Molecular characterisation of the effects of classical chemotherapy on *Candida albicans* and systemic candidiasis

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**Molecular characterisation of the effects
of classical chemotherapy on *Candida*
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School of Biological Sciences

2017

MOLECULAR CHARACTERISATION OF THE
EFFECTS OF CLASSICAL CHEMOTHERAPY ON
CANDIDA ALBICANS AND SYSTEMIC
CANDIDIASIS

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– *Twelfth Night*, William Shakespeare

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Abbreviations

5-FU	5-fluorouracil
ANOVA	analysis of variance
ATC	anhydrotetracycline
BLAST	Basic Local Alignment Search Tool
bp	base pair
bw	body weight
cDNA	complementary DNA
CFU	colony forming unit
CSP	caspofungin
DMSO	dimethyl sulfoxide
FACS	fluorescence-activated cell sorting
FRT	FLP Recombination Target
gDNA	genomic DNA
GI	gastrointestinal
HU	hydroxyurea
IC	inhibitory concentration
I.P.	intraperitoneal
I.V.	intravenous
LB	lysogeny broth
MHC	major histocompatibility complex
MTX	methotrexate
OD	optical density
PBS	phosphate buffered saline
qPCR	quantitative polymerase chain reaction
RNR	ribonucleotide reductase
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute
WT	wild-type
YPD	yeast-peptone-dextrose
YNB	yeast-nitrogen-base

Abstract

Due to chemotherapy-induced immunosuppression and epithelial barrier disruption, cancer patients are well-known to be at increased risk of opportunistic infection, such as those caused by the dimorphic fungus *Candida albicans*. While effects of chemotherapy on the host are well documented, little is known about direct effects of chemotherapy on the pathogen. We hypothesise that chemotherapeutic agents enhance genomic variation and evolvability in *C. albicans* colonising the host, thus potentially altering its virulence and its response to antifungal drugs.

Here we found that hydroxyurea (HU) induces aneuploidy in *C. albicans*, and in particular trisomy of Chromosome 2. This aneuploidy not only conferred HU resistance, but also cross-resistance to caspofungin, a first-line antifungal drug. Therefore, chemotherapeutic drugs might negatively impact the outcome of fungal infections not only by suppressing the host's defence mechanisms but also by generating genomic variation and promoting antifungal drug resistance in a major fungal colonizer of humans.

1. Introduction

1.1. Cancer-associated immunosuppression and infection

The World Health Organisation has estimated 14 million new cases of cancer in 2012 and an increase of such cases by ~70% over the next 20 years [1]. Because the incidence of cancer increases with age, and the world population is an increasingly ageing one (defined as an increase in proportion of people aged 60 years or older), incidence rates of cancer can be expected to continue increasing [2]. In general, a higher incidence of cancer occurs in economically developed countries, such as North America, Western Europe, Japan and South Korea, although mortality rates are generally higher in developing countries due to less developed medical care infrastructure [1]. Regardless of geography, cancer patients face a multitude of medical problems arising from the malignancy itself, with cancer being the second leading cause of death worldwide [3]. In addition to these problems, cancer treatment also causes side effects that can lead to the development of other diseases. In particular, cancer patients are often in a state of immunosuppression, which predisposes them to the development of opportunistic microbial infections.

This state of immunosuppression in cancer patients firstly arises from the cancer itself. This concept stems from the theory of cancer immunosurveillance, first proposed by Ehrlich, and later by Burnet and Thomas [4,5]. The theory suggests that in healthy people, the immune system is capable of recognising and removing cells that have lost cellular controls on proliferation, thus curtailing the progress of malignancy. In cancer patients however, such surveillance and subsequent removal do not take place, allowing cancer to take root in the host. In fact, many studies have reported several tactics which cancer cells use to avert detection by the immune system – with particular focus on evasion of the adaptive arm of the immune system (a topic which has been extensively reviewed by Rabinovich *et al.* and Kim *et al.*) [6,7]. In blood cancers, the immunosuppression arising from the cancer itself can be more pronounced than

in the case of solid tumours. Another substantial contributor to cancer-associated immunosuppression, which does not distinguish between solid and liquid types of cancer, is the use of chemotherapy, common to the treatment regimens of nearly all cancers [8,9]. Chemotherapy acts on several levels to suppress different players of the immune system, as will be discussed in later sections of the Introduction.

These immunosuppressive elements combined lead to a higher risk of developing opportunistic microbial infections. A leading cause of such infections in cancer patients is the group of fungi belonging to the *Candida* genus, with *Candida albicans* being the top cause of systemic candidiasis around the world [10-16]. This introduction will first present epidemiological findings of systemic *C. albicans* infection in cancer patients, followed by cell biology and genomic plasticity of *C. albicans*, and the mode of action of different chemotherapeutic drugs and known effects of chemotherapy on the human host and *C. albicans*.

1.2. Systemic candidiasis

1.2.1. Epidemiology of systemic candidiasis in cancer patients

C. albicans is the most prevalent cause of candidaemia around the world, even in developed countries [10-16]. This trend is also present in bloodstream infections in cancer patients [17]. The average annual incidence of systemic candidiasis in cancer patients is higher than that of the general hospital population, with the former ranging from 71 – 2,400 per 100,000 admissions, compared to the latter with 10 – 370 per 100,000 admissions [18]. Crude mortality rates arising from systemic *C. albicans* infection have been estimated at around 30 – 40%, but some have estimated even higher rates at 46 – 75% [10,14,15,19]. Systemic *C. albicans* infection is associated with longer hospital stays and increases the cost of illness, thereby increasing the burden on healthcare systems. In 2010, Moran *et al.* reported the cost to range from \$32,810 – 52,112 [20]. Neutrophils are a first-line defense against pathogens in general. A reduction in the number of neutrophils (termed neutropenia, defined as having less than 500 neutrophils/mm³ of blood) leads to an increased risk of developing opportunistic

microbial infections [21]. Neutropenia is also recognised as an independent risk factor for the development of systemic candidiasis [18,22]. Neutropenia is a common side effect of classical chemotherapy but can also arise as a result of certain types of blood cancers or cancers infiltrating the bone marrow [23,24].

However neutropenia does not seem to be the sole predisposing factor for systemic *C. albicans* infection. Studies have shown that only 10 % or less of all patients with systemic *C. albicans* infection are actually neutropenic [14-16]. Also, while the risk of neutropenia-related infections is greater in patients with haematological malignancies than those with solid tumours, it is instead the latter group which is far more likely to develop systemic *C. albicans* infection [10,14-16,25].

This disparity between patients with liquid tumours and those with solid tumours may result from differences in choice of chemotherapeutic agents, which may differ in their effect on the host immune system. The variation could also be the result of differences in the use of certain medical interventions, such as catheter use and surgical procedures which are in and of themselves, risk factors for systemic *C. albicans* infection [18]. Other risk factors for fungal infections, which are also commonly found in cancer patients, are mechanical ventilation, corticosteroid therapy, and renal failure [26-28]. In addition to all these external risk factors, another angle that has yet to be studied is whether chemotherapy has direct effects on opportunistic pathogens residing in the human host, in this case *C. albicans*, and whether such effects lead to increased virulence or decreased response to therapy.

1.2.2. Cell biology of *C. albicans*

C. albicans is one of the major colonisers of the skin, gastrointestinal (GI) tract and genitourinary tract. It is a dimorphic fungus, a member of the *Saccharomycetes* class, and a distant evolutionary relation of *Saccharomyces cerevisiae*, the baker's yeast. It is normally present in a diploid state, with eight pairs of chromosomes (named 1 – 7, and the eighth pair called 'R' for 'ribosomal'), comprising a total haploid genome size of approximately 14 megabases [29,30].

As we shall see later, *C. albicans* has a high plasticity with respect to ploidy, and other aspects of its genome. This plasticity is not limited to its genotype, and is also demonstrated in its ability to change several aspects of its phenotype and morphology, depending on the environment. The genomic, phenotypic and morphological plasticity of *C. albicans* combined make it an organism readily adaptable to a wide range of environmental niches.

1.2.2.1. Morphological plasticity and its link to virulence

C. albicans is capable of taking on several morphological forms. It can form budding yeast similar to *S. cerevisiae*, or filamentous forms called pseudohyphae and hyphae similar to moulds. This ability to change morphological forms is essential for virulence. Both yeast-locked and hyphal-locked mutants show reduced virulence in mouse models of systemic *C. albicans* infection [31,32]. The yeast form is believed to facilitate spread of *C. albicans* through the circulation, while the filamentous form is required for invading tissues and avoiding immune defenses.

Many stimuli are known to induce filamentation [33]. Environmental factors are paramount in regulating morphological switching in *C. albicans*. Some of these factors are alkaline pH, bacterial peptidoglycan fragments in serum, plastic, and temperature shift (from 30 to 37°C) [33-35]. DNA damage such as those induced by hydroxyurea (HU) and other chemotherapeutic compounds are also known to cause filamentation *in vitro*, a process mediated by proteins in the DNA damage checkpoint, namely Rad53 and Mrc1 [36,37]. However the appearance of these filaments differs somewhat from true hypha, resembling pseudohyphae more. DNA damage-induced filaments do express some hypha-specific genes at later stages of filamentation, but it remains to be seen if such filaments retain a full repertoire of properties associated with true hypha, especially those related to virulence, such as the ability to invade mammalian tissues [37,38].

1.2.2.2. Mating ability and the white-opaque switch

C. albicans is capable of mating, a process under the control of the mating type locus (*MTL*) located on Chromosome 5 [39]. As with *S. cerevisiae*, mating occurs

between cells of opposite mating type. Cells of the same mating type do not mate with each other, nor do a/a cells [40]. However most strains of *C. albicans* are heterozygous at this locus, and since this species is incapable of undergoing meiosis, loss of heterozygosity at this locus is generally a prerequisite for mating [41]. The second prerequisite for mating is a phenotypic switch from the white to opaque form [42]. The white-opaque transition, first described by Slutsky *et al.*, is also under partial control of *MTL* [43]. Interestingly the chemotherapeutic drug HU is also capable of increasing the rate of switching in *MTL*-homozygous cells [44]. Under certain circumstances, *MTL*-heterozygous cells are also capable of white-to-opaque switching, although they are not capable of mating [45].

As mentioned above, *C. albicans* does not undergo meiosis, possibly due to the lack of a complete genetic repertoire needed for meiosis [46]. As such, *C. albicans* lacks a true sexual cycle. Work by Hickman *et al.* using next generation sequencing has shown that tetraploid cells produced by mating undergo a random chromosome loss process, in order to approach the diploid state [47]. The randomness of this process is one way to introduce genetic variation within a mitotically-dividing population.

Transcriptional profiling has revealed that white and opaque cells differ from each other greatly in terms of metabolic processes [48]. More specifically, white cells appear to favour a fermentative process, while opaque cells rely on oxidative metabolism. This may facilitate the colonization of different host niches. Additionally, white and opaque cells may also induce different immune responses, as the former secretes a chemoattractant for polymorphonuclear leukocytes (a group that encompasses neutrophils, basophils, and eosinophils), while opaque cells appear to suppress such secretion, which may better allow them to evade immune detection [49].

Figure 1. An illustration of the morphological variation (A) and the possible pathways to genomic variation arising from mating (B) in *C. albicans*.

1.2.2.3. Genomic plasticity of *C. albicans*

C. albicans exhibits an unusually high tolerance for genomic changes [50]. Common laboratory strains of *C. albicans* have been found to carry loss of heterozygosity, arising from small changes, such as those produced by gene

conversion, as well as very large changes, such as aneuploidies (changes in chromosome copy number) [51]. Although such genetic changes can take their toll on fitness, they provide genomic variation, which in turn provides the capacity for adaptation to environmental changes [52-54]. For example, some clinical isolates of *C. albicans* have been shown to carry an extra copy of the left arm of chromosome 5, which confers fluconazole resistance, while other isolates showed relatively smaller changes such as point mutations [55,56]. Other aneuploidies are also linked to resistance against DNA-damaging agents such as methyl methanesulfonate [57].

