et al.*, 2010) including quartile normalisation and a reference annotation (C_albicans_SC5314_version_A21-s02-m07-r10; http://www.candidagenome.org). The annotation of the coding sequence assembly was used as transcript coordinates. For short non-coding RNAs (tRNAs, snRNAs, snoRNAs and ncRNAs) 20bp up- and downstream of their chromosomal coordinates were added for RPKM calculation (Hnisz *et al.*, 2012). Differential expression values were determined using Cuffdiff with library normalization method classic-fpkm and dispersion estimation method pooled (Trapnell *et al.*, 2010). Only genes with RPKM values >=5 for the corresponding condition in all mutants were included in the analysis. Venn diagrams were created using VennPlex (Cai *et al.*, 2013). Clustering was carried out with Cluster 3 (de Hoon *et al.*, 2004) and visualized with TreeView (Saldanha, 2004). Gene ontology (GO) term enrichment was determined using the GO Term finder (http://www.candidagenome.org). Overlapping GO terms were merged manually.

Catalase assay

Cells were grown overnight to an OD_{600} of 1 at 30°C. For hydrogen peroxide treatment 1.6 mM H_2O_2 was added to the culture for one hour. Before and after treatment 20 ml culture were harvested at 1500 g for 3 min at 4°C and washed once with 20 ml cold H_2O. Pellets were resuspended in 250 µl lysis buffer [50 mM Tris-HCl pH 7.5; 10% glycerol, complete protease inhibitor cocktail (Roche, Basel, Switzerland)] and an equal volume of glass beads (425–600mm, Sigma Aldrich, St Louis, MO) was added. Cells were lysed by shaking 5 times at 6 m s^{-1} for 30 s on a FastPrep instrument (MP Biomedicals, Illkirch, France). Extracts were cleared by centrifugation at 14000 g for 5 min at 4°C. Protein concentration in the extracts was determined by measuring absorption at 280 nm. For catalase activity measurement 3-5 µl whole cell extract were added to 3 ml of catalase assay buffer [384 mM Na_3PO_4; 0.015 mM Triton x-100 (Sigma Aldrich, St Louis, MO); 11.4 mM H_2O_2] and degradation of H_2O_2 was determined by measuring absorbance at 240 nm for up to 2 min. Catalase activity was calculated in µM H_2O_2 per minute per mg of whole cell extract as described previously (Durchschlag *et al.*, 2004).

Mouse strains and isolation of bone marrow-derived macrophages

For all experiments 7-10 week old C57BL/6 wild-type mice were used. Isolation and cultivation of primary bone marrow-derived macrophages (BMDMs) was done as described previously (Frohner *et al.*, 2009).

ROS assay

ROS assays were done exactly as described previously (Frohner *et al.*, 2009). The multiplicity of infection (MOI) for all ROS assays was 5:1 (fungi to immune cells).

Phagocytosis assay

Phagocytosis assays were performed essentially as described with some modifications (Bourgeois *et al.*, 2011). *C. albicans* cells were grown overnight to an OD_{600} of around 1, washed twice in PBS and stained with 10 mg ml^{-1} Alexa Fluor 488 (Life Technologies, Carlsbad, CA) in 100 mM HEPES buffer (pH 7.5) for 60 min at 30°C shaking in the dark. After staining cells were washed 3 times, resuspended in HEPES buffer and used for interaction with BMDMs. Stained *Candida* cells were added to macrophages and incubated for 45 min at 37°C and 5% CO_2. Control reactions were kept on ice during the whole procedure. A MOI of 2:1 (fungi to macrophages) was used. Phagocytosis was terminated by chilling on ice. Plates remained on ice during subsequent detaching and fixation in 1% formaldehyde. Extracellular fluorescent *C. albicans* cells were quenched by addition of 0.4% trypan blue. Samples were subject to flow cytometry analysis with FL1-H on a FACSCalibur instrument (BD, Franklin Lakes, NJ).

Western Blot analysis

Sample preparation and western blot analysis were essentially carried out as described previously (Bourgeois *et al.*, 2011). A MOI of 5:1 (fungi to macrophages) was used and samples were harvested after 30 min of interaction. Activated NADPH oxidase was detected using an antibody against the phosphorylated p40phox subunit (Cell Signaling 4311, Danvers, MA). A panERK antibody (BD 610123, Franklin Lakes, NJ) was used as loading control.