One question that might arise is why cells would develop aneuploidy if much smaller changes already permit adaptation, especially since aneuploidy is associated with greater fitness costs than smaller mutations. One postulated reason is that aneuploidy is much easier to acquire than smaller mutations, such as point mutations, which need to hit a precise location. For instance, rate of chromosome loss in yeast averages 2.6×10^{-4} per cell division, while rate of nucleotide substitution averages 8.1×10^{-6} per cell division, showing more than a thirty-fold difference between the two [58]. Hence aneuploidy is more likely to provide a quicker response to environmental changes, enabling cells to survive, if not thrive, until they evolve more refined (i.e. less disadvantageous for fitness) solutions to such environmental challenges [59]. Aneuploidy is also capable of affecting more genes at one time than smaller-scale mutations. This is a double-edged sword, as genomic imbalances arising from aneuploidy lead to severe growth defects, but at the same time produce much greater variation in gene expression, and it is this variation that provides the organism a chance to survive in adverse conditions ('a phenotypic leap' as described by Chen *et al.*) [52,53,60]. Hence an increased generation of aneuploidy can promote adaptation to various stresses [61].

1.3. Chemotherapy

In modern times, chemotherapy is one of the first-line therapies for cancer, often used in combination with radiotherapy and/or surgery. In the case of blood

cancers, chemotherapy was also the very first form of therapy shown to be effective. Continuing work on the discovery of chemical compounds that could potentially cure cancer has spawned a range of such drugs. The table below shows some classes of chemotherapeutic compounds and their mechanisms of action.

1.3.1. Different classes of chemotherapy

Class	Drug name	Mechanism of action	Used in
Antimetabolites	Methotrexate (MTX)	Modified in vivo into several different products, which are all able to block the conversion of dihydrofolate to tetrahydrofolate. Reducing the amount of tetrahydrofolate results in reduced thymidylate synthesis, which is required for DNA synthesis.	Leukaemia, lymphoma, lung cancer
	5-fluorouracil (5-FU)	In vivo products lead to RNA and DNA damage, such as the inhibition of thymidylate synthase by FdUMP, and the incorporation of FUTP into RNA, which inhibits the processing of pre-RNA into mature RNA and disrupts post-transcriptional modification of tRNA.	Stomach cancer
	Hydroxyurea (HU)	Inhibits ribonucleotide reductase (RNR), an enzyme required for DNA synthesis, stalling the cell cycle in the S phase.	Leukaemia, melanoma, ovarian cancer
Cross-linking agents	Cisplatin	The platinum complexes bind to nucleophilic groups in DNA (such as GC-rich regions), leading to DNA crosslinking and ultimately cell death.	Lung cancer, ovarian cancer
	Cyclophosphamide	A nitrogen mustard that is metabolised in the liver to become aldophosphamide and phosphoramide mustard, which bind to DNA, resulting in DNA crosslinking and inhibition of DNA replication, ultimately leading to cell death.	Leukaemia, lymphoma, ovarian cancer
Spindle inhibitors	Paclitaxel	Binds to tubulin and prevents microtubule disassembly, blocking cell division.	Lung cancer, breast cancer, ovarian cancer
Topoisomerase inhibitors	Doxorubicin	Binds to topoisomerase I when the enzyme is bound to other protein complexes during DNA replication, and prevents the complex from dissociating. Religation after cleavage of DNA strand cannot take place and the double-strand break remains unrepaired, resulting in DNA damage.	Bladder cancer, breast cancer, lymphoma
	Topotecan	Intercalates between base pairs during DNA synthesis, blocking religation of strands after cleavage by topoisomerase I. Results in double-stranded DNA breaks.	Lung cancer, ovarian cancer

Table 1. List of chemotherapeutic compounds used in our study, along with their mechanism of action, and applications in cancer treatment.

Table 1 shows a list of chemotherapeutic drugs and the types of cancer they can be used in. The list is neither definitive nor exhaustive. Choice of chemotherapy depends on the subtype and stage of cancer, and it is also important to note that regimens commonly involve combination therapies, i.e. more than one chemotherapeutic drug is used in treatment (sometimes in conjunction with radiotherapy, another DNA-damaging and immunosuppressive agent) [9].

As HU is the focus of the thesis, its properties require further elaboration here. It is used in the treatment of melanoma, cervical cancer and chronic myelogenous leukaemia [62]. It is also used for the treatment of vaso-occlusive crises resulting from sickle-cell anaemia, despite its being a non-malignant disease. In this case HU's beneficial effects appear to be mediated by an increase in fetal haemoglobin, although whether the exact mechanism behind this effect involves the ribonucleotide reductase – the known drug target of HU – is not clear [63,64]. The drug is highly water-soluble, as can be deduced from its chemical structure [62]. It is normally administered orally, and achieves a high bioavailability rapidly [65]. HU is metabolised by the liver to urea and carbon dioxide, which are excreted primarily in urine [66].

Figure 2. Chemical structure of HU.

1.3.2. Inhibition of ribonucleotide reductase (RNR) by HU

Deoxynucleotide triphosphates (dNTPs) are the building blocks required for DNA synthesis. These are in turn generated using deoxyribonucleoside diphosphates (dNDPs), which are synthesized *de novo* by the enzyme ribonucleotide reductase (RNR). This process is also the rate-limiting step in the *de novo* biosynthesis of

dNTPs. All organisms have one or more forms of RNRs. These multiple forms are classified according to properties such as: oxygen dependence, tertiary structure of the enzyme complex, the metallic cofactors required, and the location of the free radical required for its activity [67]. Namely, the different categories are: Classes I (itself divided into three sub-classes, Ia, Ib and Ic), II, and III.

This introduction will focus on the class of enzyme present in eukaryotes, which is class Ia. Class Ia enzymes require oxygen, generate the free radical at the tyrosyl residue, and are heterodimeric, comprising of two dimers, called the α - and β -subunits. The enzyme structure has been extensively reviewed by Nordlund and Reichard [68]. Briefly, the α -subunit is larger than the β -subunit, and comprises of the three different sites. One is the catalytic site where the reduction of NDPs take place, second is the allosteric specificity site which ensures that equivalent levels of each dNTP is produced, and lastly the allosteric regulatory site which controls the activity of RNR itself depending on whether ATP (stimulatory) or dATP (inhibitory) binds to the site [68]. The β -subunit on the other hand, contains the tyrosyl free radical, which is the chemical basis for RNR's reduction of NDPs [68].

Owing to its central role in DNA replication, cell division and growth, RNR activity is tightly regulated. These regulatory mechanisms occur at several levels: allosteric, temporal, spatial, transcriptional, post-transcriptional (reviewed by Guarino *et al.*) [69]. Expression of *RNR* genes is cell cycle-regulated and peak close to S-phase [68]. In *S. cerevisiae*, some components of the RNR are normally located in the cytoplasm, while others are localised to the nucleus [70]. This spatial separation contributes to the regulation of RNR activity, as the different components need to come together to produce the enzymatically active complex. This strict regulation on the levels of enzyme activity and substrate production is crucial, as imbalances in the levels of dNTPs result in genomic instability [71].

The integral role of RNR in cell division makes it a useful target for cancer treatment. A few established drugs have the ability to inhibit RNR activity through various mechanisms of action. Most act as irreversible inhibitors of RNR by

binding to its catalytic site (gemcitabine, fludarabine, cladribine) [72]. HU is unusual among RNR inhibitors for its difference in mechanism of action. HU behaves as a free radical scavenger, removing the free radical from the tyrosyl residue of the RNR β -subunit. This inhibits generation of dNTPs by RNR, which eventually triggers the intra-S checkpoint and leads to cell cycle arrest [73,74]. In mammalian cells, if DNA damage is too extensive for repair, apoptosis is the final result of checkpoint activation, although yeast cells can override the checkpoint and resume the cell cycle [75,76]. Although the different types of dNTPs show a general trend in quantitative reduction during HU treatment, purine dNTP pools appear to be more affected by HU treatment than pyrimidine dNTP pools [77]. Another property that sets HU apart from other RNR inhibitors is that HU's effect is reversible; cells that have been arrested in S-phase due to activation of the intra-S checkpoint by HU treatment can resume cell cycle progression after the drug has been withdrawn [78]. In fact, this reversibility of action in HU is exploited in yeast research for routine synchronization of the cell cycle of an entire population [79].

Figure 3. A representation of the RNR enzyme subtype commonly found in eukaryotes [80]. Note the heterodimeric α - and β -subunits, as well as the ATP cone involved in allosteric regulation of enzyme activity.

1.3.3. RNR in *C. albicans*

There are four known genes encoding for components of the RNR complex in *C. albicans*: *RNR1*, *RNR3*, *RNR21* and *RNR22* [29]. *RNR1*, *RNR21* and *RNR22* are located on Chromosome 2, while *RNR3* is located on Chromosome R. In terms of sequence identity, *RNR1* and *RNR3* correspond to the large subunit in *S. cerevisiae* (*RNR1* and *RNR3*), while *RNR21* and *RNR22* correspond to the small subunit (*RNR2* and *RNR4*). Unlike *C. albicans*, the *RNR* genes in *S. cerevisiae* are distributed more evenly across the entire genome [81-85].

Multiple sequence alignment with genetic sequences coding for RNR components from *S. cerevisiae* and humans show fairly high identity ranging from 60 – 70% (see Appendix for alignments). Humans have three genes coding for RNR components as opposed to four. Like *S. cerevisiae* and *C. albicans*, humans have two genes encoding for the small subunit (one of them being TP53 inducible), but only one gene coding for the large subunit [86]. It is interesting to note that in the case of *C. albicans* and *S. cerevisiae*, only one of the two genes coding for the large subunit is highly expressed at baseline conditions (*RNR1*), while the other gene (*RNR3*) tends to be nearly undetectable except in the presence of DNA damage [87]. While there is a strong sequence identity between *RNR1* and *RNR3*, studies in *S. cerevisiae* have shown that *RNR1* is essential for viability, whereas *RNR3* is not [81]. Studies on RNR in *S. cerevisiae* also show that the RNR complex is slightly different from other class Ia RNRs, in that the active small subunit is a heterodimer of the proteins encoded by *RNR2* and *RNR4*, instead of a homodimer [88]. *C. albicans* possibly also exhibits the same difference from other Class Ia enzymes in this respect.

In *S. cerevisiae*, regulation of RNR activity takes place on several levels: transcriptional, post-transcriptional and spatial [70]. Rnr2 and Rnr4 mainly localise to the nucleus, and under HU stress, an increased localisation to the cytoplasm is observed, mediated by Mec1/Rad53/Dun1 pathway, a G2/S checkpoint [70]. The protein Sml1 is also a key regulator of RNR activity, binding to the large subunit of RNR, which prevents the small subunit of RNR from binding, thus preventing

formation of the active complex [89]. Sml1 is regulated in turn by Mec1, Rad53 and Dun1 [90]. *SML1* has no known ortholog in *C. albicans* but possibly other proteins may be involved in a similar regulation of RNR.

1.3.4. Side effects of chemotherapy on the human host

Classical chemotherapeutic agents target rapidly-dividing cells without discriminating between healthy and cancerous tissues. Their non-specificity results in many side effects, ranging from relatively mild (e.g. hair loss, fatigue and nausea) to potentially life-threatening (e.g. immunosuppression and organ damage). Aside from reducing quality of life, these side effects also increase the patient's risk of death from other causes, one of which is opportunistic microbial infections [91,92]. The increased risk of opportunistic microbial infections in cancer patients is attributed to disturbances in immune cell development and epithelial barrier maintenance [93,94]. The epithelial barrier is the primary line of antimicrobial defense, a physical barrier that prevents invasion by microbes and bars their entry into the blood and producer of chemicals to regulate microbial growth [95]. In the event that the barrier is breached and invasion occurs, neutrophils keep infection from becoming rampant throughout the system by direct action on microbial pathogens [96-98].

1.3.4.1. Effects of chemotherapy on the epithelial barrier

Chemotherapy is well-known for eroding epithelial layers [99,100]. Its action on epithelial cells and progenitors is known to lead to cell death [101,102]. The gut epithelium is no exception, as increased apoptosis in intestinal crypts has been observed post-chemotherapy [103]. This disruption of the gut epithelium may enable *C. albicans* to invade and reach the bloodstream more easily [104].

Cell death is not the only contributing factor in chemotherapy-induced disruption of the epithelial barrier. Newer studies have found evidence of chemotherapy-induced changes in tight junctions of the gut epithelial layer (reviewed by Wardill and Bowen), which help to maintain barrier integrity [105]. Beutheu Youmba *et al.* found that methotrexate reduces expression of the tight junction proteins occludin and claudin in rats [106]. Treatment of Caco-2 cell monolayers curiously did not

show increased apoptotic cells, contradicting earlier reports that chemotherapy produces apoptosis in rapidly dividing epithelial cells. Pre-treatment of cell monolayers with inhibitors of NF- κ B, JNK, MEK1 and MEK2 pathways improved barrier integrity post-methotrexate treatment, suggesting that these pathways are involved in methotrexate-induced epithelial barrier disruption, and providing possible mechanisms to improve barrier integrity [106]. However, these observations of poorer barrier integrity and reduced tight junction protein expression were more pronounced in *in vitro* experiments, compared to *in vivo* experiments in rats.

1.3.4.2. Effects of chemotherapy on innate immunity

As mentioned previously, neutropenia is a common side effect of classical chemotherapy, and has been cited as an important risk factor for systemic *C. albicans* infection [18,22,23]. Neutrophils are one of the first responders among immune cells at the site of infection, directed by pro-inflammatory cytokines produced by epithelial cells and macrophages. Neutrophils combat *C. albicans* infections in several ways. One way is the production of antimicrobial peptides (AMPs) and reactive oxygen species (ROS) [107]. Another way is phagocytosis of the yeast form of *C. albicans* [108]. The third way is the formation of neutrophil extracellular traps (NETs), which help to kill the hyphal form of *C. albicans*, which are able to escape phagocytes unlike yeast, or are too large to be phagocytosed in the first place [31,97,109,110].

Neutrophils' multiple contributions make them essential to antifungal defense. This is demonstrated by experiments in which neutrophil depletion led to more extensive tissue damage in a mouse model of systemic *C. albicans* infection, as well as a greater fungal burden in the kidneys (which are a common site of invasion in the mouse model for acute systemic *C. albicans* infection) [98]. Hence chemotherapy-induced neutropenia can have profound effects on the ability of the host to defend against *C. albicans* invasion. However, neutrophil depletion alone cannot produce dissemination of *C. albicans* from the GI tract, and that

neutropenia needs to co-occur with epithelial barrier disruption for dissemination to take place [104].

While having an adequate neutrophil count is important for host defense, intact neutrophil function is also important for their efficacy. Patients can have a normal neutrophil count, but still be at an increased risk of bacterial and fungal infections if neutrophils are dysfunctional [111,112]. In this regard, chemotherapy can also induce decreased migratory and phagocytic activity in neutrophils, which has been correlated with reduced candidacidal activity [113].

Monocytes and macrophages are another important group of cells for defense against *C. albicans*, as their depletion results in more rapid mortality upon systemic *C. albicans* infection in the mouse model [114,115]. Monocytes and macrophages phagocytose *C. albicans* as neutrophils do, as well as secrete pro-inflammatory cytokines that contribute to the antifungal response [107,108].

They also confer a memory-like protection against systemic *C. albicans* infection, after priming with a sublethal dose of *C. albicans* [116]. This phenomenon is termed "trained immunity", and is triggered by Dectin-1 recognition of β -glucan present in the cell wall of *C. albicans*. Triggering of Dectin-1 leads monocytes to differentiate into "trained macrophages", which is the result of a combination of epigenetic and metabolic re-programming, crucial for the protective effects of trained immunity. Thus monocytes and macrophages play an important role in protecting the host from systemic *C. albicans* infection and re-infection [117,118]. Chemotherapy-mediated depletion of monocytes and macrophages therefore represents one plausible mechanism that predisposes patients to a higher risk of *C. albicans* infection.

1.3.4.3. Mutagenic effects of chemotherapeutic drugs in mammalian cells and *C. albicans*

Chemotherapeutic drugs induce DNA damage that lead to cell cycle arrest and apoptosis [72,119-121]. Through this mode of action, cancer cells are eliminated. However the possibility that chemotherapy can also inflict mutations that persist

and produce deleterious effects in future has long been a clinical concern. This is particularly so for paediatric and young adult cancer patients, as the likelihood of developing a secondary malignancy in these populations is much greater.

Hints that this concern was justified were emerging already in the 1970s. A study of more than 400 Hodgkin's lymphoma (HL) patients found that the risk of secondary malignancies, which were not associated with the lymphoma itself, were significantly higher in patients receiving intensive radiotherapy and chemotherapy [122]. However, those with HL were observed to have a higher risk of secondary cancer compared to those without HL, and this could be the result of an inherent genomic instability in these patients, or HL itself. A more recent study in HL patients (under different chemotherapeutic regimens) has shown that chemotherapy treatment alone does significantly raises the risk of secondary cancers, more specifically for leukaemia, lung cancer and non-Hodgkin's lymphoma, and this also correlated with the number of cycles of alkylating chemotherapy that patients received [123].

An increased occurrence of mutations has also been found in T-lymphocytes isolated from patients who underwent chemotherapy, as compared to untreated patients, although the population sizes were small and comprised of patients with varying medical conditions [124]. Increased frequency of mutations in p53 as well as mitochondrial DNA have also been observed in chronic lymphocytic leukaemia (CLL) patients who had undergone chemotherapy [125,126].

Exactly what types of mutations are likely to be produced depends on the type of chemotherapeutic drug used. Alkylating agents chemically react with DNA bases, producing DNA adducts, which interfere with uncoiling of DNA during replication, obstructing the progression of replication machinery, and introducing base mismatches [121]. Repair of DNA adducts can occur through direct mechanisms which simply remove the adduct, restoring the base to its normal state. It can also occur through base excision repair, nucleotide excision repair, and mismatch repair. These three mechanisms involve the excision of a segment including the damaged DNA, and then re-synthesising the original segment based on the sister

strand [121]. From these processes, the likelihood of sequence changes is expected to be small. One of the main causes of base mismatches arising from alkylating agents is 6-O-methylguanine, which pairs with thymine as opposed to cytosine, and can potentially lead to sequence changes in the genome (reviewed by Kaina *et al.*) [127]. 6-O-methylguanine is also involved in increased chromosomal instability in repair-deficient cells [128].

The spindle inhibitor paclitaxel causes mitotic arrest in cells (at least in cell culture conditions), by stabilizing microtubules and interfering with assembly of the mitotic spindle [120,129,130]. While mitotic arrest can lead to apoptosis, there is also the possibility of mitotic slippage, leading to production of tetraploid cells, which are linked to increased chromosomal instability [58,120,131]. More recent studies have shown that clinically relevant concentrations of paclitaxel cause the formation of multipolar spindles, as opposed to mitotic arrest, contributing to incorrect chromosomal segregation and producing aneuploidy [132].

Antimetabolites (such as HU) interfere with replication progress, leading to stalled replication forks. These produce ssDNA, which are stabilized by the ATM/ATR pathway. This stabilization helps to ensure that replication can resume after the stress is resolved. However if the stress occurs over a prolonged period of time, stalled replication forks collapse, leading to double strand DNA breaks (DSBs) [133]. Repair of DSBs can take place through homologous recombination (HR) and non-homologous end joining (NHEJ). Of the two, HR is less likely to produce mutations as it relies on a template to restore damaged DNA (mechanism of HR reviewed by Jasin and Rothstein) – although it can lead to loss of heterozygosity – while NHEJ involves trimming off the ends of DSBs, which will produce deletions (reviewed by Lieber) [134,135]. Thus, depending on the dominant repair pathway, HU (and other antimetabolites) have increased likelihood of introducing sequence changes to the genome, and possibly even segmental aneuploidies (the mechanisms behind which pathway is dominant is reviewed by Chapman *et al.*) [136].

The increased mutagenesis seen in chemotherapy-treated mammalian cells could also be mirrored in *C. albicans*, as fungi and mammals are both eukaryotes, hence chemotherapy is likely to have similar effects on structures common across this domain of life. As mentioned earlier, HU is a common chemical used in cell cycle synchronisation using both yeast and mammalian cells [79,137]. This shows how the targets of chemotherapy can be highly conserved between humans and their fungal inhabitants.

1.3.4.4. Stress-induced mutagenesis

In addition to the intrinsically mutagenic properties of chemotherapeutic agents on eukaryotes, another manner in which increased mutagenesis may occur is through a process called stress-induced mutagenesis. Genomic stability is involved in maintaining protein homeostasis and the normal function of cells. Genomic instability in mammals is associated with the development of cancer owing to the increased likelihood of deleterious mutations [138]. Unicellular organisms from genetic backgrounds with higher mutation rates also demonstrate poorer growth [139]. However, as mentioned earlier, mutations are highly useful for adaptation to novel conditions, meaning that genomic instability can be a useful thing in stressful conditions. The concept of stress-induced mutagenesis proposes that in adverse environments, the basal rate of mutagenesis is increased [140,141]. This leads to an increase in the generation of mutations that facilitate adaptation to the adverse environment, helping to ensure that at least a segment of the population will survive.

This concept has been studied in a range of organisms, from *Escherichia coli*, to budding yeast, to *Arabidopsis* [142-144]. There has been much work involved in understanding exactly how stress itself triggers increased mutagenesis. In *E. coli*, environmental sensors are integrated into networks that decide the level of fidelity in replication mechanisms [145]. In times of stress, a switch in preference from high fidelity to low fidelity replication takes place, demonstrating the intrinsic link between mutagenesis and environmental conditions [143,145,146].

A similar integration of environment-sensing networks with networks involved in genomic stability also takes place in yeast such as *S. cerevisiae* and *C. albicans*, mediated by the heat shock protein Hsp90, a ubiquitous and highly conserved protein chaperone. Its main function is in promoting the stability of proteins. In times of environmental stress (e.g. elevated temperature), especially those resulting in elevated protein instability, increased levels of Hsp90 are required to maintain client proteins in their correctly folded state. Should the demand for Hsp90 exceed the supply (such as in times of heat stress), protein homeostasis within the cell is disturbed. Hsp90 is involved in the formation of kinetochores (the protein structure on chromatids to which mitotic spindle fibers attach in order to separate sister chromatids), making it an important factor in faithful chromosomal segregation [147,148]. In *S. cerevisiae*, chemical inhibition of Hsp90, and heat shock have both been found to increase occurrence of aneuploidy, and such induced aneuploidies have been found to promote adaptation to diverse stresses [144,149]. In *C. albicans*, different kinds of stresses (namely reactive oxygen species, elevated temperature, and fluconazole treatment) have been found to increase the rate of LOH, with particular stresses also correlating with an increase in certain types of LOH [149]. An increased rate of aneuploidy has also been observed during fluconazole treatment, arising from the formation of ‘trimeras’, a conjoined structure of three yeast cells with two nuclei, and the aberrant mitotic events within such trimeras [150].

Figure 4. Various ways in which environmental stress can lead to increased mutagenesis, providing the possibility for selection of adaptive mutants.

1.3.4.5. Effects of chemotherapeutic drugs on the phenotype of *C. albicans*

Besides effects on the genome of *C. albicans*, chemotherapeutic drugs have also been observed to trigger phenotypic changes in *C. albicans*. *C. albicans* is a coloniser of the GI tract of most humans, and thus can be exposed to chemotherapeutic drugs in cancer patients undergoing treatment, and be similarly affected [151,152]. Several recent studies have found that HU and other chemotherapeutic drugs induce morphological and phenotypic changes in *C. albicans* which may influence its virulence. These virulence factors (reviewed by Calderone *et al.*) are: ability to change morphological form, ability to adhere to mammalian tissues, production of certain proteinases, and resistance to antifungal drugs [153]. In this regards, some chemotherapeutic drugs – namely cisplatin and 5-fluorouracil – reduce the sensitivity of *C. albicans* to amphotericin B and miconazole, in addition to improving adherence to HeLa cells [154]. HU can induce filamentation in *C. albicans*, and induce phenotypic switching a process involved in mating, biofilm formation, metabolic adaptation to different host microenvironments, and immune evasion, as detailed earlier [36,41,44,48,49,155].

1.4. Summary and aims

Cancer patients have an elevated risk of acquiring systemic candidiasis associated with immunosuppression arising from malignancy and more importantly, from chemotherapeutic regimens. However chemotherapeutic drugs can have pleiotropic effects, with its sphere of influence extending beyond the human host, and possibly touching on the host microbiota, such as *C. albicans*. The highly conserved nature of chemotherapeutic drug targets make it likely that these drugs can also act on *C. albicans*. Given the strong mutagenicity of chemotherapeutic drugs, and the high receptivity of *C. albicans* towards mutations, we hypothesise that the treatment of a human host with chemotherapeutic drugs also leads to a domino effect on the resident *C. albicans* in the host. This leads to an increased mutation rate in the fungus, which could change its virulence and improve its adaptation to various clinically relevant

conditions, such as antifungal treatment. This is a yet unexplored mechanism through which chemotherapeutic drugs could modulate the risk of systemic candidiasis, and possibly other forms of opportunistic fungal infections.