Neutrophil survival assay

Isolation of bone marrow neutrophils and subsequent *C. albicans* survival assays were done as described previously (Majer *et al.*, 2012). A MOI of 1:10 (fungi to neutrophils) was used and cells were harvested after 1 hour interaction.

Mouse model of systemic candidiasis

Mouse infection was carried out as described previously with some modifications (Majer *et al.*, 2012). Briefly, *C. albicans* strains were grown overnight to an OD_{600} of around 1, washed twice and resuspended in PBS. For infection 1 x 10^5 *Candida* cells per 21 g mouse body weight were injected via the lateral tail vein. For survival experiments mice were monitored for 32 days. Analysis

of fungal burdens in the kidneys at day 1, 3 and 7 post infection as well as MPO level measurement was done as described previously (Majer *et al.*, 2012). Statistical analysis was carried out using the Prism software (Graphpad Software Inc., San Ciego, CA).

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9 Appendix

9.1 Additional Publications

Pharmaceuticals 2011, 4, 169-186; doi:10.3390/ph4010169

Pathogenesis and Antifungal Drug Resistance of the Human Fungal Pathogen *Candida glabrata*

Michael Tscherner, Tobias Schwarzmüller and Karl Kuchler

Candida glabrata is a major opportunistic human fungal pathogen causing superficial as well as systemic infections in immunocompromised individuals and several other patient cohorts. *C. glabrata* represents the second most prevalent cause of candidemia and a better understanding of its virulence and drug resistance mechanisms is thus of high medical relevance. In contrast to the diploid dimorphic pathogen *C. albicans*, whose ability to undergo filamentation is considered a major virulence trait, *C. glabrata* has a haploid genome and lacks the ability to switch to filamentous growth. A major impediment for the clinical therapy of *C. glabrata* infections is its high intrinsic resistance to several antifungal drugs, especially azoles. Further, the development of antifungal resistance, particularly during prolonged and prophylactic therapies is diminishing efficacies of therapeutic interventions. In addition, *C. glabrata* harbors a large repertoire of adhesins involved in the adherence to host epithelia. Interestingly, genome plasticity, phenotypic switching or the remarkable ability to persist and survive inside host immune cells further contribute to the pathogenicity of *C. glabrata*. In this comprehensive review, we want to emphasize and discuss the mechanisms underlying virulence and drug resistance of *C. glabrata*, and discuss its ability to escape from the host immune surveillance or persist inside host cells.

Contribution:

I contributed extensively to the writing and the preparation of the manuscript.

Methods in Molecular Biology 2011, 734, 303-315; doi:10.1007/978-1-61779-086-7_15

Morphological and Molecular Genetic Analysis of Epigenetic Switching of the Human Fungal Pathogen *Candida albicans*

Denes Hnisz, **Michael Tscherner**, Karl Kuchler

Candida albicans is a pleiomorphic fungal pathogen whose morphogenetic plasticity has long been considered as a major virulence factor. In addition to the yeast-filament transition, *C. albicans* cells also have the unique ability to switch between two epigenetic phases referred to as white and opaque. White and opaque cells harbor identical genomes yet they differ in cellular morphologies, gene expression profiles, mating abilities, and virulence properties. The switching process is regulated by a small network of transcription factors and is suggested to be driven by stochastic fluctuations of the regulatory components, which correlates with altered switching frequencies. Traditionally, phase variants have been identified based on cellular morphologies and expression levels of a few marker transcripts, yet it has recently become clear that several other criteria are also essential and relevant, because phase markers are regulated at multiple branching sites of transcriptional circuitry regulating switching. Here, we describe basic methods to discriminate between white and opaque switching variants, based on cellular and macroscopic morphologies, expression levels of phase-specific transcripts, Wor1 protein levels, as well as quantitative mating assays.

Contribution:
For this study I prepared the microscopy pictures of *C. albicans* white and opaque cells grown in different media.