In this study, we aim to understand how chemotherapy treatment affects the genomic, and in turn, phenotypic variation of *C. albicans*, both *in vitro* and *in vivo*, and whether such changes have any impact on adaptation to stress. Our specific goals are to:

- I. Determine which chemotherapeutic compounds have the potential to influence the virulence of *C. albicans* in the host through modulation of viability and growth and the induction of morphological and phenotypic changes involved in virulence;
- II. Assess the potential of chemotherapeutic compounds to improve the adaptability of *C. albicans* to hostile environments through increased mutagenesis and study the mechanisms that facilitate adaptation

We begin by verifying the effect of chemotherapy treatment, specifically HU, on the outcome of systemic *C. albicans* infection in an *in vivo* mouse model. We found that HU treatment in general worsens the outcome of systemic candidiasis, resulting in accelerated mortality. Further investigation into neutrophil and monocyte levels (the primary effector cells in antifungal defense) and kidney fungal burdens (the kidney being a focus of infection in the mouse model) showed that while immunosuppression correlated with mortality, mortality did not correlate with kidney fungal burden. This led us to question if chemotherapy also acts on *C. albicans*, in addition to the mammalian host, and whether this leads to increased virulence during infection.

We explored this possibility through *in vitro* screening of chemotherapeutic drugs from different drug families, to find out which drugs are capable of acting on *C. albicans*. The spotlight of our studies eventually falls on HU, which produces increased karyotypic variation under *in vitro* and *in vivo* conditions, in addition to a recurrent aneuploidy – trisomy of chromosome 2 - which confers increased resistance to HU, as well as resistance to caspofungin, a first-line antifungal drug.

Using genetic screens, we also find that trisomy 2 mediates HU resistance through increased copy numbers of specific *RNR* genes on Chromosome 2, although these genes are not required for caspofungin resistance. These results show how aneuploidy is capable of conferring pleiotropic effects mediated by different genes on the same chromosome.

Overall, this body of work demonstrates that certain chemotherapeutic compounds induce genomic instability in *C. albicans*, negatively affect the outcome of systemic candidiasis, as well as of treatment response towards a first-line antifungal drug. These results have important implications for the clinical management of cancer patients undergoing chemotherapy, especially in relation to their risk of opportunistic fungal infections.

2. MATERIALS AND METHODS

2.1. Dose-response relationship of *C. albicans* and chemotherapeutic drugs

2.1.2. Overnight culture

The wildtype strain of *C. albicans* SC5314 was grown overnight in liquid yeast peptone dextrose (YPD) medium at 37°C on a shaker (150 rpm) for about 15 – 16 hours. Where the chemotherapeutic drug being tested was dissolved in dimethyl sulfoxide (DMSO) instead of water (as in the case of doxorubicin, paclitaxel, topotecan and 5-fluorouracil), the YPD medium used contained 1 % DMSO (v/v) (Sigma-Aldrich, MO, USA) in this step and in subsequent steps.

The culture was then reinoculated into fresh medium, incubating for 2 hours at 37 °C, in order for cells to restart entry into the cell cycle. The cell density was then measured by spectrophotometer, and cells were then harvested to a volume that would give a final optical density at 600 nm (OD₆₀₀) of 0.03 – 0.05 in 20 mL YPD medium. Cells were centrifuged at 13,000 rpm for 1 minute and the medium removed. Each aliquot of cells was then resuspended in 20 mL of YPD medium either with or without the drug.

In the case of experiments involving methotrexate, liquid yeast nitrogen base (YNB) medium (without amino acids) was used instead of YPD medium. Steps involved in the culturing and processing of the cells remain identical.

2.1.2. Growth rate measurement

In order to chart cell division over time, OD₆₀₀ readings were measured on a spectrophotometer on an hourly basis for at least 6 hours. These readings were then converted to log form and used to plot a growth curve over time. The slope of these curves was obtained to measure the half maximal inhibitory concentration (IC₅₀) using GraphPad Prism 6.

2.1.3. Viability measurement

For determining viability changes in *C. albicans* after acute chemotherapeutic treatment, 1 mL of culture was taken at 6 hours post-treatment with the drug, spun down at 13,000 rpm for 1 minute and the medium removed. Cells were washed twice by resuspending in PBS and then centrifuging to collect a cell pellet. After the final wash, 1 mL of solution containing 0.0015 % (v/v) propidium iodide (Sigma-Aldrich, MO, USA) in PBS was added to each aliquot of cells. The suspension was mixed and allowed to incubate for 15 minutes at room temperature. The number of unstained cells and stained cells was measured using flow cytometry on the MACSQuant VYB (Miltenyi Biotec, Germany), and data analysed using FlowJo v7.6.5. Gating is shown in Figure 5.

Figure 5. Gating for viable and non-viable cells as determined by propidium iodide staining.

2.1.4. Morphological changes

At 6 hours post-treatment with a chemotherapeutic drug, cells were taken and diluted to an appropriate concentration for cell counting using a haemocytometer. The total cell number was counted and the number of hyphae, pseudohyphae and other elongated cells were quantified using light microscopy.

2.2. Generation of genomic changes in *C. albicans* by acute exposure to chemotherapeutic drugs

An overnight culture of SC5314 and reinoculation of cells into fresh medium was performed exactly as in the dose-response experiment (see above). Cells from the reinoculated medium were then taken from the culture and aliquoted to give a final OD₆₀₀ of 0.03 – 0.05 in 20 mL of YPD medium. The aliquots were then spun down at 13,000 rpm for 1 minute, and the medium removed. Cells were then resuspended in 20 mL of liquid YPD medium either without chemotherapeutic drugs (control), or containing an amount of drug equivalent to the drug's IC₅₀ or IC₉₀. In cases where calculation of the IC₅₀ and IC₉₀ was not possible (as with cisplatin), the highest concentration used in determining the dose-response relationship was used in lieu of the IC₉₀.

Growth rates were measured as in the dose-response experiment to ensure that growth inhibition by the drugs was within the expected ranges. At 6 hours after treatment with a chemotherapeutic drug, cells were taken and counted under a haemocytometer. They were then diluted with PBS to give a cell density of 2,000 cells/mL. 100 µl of this suspension was then spread on YPD agar without any chemotherapeutic drug to obtain single colonies. The plates were incubated overnight at 37°C. After overnight incubation, plates were then scanned using a desktop scanner. The images were processed by Fiji and then by a custom R script to randomly select for colonies [156,157]. These colonies were reinoculated in YPD contained in a deepwell block to obtain cells for FACS analysis of ploidy (described below in Figure 7) and karyotyping by quantitative PCR (described below). A detailed experimental workflow is shown in Figure 6.

Figure 6. The experimental workflow for examining genomic variation in *C. albicans* after acute short-term chemotherapeutic treatment.

2.2.1. Analysis of ploidy changes in *C. albicans* using flow cytometry

For this analysis, we used a protocol essentially as described by Pavelka *et al* [53]. Briefly, cells in the logarithmic phase were fixed with 70% ethanol (in distilled water) for 15 minutes at room temperature. Fixed cells were then treated with RNase A (Macherey-Nagel AG, Switzerland; 50 µg/ml RNase A in 0.05 M sodium citrate) for at least 3 hours and incubated at 37°C, then followed by overnight treatment with proteinase K (Promega, WI, USA; 50 µg/ml proteinase K in TRIS-CaCl₂). Cells were stained with Sytox Green (Invitrogen, CA, USA; 5 µM of Sytox Green in 0.05 M sodium citrate) for 15 minutes at room temperature, then measured using MACSQuant VYB (Miltenyi Biotec, Germany) and analysed with FlowJo version 7.6.5. The gating method is shown in Figure 7.

Figure 7. Gating method for FACS analysis of ploidy.

2.2.2. Karyotyping of *C. albicans* by quantitative PCR (qPCR)

Karyotyping was performed using a method as described by Pavelka *et al.* [53]. DNA from frozen cell pellets was extracted using the following method: cells were resuspended in PBS, and then washed twice with PBS. A digestion buffer made up of 1 μ l of 0.1 M dithiothreitol (Invitrogen, CA, USA) and 13 μ l of 10 mg/mL zymolyase (US Biologicals, MA, USA) in every 30 μ l PBS were then added to the cells and incubated at 37 °C for at least 1 hour. Cells were then lysed at 95 °C for 5 minutes and spun down at 3,500 rpm for 5 minutes. The supernatant was collected and stored at -80°C.

qPCR assays were designed with primers in non-coding regions on each arm of the 16 yeast chromosomes. FASTA sequences of intergenic regions were obtained from the CGD project “Candida Genome Database” <http://www.candidagenome.org/> (version 6 June 2008) and prospective loci of at least 500 bp in size were chosen from areas proximal (≤ 25 kb) to the centromere while specifically avoiding repetitive elements. Primers were designed using PrimerQuest (<http://www.idtdna.com/Scitools/Applications/Primerquest/>) with parameters set for qPCR. Amplicon sizes were limited to 75-150 bp with primer optimal T_m set at 60 °C. All primer pairs were BLAST-searched and in-silico PCRed (<http://genome.ucsc.edu/cgi-bin/hgPcr>) to ensure their uniqueness. Primer sequences can be found in Table 1 of the Appendix.

qPCR reactions were set up in 384-well plates using the Freedom EVO 150 (Tecan Trading AG, Switzerland) and cycled using an ABI 7900HT (Applied Biosystems) in 10 μ l reaction volumes. All reactions were set up in technical triplicate. A master mix for each sample was made using 625 μ l 2 $\times$ PerfeCTa SYBR Green FastMix Reaction Mixes ROX (Quanta), 244 μ l H₂O, and 6 μ l DNA from the zymolyase preparation. This master mix was aliquoted to the reaction plate. 1.3 μ M primer sets were then added to each sample. This leads to each reaction containing 5 μ l 2X SYBR Master Mix (Quanta Biosciences), 0.4 μ M each primer, and 2 μ l diluted DNA from the zymolyase extraction.

Cycling conditions were 95 °C for 5 min followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. Dissociation curves were performed to verify that no secondary products had been amplified. Ct values were determined using the Automatic thresholding feature of the ABI 7900HT SDS software version 2.3. All assays were further validated by 10-fold serial dilutions to determine amplification efficiency and dynamic range; calculated slopes based on these dilutions were all very close to the ideal value of -3.32 .

Chromosome copy numbers were determined using a modified version of the classical $\Delta\Delta$ Ct method, implemented as a custom R script [158].

2.3. Generation of isogenic strains to assess requirement of trisomy 2 for acquired HU resistance

Three trisomic 2 strains were selected from our short-term growth assays, two of which were derived from HU IC₅₀ treatment, and one from IC₉₀ treatment. These strains were passaged twice on YPD agar without any HU. Prospective colonies were selected on the basis of colony size (as aneuploid strains tend to grow slower than their euploid counterparts under the absence of relevant selective forces). After the second passage, single colonies were karyotyped using flow cytometry and qPCR (as detailed above). See Figure 8 below for details of the experimental workflow.

Figure 8. Experimental design for the derivation of isogenic strains from trisomic 2 strains.

2.3.1. Spotting assays for screening HU resistance

The protocol used was identical to that for high-throughput phenotypic profiling as essentially described by Sem *et al.* [159]. Strains were grown overnight at 37°C in liquid YPD medium, 150 rpm. OD₆₀₀ of overnight cultures were measured, and diluted to obtain an OD₆₀₀ of 2 in 1 mL of PBS. Cells were then washed once by centrifuging cells at 13,000 rpm for 1 minute, removing the supernatant, and then resuspended to a final volume of 1 mL in PBS. 100 µl of this cell suspension was then aliquoted into the first column of 96-well flat-bottom plates. Serial dilutions of cell suspensions were performed with sterile water and spotted on plates using the Freedom EVO 150 (Tecan Trading AG, Switzerland). Table 11 in the Appendix lists the strains we used in these experiments.

2.4. Adaptive evolution experiments in *C. albicans*

SC5314 was cultured overnight at 37°C, 150 rpm. Cells were then diluted to an OD₆₀₀ of 0.01 in 20 mL liquid YPD medium, containing varying concentrations of HU. At approximately 24 hours after the previous passage, cell morphology was assessed as described in 2.1.4., and cultures were diluted in PBS and spread on YPD agar to obtain single colonies for subsequent analysis of ploidy and karyotyping, as well as to assess colony size variation of the population using a

custom R script. Cultures were passaged daily by diluting cells to an OD₆₀₀ of 0.01 into fresh YPD medium, containing the same concentration of HU it was exposed to. The one-week experiment comprised of a total of 7 passages, and the two-week experiment comprised of a total of 14 passages.

2.5. Effect of HU treatment on *C. albicans* colonising the adult murine GI tract

An inoculum of *C. albicans* for oral gavage was prepared as follows: an overnight culture of SC5314 was incubated at 37°C, 150 rpm. Cells were washed thrice with 50 mL PBS, counted using a haemocytometer, then diluted with PBS to obtain a cell concentration of 10⁸ cells/mL. 3 days prior to gavage with *C. albicans*, male C57BL/6J mice aged 6 – 8 weeks old were provided with drinking water containing an antibiotic cocktail of 2 mg/L streptomycin and 1 mg/L of penicillin, and standard diet *ad libitum*. Mice were gavaged with 1 x 10⁷ cells suspended in 100 µl PBS. Mice were maintained on antibiotic treatment throughout the entire duration of the experiment. A detailed timeline of procedures can be found below in Figure 9.

The dose of HU (1000 mg/kg bw every 3 days) was obtained from a conversion (as calculated by Reagan-Shaw *et al.*) of the human dosage guideline provided in the product monograph of its commercial preparation by Bristol-Myers Squibb [62,160]:

$$\text{Human equivalent dose (mg/kg bw)} = \frac{\text{Animal dose (mg/kg bw)} \times \text{Mouse } K_m}{\text{Human } K_m}$$

$$80 \text{ mg/kg bw} = \text{Animal dose} \times (3/37)$$

$$\text{Animal dose (mg/kg bw)} = 80/(3/37)$$

$$\text{Animal dose} = 986 \text{ mg/kg bw (approximated to 1000 mg/kg bw)}$$

Figure 9. Experimental timeline for assessing the effect of HU on neutrophils and monocytes, as well as the karyotypic variation of GI-colonising *C. albicans*.

Blood collection via the cheek vein was performed to quantify levels of circulating neutrophils and monocytes, using sodium citrate as an anticoagulant. First, red blood cells were lysed using the RBC Lysis Buffer (BioLegend, CA, USA) according to the manufacturer's protocol. Cells were then washed with 1 mL of PBS, then stained with the following antibodies: CD45-FITC (Clone 30-F11, eBioscience), CD11b-APC (Clone M1/70, BioLegend), Ly6C-PE (Clone HK1.4, eBioscience), and Ly6G-PB (Clone 1A8, BioLegend). Neutrophils and monocytes were detected using the gating strategy shown in Figure 10.

Figure 10. Gating strategy for detecting neutrophils and monocytes in the blood.

For the detection of *C. albicans* dissemination, organs (spleen, liver, and kidney) were manually homogenised and plated on YPD agar containing 1 g/L penicillin and 2 g/L streptomycin. For the quantification of *C. albicans* colonisation level and detection of karyotypic changes in *C. albicans* colonising the GI tract (stomach, cecum, and stool), samples were manually homogenised in PBS, and then plated on YPD agar in various dilutions containing penicillin and streptomycin (as above). Colonies were selected randomly using a custom R script, inoculated into YPD medium and grown overnight at 37°C to obtain cells for preservation in glycerol stock and for gDNA extraction for karyotyping. The overnight cultures were also inoculated into fresh YPD medium and grown for 3 – 5 hours to obtain cells for analysis of ploidy change by flow cytometry.

2.6. Effect of HU treatment on the outcome of systemic candidiasis in the adult mouse

The preparation of the inoculum is identical to that for the adult mouse colonization model. 6 – 8 week old C57BL6/J male mice were pre-treated with various doses of HU (Sigma-Aldrich, MO, USA) dissolved in sterile water through the oral route beginning on Day -4. Figure 11 shows the detailed experimental timeline. Blood was collected via facial vein before infection to check for levels of circulating neutrophils and monocytes. The mice were infected intravenously by retro-orbital injection with 1×10^5 *C. albicans* yeast cells, and monitored daily for weight loss and mortality. Moribund mice were euthanized with CO₂, and organs (spleen, liver and kidney) harvested to quantify fungal burden. Neutrophils and monocytes were gated as in the experiment for benign colonization, and quantified using the CountBright Absolute Counting beads (ThermoFisher, MA, USA). Gating strategy for blood neutrophils and monocytes is same as that in Figure 10.

Figure 11. Experimental timeline for assessing the outcome of HU treatment during systemic candidiasis.

To obtain the levels of resident neutrophils and monocytes in the kidneys, both kidneys from each mice were harvested, and then individually homogenised by first cutting them into small pieces. Then the pieces were incubated in digestion buffer containing 1 mg/mL Type IV collagenase (Sigma-Aldrich, MO, USA) and 20,000 U/mL DNase I (Roche Diagnostics, Switzerland) at 37°C for 30 minutes, 150 rpm. Further homogenisation was manually performed on the pieces using a 70 µm filter and syringe plunger. The resulting cell suspension was centrifuged at 1,500 rpm for 5 minutes at 4°C. The supernatant was aspirated, and cells resuspended in 10 mL of RPMI medium. Cell suspensions of two kidneys from the same mouse were then combined, giving a total volume of 20 mL. 20 mL of 40% Percoll was then added to the cell suspension and mixed by inverting the tube. The cell suspensions were centrifuged for 20 minutes, at 1,900 x g, room temperature, without braking. The supernatant was aspirated, and the cell pellet resuspended in 1 mL of RPMI, followed by another 4 mL of RPMI added to the suspension. The suspension was then spun at 1,500 rpm, for 5 minutes at 4°C. The supernatant was removed. Then red blood cell lysis was performed using the RBC Lysis Buffer from BioLegend according to the manufacturer's protocol. After red cell lysis, cells were washed in 1 mL PBS. Cells were then stained with the following antibodies: CD45-FITC (Clone 30-F11, eBioscience), CD11b-APC (Clone M1/70, BioLegend), CD11c-BV510 (Clone N418, BioLegend), Ly6C-PE (Clone HK1.4, eBioscience), Ly6G-PB (Clone 1A8, BioLegend), F4/80-PE Dazzle

594 (Clone BM8, BioLegend), MHC II-APC/Cy7 (Clone M5/114.15.2, BioLegend), and flow cytometry performed on the MACSQuant VYB (Miltenyi Biotec, Germany). Data was analysed using FlowJo v7.6.5. Figure 12 below shows the gating strategy for kidney monocyte and neutrophil subsets, as described by Sheng *et al.* [161].

Figure 12. Gating strategy for kidney neutrophil and monocyte populations (Sheng *et al.*, Immunity 2015) [161].

In assessing the effect of CSP treatment during systemic *C. albicans* infection, in the presence or absence of HU treatment, we prepared the inoculum as described above, using an infectious dose of 1×10^5 yeast cells (in 100 μ l). CSP was dissolved in sterile MilliQ water to a concentration of 10 mg/mL, and diluted with PBS for intraperitoneal administration to a concentration of 0.01 mg/mL. CSP-treated mice received 0.063 mg/kg bw of CSP one day after i.v. infection, daily for 7 days. HU was administered via oral gavage (1000 mg/kg bw) as in colonisation experiments described above. Treatment regimens are described

below in Figure 13. Mice not on CSP treatment were instead administered 100 μ l of PBS i.p.

Figure 13. Experimental timeline for assessing effect of CSP treatment during systemic infection.

2.7. Generation of knockout mutants for *RNR* genes located on Chromosome 2

To generate null mutations in the individual *RNR* genes of *C. albicans*, we used the SAT1-Flipping strategy as described previously by Sasse and Morschhauser [162]. First, primers were designed to amplify regions within 1000 bp of the coding region of each gene, at the 5' and 3' ends, using genomic DNA from SC5314 (see Table 9 in the Appendix for primer sequences used in cloning each gene) extracted with the phenol-chloroform method as described by Rancati *et al.* [54]. Specific restriction digest sites (ApaI & KpnI in the 5' segment and SacII & SacI in the 3' segment) were introduced into each cloned segment. The 5' and 3' segments were then digested with the relevant restriction enzymes overnight at 37 °C.

These segments were then purified using the QIAGEN PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's protocol. The purified segments and the plasmid pSFS2 containing the SAT1-flipper cassette (see Figure 14 for the plasmid map) were then digested with the respective restriction enzymes.

Figure 14. The SAT-Flipper cassette in the pSFS2 plasmid, as described by Sasse and Morschhaeuser [162]. containing the *SAT1* (conferring nourseothricin resistance) and *FLP* genes adapted for *C. albicans* codon usage, flanked at both ends by a FLP Recombination Target (FRT). Expression of the *caFLP* gene is under the control of the *MAL2* promoter, enabling conditional expression of the *caFLP* gene only when in presence of maltose.

The digested plasmid was first dephosphorylated overnight at 37 °C with alkaline phosphatase to prevent re-ligation of the digested ends. The plasmid was then combined with the 5' and 3' segments of the cloned *RNR* genes for ligation. The ligated plasmids were transformed into *E. coli* DH5α via the heat shock method. Transformants were selected based on ampicillin resistance (carried by the plasmid) using LB plates containing 0.1 mg/mL ampicillin, and PCR was used to check for successful insertion of segments in transformants. Successful clones were then grown overnight in 50 mL LB broth containing 0.1 mg/mL ampicillin for plasmid extraction using the QIAGEN Plasmid Midiprep Kit (QIAGEN, Germany) according to the manufacturer's protocol.

The extracted plasmid DNA was then digested with the restriction enzymes KpnI and SacI and purified using the QIAGEN PCR Purification Kit. The purified insert was then transformed into *C. albicans* strain SC5314 using electroporation. Transformants were screened for nourseothricin resistance on YPD plates containing 200 µg/mL nourseothricin (Jena Biosciences, Germany). Successful integration of the plasmid was confirmed by PCR with primers listed in Table 1 of the Appendix. Positive transformants were then grown in YNB media supplemented with maltose to induce the loss of the NAT selection marker and replica-plated onto YPD agar and YPD-NAT agar to identify heterozygous NAT-sensitive transformants, as described by Cottier *et al.* [163].

2.8. Overexpression of individual *RNR* genes in *C. albicans*

Conditional overexpression of each *RNR* gene was attempted using the Tet_{ON} system and the repressible methionine promoter attached to an ectopic copy of the gene as essentially described by Chauvel *et al.* [164]. For the Tet_{ON} system, plasmids were kindly provided by Christophe d'Enfert's group at Institut Pasteur. The plasmid pNIMX (see Figure 15 for plasmid map) was digested with KpnI and SacII, then transformed into *C. albicans* strain SN148 (a uracil auxotroph). Transformants were selected for nourseothricin resistance and verified for integration with PCR using the primers NIM1_verif and ADH1_verif. The second plasmid carrying the gene to be overexpressed (see Figure 15B for plasmid map) was then digested with StuI, and then transformed into the successful pNIMX-carrying strains. Transformants were then selected for uracil prototrophy and integration verified using the primers ClpUL and ClpUR.

Figure 15. Schematics of the two cassettes used for the TetON overexpression system (adapted from Chauvel *et al.*) [164]. The pNIMX cassette (A) introduces the *C. albicans*-adapted reverse Tet-dependent transactivator which binds to and activates the P_{TET} promoter in presence of tetracycline. In this cassette *cartTA* is

under the control of the constitutive *TDH3* promoter. The Clp10-P_{TET}-Gateway cassette (B) introduces a single copy of the gene to be overexpressed, between the *attR1* and *attR2* sites, under control of P_{TET}.

2.9. Quantification of *RNR* gene expression levels by quantitative PCR

Primers were designed using the GenScript Real-Time PCR (TaqMan) Primer Design website (<https://www.genscript.com/ssl-bin/app/primer>). The various primer parameters were set at the following values:

Number of Primer/Probe Sets to Return (Limit 20 Sets)

PCR Amplicon Size Range

Primer Tm Minimum Optimum Maximum °C

Probe Tm Minimum Optimum Maximum °C

Figure 16. Settings for the generation of qPCR primers in the GenScript website.