Epigenomics, April 2011, Vol. 3, No. 2 , Pages 129-132; doi:10.2217/epi.11.7

Targeting chromatin in fungal pathogens as a novel therapeutic strategy: histone modification gets infectious

Denes Hnisz, **Michael Tscherner**, and Karl Kuchler

Contribution:

I was involved in the writing and preparation of the manuscript.

Bio-Protocol 2013, October 20, 3(20)

Immunoblot Analysis of Histone H4 Acetylation and Histone H2A Phosphorylation in *Candida albicans*

Michael Tscherner and Karl Kuchler

Posttranslational modifications of histones are required for different processes including transcription, replication and DNA damage repair. This protocol describes the preparation of a whole-cell extracts for the fungal pathogen *Candida albicans*. Furthermore, the extract is used to detect lysine acetylation of histone H4 as well as serine 129 phosphorylation of histone H2A by immunoblot analysis.

Contribution:
I developed the protocol explained in the publication and wrote the manuscript.

Revised version submitted to PLoS Pathogens in March 2014

Systematic Phenotyping of a Genome-Scale *Candida glabrata* Deletion Collection Reveals Novel Antifungal Tolerance Genes

Tobias Schwarzmüller, Biao Ma, Ekkehard Hiller, Fabian Istel, **Michael Tscherner**, Sascha Brunke, Lauren Ames, Arnaud Firon, Brian Green, Vitor Cabral, Marina Marcet-Houben, Ilse D. Jacobsen, Jessica Quintin, Katja Seider, Ingrid Frohner, Walter Glaser, Helmut Jungwirth, Sophie Bachellier Bassi, Murielle Chauvel, Ute Zeidler, Dominique Ferrandon, Toni Gabaldón, Bernhard Hube, Christophe d'Enfert, Steffen Rupp, Brendan Cormack, Ken Haynes & Karl Kuchler

The opportunistic fungal pathogen *Candida glabrata* is a frequent cause of candidiasis, causing infections ranging from superficial to life-threatening disseminated disease. The inherent tolerance of *C. glabrata* to azole drugs makes this pathogen a serious clinical threat. To identify novel genes implicated in antifungal drug tolerance, we have constructed a genome-scale *C. glabrata* deletion library consisting of 619 unique, individually bar-coded mutant strains, each lacking one specific gene. Functional analysis of this library in a series of phenotypic and fitness assays identified numerous genes required for growth of *C. glabrata* under normal or specific stress conditions, as well as a number of novel genes involved in tolerance to clinically important antifungal drugs such as azoles and echinocandins. We identified 39 deletion strains displaying increased susceptibility to caspofungin, 28 of which encoding proteins that have not previously been linked to echinocandin tolerance. Our results demonstrate the potential of the *C. glabrata* mutant collection as a valuable resource in functional genomics studies of this important fungal pathogen of humans, and to facilitate the identification of putative novel antifungal drug target and virulence genes.

Contribution:

For this study I constructed *C. glabrata* mutants, participated in the verification and phenotypic screening of mutants created by other people and was involved in writing the manuscript.

Submitted to Antimicrobial Agents and Chemotherapy in January 2014

Position and numbers of FKS mutations in *C. albicans* selectively influence in vitro and in vivo susceptibility to echinocandin treatment

M. Lackner, **M. Tscherner**, M. Schaller, K. Kuchler, C. Mair, B. Sartori, F. Istel, M. C. Arendrup and C. Lass-Flörl

Candidemia is the fourth most common microbial bloodstream infection, with *Candida albicans* being the most common causative species. The echinocandins are employed as first line treatment for invasive candidiasis until fungal speciation is confirmed by clinical diagnosis. Echinocandins block the FKS glucan synthases responsible for embedding β-(1,3) D-glucan into the cell wall. The increasing use of these drugs has led to the emergence of antifungal resistance, and elevated MICs have been associated with single residue substitutions in specific hot spot regions of *FKS1* and *FKS2*.