Primer sets were then evaluated for specificity to the gene of interest by using BLAST on the “Candida Genome Database” <http://www.candidagenome.org/> (version dated 06 January 2015), and ensuring that only a single sequence (the expected amplicon) was obtained from the BLAST results. Next, the expected amplicon was also BLASTed against the entire *C. albicans* genome, to ensure that there was only a single non-redundant hit obtained. To screen for the most efficient primers (i.e. closest to 100% amplification efficiency), logarithmically growing cell pellets from SC5314 were obtained for RNA extraction and cDNA generation. cDNA samples were then serially diluted to obtain a standard curve. Primer efficiencies were then determined by measuring the linearity of the standard curve in three technical replicates, for three biological replicates. Efficiency (%E) was calculated by the following formula: $\%E = [10^{(-1/\text{slope})} - 1] * 100$

Primer sequences used in the gene expression qPCR are listed in Table 10 of the Appendix.

RNA samples were obtained from cells using the QIAGEN RNeasy Kit (QIAGEN, Germany) according to the manufacturer's protocol. cDNA from these samples was performed using the SuperScript III Reverse Transcriptase set (ThermoFisher Scientific, USA), according to manufacturer's protocol. The cycling conditions used in the qPCR are identical to those detailed in the karyotyping qPCR (described above).

3. RESULTS

3.1. HU induces immunosuppression and increased mortality during systemic *C. albicans* infection

Chemotherapy-induced immunosuppression is associated with an increased risk of systemic *C. albicans* infection [18,23]. We first set out to investigate if treatment with a chemotherapeutic drug, HU, in mice systemically infected with *C. albicans* led to poorer outcome in the course of infection, and if any differences in outcome were dose-dependent. We used a lethal dose of *C. albicans* (5×10^5 cells) for infection, but with mortality only occurring approximately 9 days post-infection in untreated mice. This allowed room for more rapid mortality (which we expected from HU treatment) to be observed (Figure 17). Figure 11 in the Materials and Methods shows the detailed experimental timeline for this experiment.

Figure 17. Survival curves from pilot experiment of systemic infection with SC5314 (5×10^5 yeast cells).

Figure 18. A: Survival curves of systemically infected mice under different doses of HU treatment (data analysed by log-rank test). B: Kidney fungal burden at 250 and 1000 mg/kg bw HU (one-way ANOVA, Holm-Sidak multiple comparisons test).

We observed that HU-treated mice in general showed a much poorer outcome for systemic *C. albicans* infection (Figure 17 and 18A). The increased mortality seen in HU-treated mice was also dose-dependent: as HU dose increased, so did mortality. Nevertheless, even the lowest dose group (250 mg/kg) exhibited a much more accelerated mortality compared to untreated mice ($p < 0.005$). At 500, 1000 and 2000 mg/kg bw HU, this rapidity in mortality was exacerbated ($p = 0.0001$ for all three dose groups). This was in spite of the less rapidly lethal dose of *C. albicans* (1×10^5 yeast cells) we used in systemic infection. This result shows that HU in itself is capable of causing an increased mortality during systemic *C. albicans* infection. In this model of systemic infection, the kidney is one of the main organs affected and kidney fungal burden is correlated with severity of renal failure [165]. Renal failure is also speculated to be the cause of death in this model of infection [165,166]. As such, we also obtained the kidney fungal burden of mice treated with HU at the lowest and highest concentrations used in our experiments (250 and 1000 mg/kg bw respectively), 2 days post-infection, to see if there were differences in fungal burden that correlated with mortality. HU-treated mice showed a higher kidney fungal burden compared to untreated mice, correlating with the poorer outcome of systemic candidiasis

(Figure 18B). Curiously, no differences were observed between the two HU treatment groups despite the difference seen in mortality.

We continued by investigating whether immunosuppression caused by HU treatment was correlated with a poorer outcome in systemic candidiasis, and whether this immunosuppression was also dose-dependent. We examined the level of neutrophils and monocytes present in the blood, as these two groups of cells are involved in direct clearance of *C. albicans* [97,107-110]. We found a reduction in the percentage of myeloid cells in the blood, suggesting myelosuppression. While we did not observe changes in proportions of neutrophils and monocytes, absolute numbers of neutrophils and monocytes were reduced, likely a result of myelosuppression (Figure 19). The decrease in numbers of circulating neutrophils and monocytes is consistent with the commonly observed side effect of chemotherapy-induced immunosuppression. However, while survival correlated with immunosuppression, neither of these correlated with the kidney fungal burden, as mice treated with 250 mg/kg of HU had similar kidney fungal burdens to those treated with 1000 mg/kg HU, even though both groups had a markedly different survival.

Figure 19. Relative percentages (to total number of cells for myeloid cells, and to total number of myeloid cells for neutrophils and monocytes) and absolute cell numbers of myeloid cells, neutrophils and monocytes in the blood. Top panel (A) shows the relative percentages, while the bottom panel (B) shows the absolute numbers. Headings at the top indicate the cell type measured (left to right: myeloid cells, neutrophils, monocytes). Data analysed using one-way ANOVA with Dunn's multiple comparisons test.

This led us to study if the number and proportion of the relevant immune cell subsets in the kidney differed from that in the circulation, and if this was the reason for the similar fungal burdens at 250 and 1000 mg/kg HU, despite the different degrees of immunosuppression these doses produce in the circulation.

Figure 20. Left: Frequency of kidney monocytes (defined as $CD45^+ F4/80^{int} CD11b^{hi} Ly6C^{hi} MHCII^{-to\ hi}$). Right: Frequency of kidney neutrophils (defined as $CD45^+ CD11b^{hi} Ly6G^{hi} Ly6C^{int}$) after 2 doses of oral HU treatment. Kruskal-Wallis test, Dunn multiple comparisons test (* : $p < 0.05$).

As can be seen from Figure 20, HU treatment appears to affect the kidney monocytes population significantly, although the neutrophil population appears not to be affected. This result mirrors that of our observations in blood (Figure 19). Furthermore, the level of monocyte depletion in the kidney correlates with the similar levels of CFUs and in both the 250 mg/kg and 1000 mg/kg group (Figure 18B). These results suggest that it is the reduction in kidney monocytes – and not circulating neutrophils and monocytes – that leads to an increased kidney fungal burden in HU-treated mice. However, this depletion of kidney monocytes still did not correlate with the difference in mortality between the different dose groups.

Our findings from these experiments suggest that while HU causes immunosuppression, which is an important component in determining the outcome of systemic candidiasis, immunosuppression alone may not be the sole

contributing factor to the poorer outcome of infection. This led us to consider whether chemotherapeutic treatment could have effects on other elements besides the host which influenced mortality, perhaps the pathogen itself, in this case *C. albicans*.

3.2. Effect of chemotherapeutic drugs on the growth, morphology and viability of *C. albicans*

Chemotherapeutic agents are well-known for disrupting cell division, growth, as well as triggering apoptosis in mammalian cells. As there is no systematic study of which chemotherapeutic drugs exert an effect on *C. albicans*, we began with an initial panel of several chemotherapeutic drugs, with different mechanisms of action and screened for possible effects on *C. albicans* by observing for acute, short-term changes in its growth, morphology and viability. (The mechanisms of action of these drugs are listed in Table 1 in the Introduction.)

We found that four of the eight drugs tested, namely HU, methotrexate, 5-fluorouracil and cisplatin demonstrated the ability to reduce the growth rate of *C. albicans* in a dose-dependent manner (Figure 21). In the case of methotrexate, its effects were only apparent in YNB medium. This is to be expected, as methotrexate – being a chemical analogue of folate – inhibits synthesis of tetrahydrofolate which is required as a cofactor in nucleotide synthesis [167,168]. The drug is therefore effective only in conditions where tetrahydrofolate is lacking (i.e. in minimal medium, as opposed to nutrient-rich medium such as YPD) [168].

The morphological change induced by HU was hyphal formation (also observed by Shi *et al.* and Bachewich *et al.*) [36,37]. Methotrexate, 5-fluorouracil and cisplatin produced a more elongated cell shape that did not correspond with the appearance of typical hyphae or pseudohyphae, producing an indeterminate morphology, but their elongated cell shape ultimately led us to classify them as filaments, together with hyphae and pseudohyphae during our quantitation of morphological changes (Figure 21). Figure 22 shows examples of these morphological changes.

Figure 21. Top: Growth rates and morphological changes of *C. albicans* within a 6-hour growth assay at different drug concentrations, for HU (A), 5-fluorouracil (B),

methotrexate (C), and cisplatin (D). Bottom: Viability of *C. albicans* after 6 hours' treatment with HU, 5-FU, MTX and cisplatin.

Figure 22. Pictures of cells exposed to HU, methotrexate, cisplatin, and 5-fluorouracil. Top left: HU, IC_{50} ; top right: cisplatin [1 mM] ; bottom left: MTX [0.0025 mM]; bottom right: 5-fluorouracil [1,000 μ M].

However doxorubicin, topotecan, cyclophosphamide and paclitaxel showed no effects on *C. albicans* with respect to our observed parameters during the dose-response experiments (Figure 23). No significant differences in growth, morphology and viability from the untreated controls were detected. From these results we infer that the drugs are also unable to exert any effects on the genome of *C. albicans*, and therefore excluded these drugs from consideration in our future experiments involving genomic variation and evolution.

Figure 23. Doxorubicin, topotecan, cyclophosphamide and paclitaxel do not produce any changes in the short-term growth rate of *C. albicans*. Viability was also not affected even at the highest concentrations tested.

Figure 24. The dose-response curve for HU and methotrexate, derived from three biological replicates of short-term growth assays. The IC₅₀ is indicated by the red line (HU IC₅₀ $\approx$ 37 mM, MTX IC₅₀: 3.8 nM).

Drug	Growth	Morphological changes	Viability	Karyotypic changes
HU	Decreased	Pseudohyphal-like	Decreased	Yes
Methotrexate	No change (YPD), decreased (YNB)	Pseudohyphal-like	No change	Yes
5-fluorouracil	Decreased	Pseudohyphal-like	No change	Yes
Cisplatin	Decreased	Pseudohyphal-like	No change	Yes
Cyclophosphamide	No change	No change	No change	No change
Doxorubicin	No change	No change	No change	No change
Topotecan	No change	No change	No change	No change
Paclitaxel	No change	No change	No change	No change

Table 2. Summary of results from short-term growth assays for each chemotherapeutic drug tested. All growth, morphological, viability and karyotypic changes were measured 6 hours post-treatment. Yellow indicates a change from untreated conditions, blue indicates no change. Number of biological replicates:

HU (n = 3), MTX (n = 3), 5- fluorouracil (n = 3), cisplatin (n = 3), cyclophosphamide (n = 2), doxorubicin (n = 5), topotecan (n = 1), paclitaxel (n = 2).

3.3. *In vitro* and *in vivo* induction of aneuploidy in *C. albicans* by chemotherapeutic drugs

After identifying some drugs capable of affecting *C. albicans* growth, morphology and viability in an acute setting, we next set out to study if these drugs were also capable of inducing mutations in *C. albicans*, as they are observed to do in mammalian cells [127,128,134]. We therefore treated *C. albicans* at the following

concentrations: IC₅₀ and IC₉₀ for HU and methotrexate. (Calculating the IC₅₀ and IC₉₀ for cisplatin was not possible, therefore we used a mid-range concentration based on the dose-response studies we performed before.) Cells were then plated after 6 hours' treatment to obtain single colonies, which were randomly selected for ploidy screening by FACS. Those with ploidy shifts were further analysed with qPCR to obtain the actual karyotype.

We observed a few aneuploidies from all the drugs we tested (Table 3), but the drug giving the widest range of aneuploidies was HU (compare karyotypes of Figures 25 and 26). Furthermore, HU also produced recurrent aneuploidies, even between different biological replicates. That HU can generate a range of aneuploidies in *C. albicans* suggests that it can drive the generation of karyotypic diversity within a population, which is the material on which selective pressures can act, and which enable a population to adapt to the selective pressures in the environment. If selective pressures such as antifungal treatment are present in the host, HU treatment may enable cells to adapt more rapidly to the treatment by virtue of generating genomic diversity. Genomic diversity can also produce populations with individuals of differing phenotypes, and some of these individuals could display more virulent traits, such as an increased propensity towards tissue invasion, increased capacity for tissue damage or immune evasion.

	Number of samples showing significant shift analysed by FACS			Aneuploid colonies detected by qPCR		
	Untreated	IC ₅₀	IC ₉₀	Untreated	IC ₅₀	IC ₉₀
Hydroxyurea	10/30	14/44	12/17	0/10	9/14	8/12
	Number of samples showing significant shift analysed by FACS			Aneuploid colonies detected by qPCR		
	Untreated	IC ₅₀	IC ₉₀	Untreated	IC ₅₀	IC ₉₀
Methotrexate	1/30	1/30	1/30	0/1	0/1	1/1
	Number of samples showing significant shift analysed by FACS			Aneuploid colonies detected by qPCR		
	Untreated	0.0625 mM	1 mM	Untreated	0.0625 mM	1 mM
Cisplatin	0/12	0/12	2/12	0/0	0/0	2/2

Table 3. Frequency of aneuploidy induced by HU, MTX and cisplatin.

Figure 25. Aneuploidies arising from HU IC₅₀ and IC₉₀ treatment. The eight clusters of bars represent each chromosome in *C. albicans*, while the four bars in the individual clusters correspond to telomeric and centromeric regions (left to right: left telomere, left centromere, right centromere, right telomere). Line refers to the individual evolution lines. Top: recurrent aneuploidy of trisomy 2 in three different colonies arising from independent cultures. Bottom: Other karyotypes isolated from various HU treatments.

Figure 26. Aneuploidies arising from treatment with cisplatin, methotrexate (MTX), and 5-fluorouracil (5-FU). Drug concentrations are indicated for each clone.

Trisomy 2 arose rapidly in HU-treated cultures, despite the very short treatment time of 6 hours (Figure 25). These trisomic 2 strains were isolated among colonies which appeared 24 hours after incubation at 37 °C. Other forms of aneuploidies found in these groups were primarily segmental: one clone appeared to have lost the right centromeric region of Chromosome 4, another clones lost one copy of Chromosome 5 together with the right centromeric region of the remaining Chromosome 5, and the third contained a tetrasomy of Chromosome 1 along with trisomy 7.

The recurrence of trisomy 2 led us to hypothesize that this particular mutation conferred a selective advantage in the presence of HU stress. To test this hypothesis, we performed evolution experiments over the course of one and two weeks (seven and fourteen passages, respectively), to see if trisomy 2 became the dominant karyotype within HU-treated populations. We also wanted to use these experiments to find out if the short-term acute effects that HU produced in *C. albicans* at high doses could also occur during long-term treatment with a lower dose of HU, particularly those which are representative of physiologically relevant concentrations. A literature search showed that the plasma concentration of HU in patients was approximately 0.4 mM, about 100 times lower than the IC₅₀ we identified [65]. We hypothesised that for such a low concentration, a more prolonged exposure to HU, as in an evolution experiment, would be required to produce some – if any – effect on *C. albicans*.

In our evolution experiments, cells were passaged daily in fresh YPD medium, maintaining a constant HU concentration in all passages. Aliquots of cells were harvested daily to monitor morphology and colony size variation (as a measure of phenotypic variation in the population) 24 hours after the last passage, in addition to isolating single colonies from each passage for later karyotyping.

Figure 27. Evolution experiments in the presence of HU. Row A shows the results of the one-week experiment, while row B shows the results of the two-week experiment. Cells were passaged daily throughout the experiment. Aliquots were collected daily, approximately 24 hours after the previous passage, to measure filamentation (left) and colony size variation (right) in the various evolution lines.

Not unexpectedly, given the findings of our earlier dose-response studies (Figure 21A), using the plasma concentration of HU *in vitro* produced no apparent effect on *C. albicans* morphology and colony size variation (Figure 27A). Nor did it produce aneuploidies that we observed from HU IC₅₀ and IC₉₀ treatment. We also performed the experiment with 4 mM HU, 10 times higher than the plasma concentration, which produced the same lack of effect as 0.4 mM.

The IC₅₀ of HU was a strong selective pressure for the emergence of trisomy 2. At the end of one week, 75 % of colonies we isolated harboured trisomy 2 (see Table 4 below). We also observed that the filamentation in the HU IC₅₀-treated culture began to decrease, beginning around day 2 post-treatment, and was sustained to the end of the one-week period (Figures 27A and B). Since *C. albicans* filaments in response to replication stress (such as from HU), our observation – correlated with the expansion of trisomy 2 in the population –

suggested to us that trisomy 2 did play a role in relieving HU-induced replication stress in *C. albicans* [36,37]. In the light of this idea however, it was curious that colony size variation, which we used as an indication of genomic variation in the population, appeared to remain at a fairly constant level throughout the entire one-week period in IC₅₀-treated cultures (Figure 27A), despite the expansion of an aneuploidy which was apparently advantageous in that condition. This suggests that while trisomy 2 relieves HU-induced replication stress, it does not protect against the mutagenic effect that HU exerts on *C. albicans*.

Owing to the lack of any observable change with 0.4 mM HU after a week, we next decided to repeat the experiment, with some changes, specifically: 1) an extension of the experimental evolution period by another week, for a total of two weeks, and 2) the use of two biological replicates per HU concentration used instead of one.

Again, we observed that 0.4 mM HU treatment and the untreated cultures produced similar trends in terms of filamentation and colony size variation over the entire course of the two week long experiment (Figure 27B). HU IC₅₀-treated cultures on the other hand, showed a difference in trends compared to the one-week evolution experiment. Despite the prolonged exposure to HU, filamentation – the indication of replication stress – began to gradually increase again after one week, although not to the same levels as day 1 post-treatment (Figure 27B). Paradoxically, colony size variation over the two-week evolution experiment gradually began to decrease after one week. If indeed the cells began to experience increased replication stress as indicated by the increasing filamentation during this period, then we should have found a constant, or even increasing level of colony size variation, but our observations were entirely to the contrary. Furthermore, an extended period in the selective stress should have led to even less experience of the stress due to improving adaptation. That both biological replicates under the HU IC₅₀ treatment demonstrated extremely similar trends in filamentation and colony size variation within this period suggests that

this is not likely to be due to a chance introduction of a random mutation in the population, but the result of identical underlying processes.

In an effort to understand what could have caused such differences in IC₅₀-treated populations at the end of one-week and two-week treatments, we looked first to karyotypic variation. Comparing the karyotypes of random colonies obtained after one- or two-week treatment at the IC₅₀ showed that most of them lost trisomy 2 by the end of two weeks (Table 4).

Day 8			Day 15		
Colony #	Untreated	HU IC ₅₀	Colony #	Untreated	HU IC ₅₀
1	2N	2N, +R, +2	1	2N	2N, +R
2	2N	2N, +2	2	2N	2N, +2
3	2N	2N, +2	3	2N	2N, +2
4	2N	2N, +2	4	2N	2N, +4
5	2N	2N, +2	5	2N	2N
6	2N	2N, +2	6	2N	2N
7	2N	2N, +2	7	2N	2N, +7
8	2N	2N, +2	8	2N	2N
9	2N	2N, +2	9	2N	2N
10	2N	2N	10	2N	2N
11	2N	2N	11	2N	2N
12	2N	2N	12	2N	2N

Increasing colony size
↓

Table 4. List of karyotypes from colonies isolated after one (Day 8; 7 passages) or two weeks (Day 15; 14 passages) at HU IC₅₀, compared to karyotypes of untreated cultures, within the same evolution experiment. Colonies are arranged according to size. 2N indicates euploidy, with additional (whole) chromosomes indicated by the plus (+) sign.

To follow up on the correlation between decreased filamentation in HU-treated cultures with the expansion of trisomy 2 in the population (Figure 27A and Table 4), we investigated the filamentation ability of trisomic 2 strains on Spider agar. Since the ability to switch between yeast and hyphae is crucial for virulence, inability to filament in response to environmental cues could lead to attenuation of *C. albicans*. The sensing of such cues is primarily mediated by the Efg1 signalling

pathway, which can be assessed through colony morphology on Spider agar [31,33]. We plated trisomic 2 strains isolated after 7 passages (when the incidence of trisomy 2 peaked) on Spider agar, and found that these trisomic 2 strains were just as capable of producing wrinkled colonies on Spider agar as the *EFG1* parental strain, while the *efg1*^{-/-} strain produced only smooth colonies (Figure 28). This shows that the decreased filamentation during the expansion in trisomic 2 strains is not the result of poorer capacity for environment-induced filamentation, and trisomic 2 strains are unlikely to be less virulent, at least in this regard.

Figure 28. Top: Representative images of colonies on Spider agar. Bottom: Percentage of all colonies on Spider agar appearing as either smooth

(filamentation-incompetent) or wrinkled (filamentation-competent). Total number of colonies assessed for each strain was at least 100 (YFT13, YFT14, YFT15: average of three biological replicates, *EFG1* and *efg1*^{-/-}: average of two biological replicates).

Having observed increased karyotypic variation in *C. albicans* treated with HU during our *in vitro* studies, we next wanted to see if a physiologically relevant concentration of HU was capable of producing increased karyotypic variation in the context of a systemic infection. Since we were unable to completely correlate HU-induced host effects with the increased mortality and fungal burden we observed, we hypothesised that HU-induced karyotypic variation might occur in the context of a systemic infection, which might lead to increased growth and infection in the kidney, contributing to increased fungal burden. To do this, we karyotyped colonies isolated from kidneys of infected mice 2 days after infection (from Figure 18B). As Table 5 shows, although aneuploidies were detected in *C. albicans* from the kidneys of HU-treated mice and none were detected in untreated mice, such aneuploidies were few, and this increase was not statistically significant.

	Untreated			250 mg/kg bw HU				1000 mg/kg bw HU		
Mouse #	1	2	3	1	2	3	4	1	2	3
Colony 1	2N	2N	2N	2N, +1	2N	2N	2N	2N	2N	2N
Colony 2	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N
Colony 3	2N	2N	2N	2N	2N, +3	2N	2N	2N	2N	2N
Colony 4	2N	2N	2N	2N	2N	2N	2N	2N, +7	2N	2N
Colony 5	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N
Colony 6	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N
Colony 7	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N
Colony 8	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N
Colony 9	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N, +R
Colony 10	2N	2N	2N	2N	2N	2N	2N	2N	2N, +R	2N

Table 5. Karyotypes of *C. albicans* in the kidney 2 days-post systemic infection.

However, since *C. albicans* is a well-known resident of the human GI tract, and HU is a drug administered orally, we hypothesised that in an *in vivo* system

(where the host is colonised with *C. albicans* and then orally given HU), *C. albicans* is more likely to encounter HU in much higher concentrations (than those found in the plasma) that are also biologically relevant [62,65,151].

For our pilot experiment, we first colonised mice with *C. albicans* in the gastrointestinal tract, which was achieved by depleting the intestinal bacteria with an antibiotic cocktail of penicillin and streptomycin in the drinking water, and then gavaging *C. albicans* yeast cells (see Figure 9 in Materials and Methods for the detailed experimental timeline). Mice were maintained on this antibiotic cocktail throughout the entire duration of the experiment. We compared colonization levels between untreated and HU-treated mice, and did not find significant changes post-HU treatment between untreated and HU-treated mice, as measured in the stool (Figure 29).

Figure 29. Colonisation levels as detected by CFUs in stool pellets. A: Colonisation levels in mice before the start of treatment; mice were allocated to treatment groups before the start of treatment. B: colonisation levels 3 days after start of HU treatment (Mann-Whitney test, two-tailed).

We randomly selected *C. albicans* derived from the stool for karyotyping, to see if HU treatment induced a greater karyotypic variation in *C. albicans* colonisers.

Most aneuploidies observed in both untreated and HU-treated groups involved an increase in copy number of Chromosome 7 (Table 6), which other colleagues in our group have also frequently observed in GI-colonising *C. albicans* (unpublished data). We hypothesise that an increase in copy number of Chromosome 7 is beneficial for adaptation to the murine GI tract, although exactly how Chromosome 7 mediates this improved adaptation is not yet known.