Here, we show for the first time, the caspofungin-mediated in vivo selection of a double mutation within one allele of the *FKS1* hot spot 1 in a clinical isolate. We created a set of isogenic mutants and used a haematogenous murine model to evaluate in vivo outcome of echinocandin treatment. Heterozygous and homozygous double mutations significantly enhance the in vivo resistance of *C. albicans* when compared with heterozygous single mutations. The various *FKS1* hot spot mutations differ in their MIC increase, substance-dependent in vivo response, and impact on virulence. Our results demonstrate that echinocandin EUCAST breakpoint definitions correlate with in vivo response at standard dosing regimen, but cannot predict in vivo response at dose escalation. Moreover, patients colonized by a *C. albicans* strain with multiple mutations in *FKS1* have a higher risk for therapeutic failure.

Contribution:

I constructed all of the *C. albicans FKS1* mutants in the SC5314 background strain used in this study.

9.2 Danksagung

Ich möchte mich hiermit bei allen Menschen bedanken, die mich während meiner Dissertation unterstützt haben.

Vor allem möchte ich mich bei meinen Eltern für ihre Unterstützung und ihr Vertrauen während meiner gesamten Ausbildung bedanken.

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9.3 Curriculum Vitae

Personal Data

Name: Michael Tscherner

Date of Birth: 6th of May 1983

Place of Birth: Bad Radkersburg, Austria

Nationality: Austria

Education

1989 – 1993 Primary School VS Deutsch Goritz

1993 – 1997 Secondary School HS Deutsch Goritz

1997 – 2001 Secondary School BORG Bad Radkersburg with focus on Biology (first class honours)

University Studies

2002 – 2005 Bachelor in Molecular Biology at the Karl Franzens University Graz (first class honours)

2005 – 2007 Master in Microbiology at the Karl Franzens University Graz (first class honours)
Diploma thesis "Enhancing heterologous protein expression in *Pichia pastoris*" at the Technical University Graz with Prof. Dr. Helmut Schwab

2008 – 2014 PhD student with Prof. Dr. Karl Kuchler, Medical University of Vienna, MFPL

Grants & Awards

2009 Young Investigator Award of the ASMM for best Poster Presentation

2010 Hans Rieth Poster Price 2010 of the German Mycological Society (DMykG)

2012 Prize for best Poster Presentation at the ISHAM Meeting 2012

2013 FEBS YTF Grant for the 5th Advanced Lecture Course "Human Fungal Pathogens"

Languages

German: native; English: fluent (written and spoken)

Membership in Professional Societies

Austrian Association of Molecular Life Sciences and Biotechnology

Teaching Experience

2010 Supervision of the Diploma Thesis of Eva Stappler: Role of Histone Modifications in DNA Repair in *Candida albicans*

2011 Supervision of the Internship of Lisa Nika: Characterisation of Azole and oxidative stress resistance of a *Candida albicans HAT1* deletion strain

2012 Supervision of the Internship of Kathrin Bracher: Deletion and tagging of *Candida albicans* Spt6 and Spt16 histone chaperones

2012 Supervision of the Internship of Philipp Bammer: The role of *Candida albicans* Hat1 and Asf1 in histone processing

2007 Tutorial assistant at the Technical University of Graz

Selected Scientific Presentations

2009 Poster Presentation, 25th Fungal Genetics Conference, Asilomar, USA

2010 Poster Presentation, 10th ASM Conference on Candida and Candidiasis, Miami, USA

2011 Poster Presentation, Yeast Cell Biology Meeting, Cold Spring Harbor, USA

2012 Poster Presentation, 11th ASM Conference on Candida and Candidiasis, San Francisco, CA

2013 Oral Presentation, 5th Advanced Lecture Course "Human Fungal Pathogens", Nice, France

Scientific Publications

Zahrl D., Wagner A., **Tscherner M.** & Koraimann G. (2007). GroEL plays a central role in stress-induced negative regulation of bacterial conjugation by promoting proteolytic degradation of the activator protein TraJ. Journal of Bacteriology 189(16): 5885-5894

Hnisz D., **Tscherner M.** & Kuchler K. (2011). Targeting chromatin in fungal pathogens as a novel therapeutic strategy: histone modification gets infectious. Epigenomics 3: 129-132

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Tscherner M. & Kuchler K. (2013). Immunoblot Analysis of Histone H4 Acetylation and Histone H2A Phosphorylation in *Candida albicans.* Bio-Protocol October 20, 3(20)

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