	Control		HU-treated					
Colony #	1	2	1	2	3	4	5	6
1	2N	2N, +3	2N	2N, 2(+7)	2N, +7	2N, +1, +7	2N	2N
2	2N, +7	2N	2N	2N	2N, +3	2N, +7	2N, +7	2N
3	2N, +7	2N	2N, +7	2N, +7	2N, +7	2N, +7	2N	2N
4	2N	2N	2N	2N	2N	2N	2N	2N
5	2N	2N, +7	2N	2N	2N, +7	2N, +7	2N, -1	2N
6	2N	2N	2N	2N, +7	2N, +7	2N	2N	2N, +7
7	2N	2N	2N	2N, +7	2N, +7	2N	2N	2N
8	2N	2N	2N, +7	2N	2N, +7	2N	2N	2N
9	2N	2N, +7	2N	2N	2N, +7	2N	2N	2N
10	2N	2N	2N	2N	2N	2N	2N, +7	2N
11	2N	2N	2N	2N	2N, +7	2N	2N	2N
12	2N	2N	2N	2N	2N	2N	2N	2N

Table 6. Karyotypes of colonies from the stool of untreated and HU-treated mice. Green boxes are euploid, while yellow boxes indicate aneuploidy. 2N, +2(7) indicates that 2 additional copies of Chromosome 7 was detected.

Since an increase in Chromosome 7 appears to be linked purely with colonisation of the murine GI tract, we decided to segregate aneuploidies depending on whether they involved only Chromosome 7 or other chromosomes as well, during analysis of karyotypic variation, to avoid the confounding factor of aneuploidies arising simply from adaptation to the gut (Figures 30C and D). Fisher's exact test comparing the number of aneuploidies involving only Chromosome 7 and those involving other chromosomes did not show a significant difference between untreated and HU-treated groups ($p = 0.5675$).

Figure 30. Statistics of karyotypic changes in *C. albicans* isolated from the stool. Absolute number (A) and percentage of aneuploid colonies detected in untreated and HU-treated mice (B), absolute number (C) and percentage of aneuploid colonies (D), divided into two groups: those involving only increase in Chromosome 7, and those that involving other chromosomes as well.

As we were unable to observe any significant changes in the stool karyotypes between untreated and HU-treated mice, we modified our experimental approach by karyotyping colonies residing in the stomach, along with those isolated from the caecum (which would be more related to stool). We reasoned that as we were administering HU orally, one of the first points of contact between *C. albicans* colonisers and HU would occur earlier in the GI tract (e.g. stomach), instead of later (e.g. the caecum, which is located at the beginning of the large intestine). Any effect that HU might have on *C. albicans* would be more likely to occur in the upper GI tract, than the lower GI tract, where most, if not all of the drug is already

absorbed into the bloodstream. Colonisers in lower parts of the GI tract therefore may not encounter HU in any biologically meaningful dose. We tested this hypothesis by comparing colonisation levels between the stomach and the caecum. Our early *in vitro* studies showed that HU causes growth inhibition in *C. albicans* at concentrations above 12.5 mM (Figure 21A). We made a theoretical calculation of the effective HU concentration within the stomach after orally gavaging the drug, and we found it to fall well within HU doses that inhibit *C. albicans* growth, shown below:

Average weight of a (male) C57BL6/J mouse at 8 weeks of age [169]: 24 g

Dose of HU administered: 1000 mg/kg body weight

Volume of mouse stomach [170]: 0.4 mL

Molecular weight of HU: 76.0547 g/mol

Effective local concentration:

$$0.025 \text{ g} / 76.0547 \text{ g/mol} = 3.287 \times 10^{-4} \text{ mol}$$

$$(3.287 \times 10^{-4}) / 0.0004 \text{ L} \approx 0.82 \text{ M}$$

Therefore we expected to see lower levels of colonisation in the stomach compared to the caecum, as a result of growth inhibition by HU, and this was indeed the result we found 11 days post-treatment (see Figure 31).

Figure 31. Levels of *C. albicans* colonisation in the stomach and caecum (Mann-Whitney test, two-tailed).

Since our results showed that HU treatment affected colonisation of *C. albicans* in the stomach, it also followed that there could be karyotypic changes induced by HU if we examined the colonies from the stomach. We karyotyped a total of 60 colonies from the stomachs of untreated mice and 60 colonies from HU-treated mice.

	Untreated									
Mouse #	1	2	3	4	5	6	7	8	9	10
Colony 1	2N	2N, +3, +5	2N, +7	2N, +7	2N, +7	2N	2N, +7	2N, +R(2RC), +1, +3, +7	2N, +7	2N, +1, +3, +7
Colony 2	2N	2N, +7	2N	2N	2N, +7	2N	2N, +7	2N, +7	2N, +7	2N, +1, +3, +7
Colony 3	2N	2N	2N, +7	2N, +7	2N, +7	2N	2N, +7	2N, +7	2N, +7	2N, +7
Colony 4	2N	2N	2N	2N	2N	2N	2N, +7	2N	2N, +7	2N
Colony 5	2N	2N, +7	2N	2N, +7	2N	2N	2N, +7	2N, +7	2N	2N
Colony 6	2N	2N, +7	2N	2N	2N	2N	2N, +7	2N	2N, +7	2N
Colony 7	2N	2N	2N	2N	2N	2N	2N, +7	2N	2N	2N
Colony 8	2N	2N, +7	2N	2N	2N	2N	2N, +7	2N	2N	2N
Colony 9	2N	2N	2N	2N, +7	2N	2N	2N, +7	2N	2N	2N
Colony 10	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N
Colony 11	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N
Colony 12	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N

Table 7. Karyotypes of colonies from stomachs of untreated mice. Green squares indicate euploid colonies, while yellow squares indicate trisomy 7 only, while blue squares indicate non-trisomy 7 only colonies. R(2RC): 2 additional copies of right centromere of Chromosome R.

	HU-treated									
Mouse #	1	2	3	4	5	6	7	8	9	10
Colony 1	2N	2N, +7	2N	2N	2N, +3, +7	2N, +R(2RC), +6	2N, +3	2N, +6	2N	2N, +1, +3, +7
Colony 2	2N, +2	2N, +7	2N	2N	2N, +3	2N, -5(LT, LC, RT), +7	2N, +7	2N, +7	2N, +7	2N, +3, +7
Colony 3	2N	2N	2N	2N	2N	2N	2N, +7	2N	2N	2N, 2(+7)
Colony 4	2N	2N, +7	2N	2N	2N, +R(RC), +7	2N, +5(LT, LC, 2RC, RT), +7	2N, +3	2N	2N	2N, +7
Colony 5	2N	2N	2N	2N	2N	2N	2N, +7	2N	2N	2N, +7
Colony 6	2N	2N	2N	2N	2N	2N, +7	2N, +7	2N	2N	2N, +7
Colony 7	2N	2N	2N	2N	2N	2N, +7	2N, +7	2N	2N	2N, +R(2RC) +7
Colony 8	2N	2N	2N	2N	2N	2N	2N, +7	2N	2N	2N, +7
Colony 9	2N	2N	2N	2N	2N	2N, +7	2N, +7	2N	2N	2N, +7
Colony 10	2N	2N	2N	2N	2N	2N	2N, +7	2N	2N	2N, +R(2RC) +7
Colony 11	2N	2N	2N	2N	2N	2N	2N, +7	2N	2N	2N, +7
Colony 12	2N	2N	2N	2N	2N	2N	2N	2N	2N	2N, +7

Table 8. Karyotypes of colonies from the stomachs of HU-treated mice.

Abbreviations of chromosomal segments: LT (left telomere), LC (left centromere), RC (right centromere), RT (right telomere). Segmental aneuploidies are indicated by the number/letter of a chromosome, followed in brackets by the relevant segments. Numbers preceding the abbreviations of chromosomal segments indicate the number of additional or missing copies of that segment.

Figure 32. Statistics of karyotypic changes in *C. albicans* colonising the stomach.

Absolute number (A) and percentage (B) of euploid and aneuploid colonies detected in untreated and HU-treated mice, absolute number (C) and percentage (D) of aneuploid colonies, divided into two groups: those involving only increase in copy number of Chromosome 7, and those that involving other chromosomes as well.

The incidence of aneuploidy in stomach-colonising *C. albicans* of HU-treated mice was not significantly higher than that of untreated mice (Figure 32A and B).

Trisomy 7, which we observed to be abundant in the stool of untreated mice

(Table 6), is also the predominant aneuploidy found in the stomach of untreated and HU-treated mice (Tables 7 and 8, Figure 32). But there was an increase in karyotypic diversity in stomach-colonising *C. albicans* of HU-treated mice, which show a greater percentage of colonies that harbour other types of aneuploidies besides an amplification of Chromosome 7 (Figure 32D). We performed Fisher's exact test to see if the number of aneuploidies excluding amplification of Chromosome 7 was significantly higher in HU-treated mice, and this was the case (Figure 32C, $p = 0.0165$). We also calculated Shannon's index for diversity in the karyotypes of these *C. albicans*, and found the HU-treated group to also have a higher Shannon's index (1.1081) compared to the untreated group (0.7419). However, besides trisomy 7, we did not observe a dominant karyotype occur in populations from HU-treated mice.

3.4. Adaptive advantage of trisomy 2 under HU-induced stress

Having observed the rapid emergence and recurrence of trisomy 2 in *C. albicans* during significant HU stress, under *in vitro* conditions, we set out to determine if the preponderance of trisomy 2 in HU-treated cultures was due to a fitness advantage conferred by this particular aneuploidy. We isolated three trisomic 2 strains arising independently from short-term acute HU treatment. Each strain was passaged twice on YPD agar in the absence of HU, to enable the spontaneous loss of the additional Chromosome 2. From this single passage, two siblings (one which retained the trisomy, and another which lost the trisomy) were isolated from the initial trisomic 2 strain. We reasoned that this would enable us to determine if it was trisomy 2, or other smaller mutations in the genetic background (which could occur with HU treatment, but likely to be retained even when passaged in the absence of stress, unlike aneuploidy) that was responsible for increased fitness under HU stress. Figure 8 in the Materials and Methods show the detailed protocol for derivation of isogenic strains.

Figure 33. Spotting assays with three pairs of of euploid and trisomic 2 strains derived from trisomic 2 strains generated by 6 hours' HU IC₅₀ and IC₉₀ treatment. Identical colours indicate sibling pairs. Images are representative of three biological replicates.

We found that despite the similarity in genetic background, the sibling strains differed markedly in terms of growth under HU stress. None of the siblings showed any visible growth differences between each other in the absence of HU, but all trisomic 2 siblings grew much better than their euploid counterparts in the presence of HU (Figure 33). This result shows that trisomy 2 is required for HU resistance in these strains.

However, we found in our *in vitro* experiments that HU-treated cultures undergoing more than seven passages gradually lost trisomy 2, and in fact more euploid colonies were isolated (see Table 4). We next set out to determine if

these euploid strains evolved in IC₅₀ for two weeks were better adapted to growth in HU IC₅₀ compared to the trisomic 2 strains obtained after one week at the IC₅₀.

Figure 34. Top: Spotting assay showing growth of different strains derived from after 14 passages in HU IC₅₀, compared to a trisomic 2 strain derived after 7 passages in HU IC₅₀. Bottom: Quantification of the growth of each strain, relative to SC5314. Euploid strains are black-and-white bars, while coloured bars refer to

aneuploid strains, with red indicating a trisomic 2 strain, and green indicating strains with non-trisomy 2 aneuploidies. Data analysed using one-way ANOVA with Holm-Sidak multiple comparisons test. Results are the average of three biological replicates.

As shown by the spotting assays, despite the loss of the additional chromosome 2 which conferred increased HU resistance in trisomic 2 strains, the euploid evolved strains from two weeks of HU treatment demonstrated much greater resistance than SC5314 and even the trisomic 2 strain derived from one week of HU treatment (Figure 34). This result is consistent with the observation that aneuploidy comes with a fitness cost, especially in the absence of the stress, and that improved adaptation to the stress involves the acquisition of other smaller mutations, which provide a similar or better fitness advantage in the presence of stress, without bearing the cost of aneuploidy [59]. These smaller mutations take a longer time to develop however, hence their appearance much later in the course of the evolution experiment.

To test if the gradual disappearance of trisomy 2 might be attributed to a fitness cost associated with aneuploidy and refinement of adaptive strategy, we performed a competitive growth assay between SC5314, YFT1 (trisomic 2) and YFT2 (euploid), to see if trisomy 2 carried a growth disadvantage in the absence of HU stress. As Figure 35 shows, trisomy 2 does indeed have a slight fitness cost in YPD when competed against SC5314, while its euploid sibling did not show such a deficit, indicating that the fitness cost in YPD is due solely to the additional Chromosome 2. We were unable to perform a similar competitive fitness assay in HU, owing to the fact that HU causes filamentation in SC5314, which would confound assessments for fitness differences using growth rate measurements via optical density. HU is also aneuploidogenic in itself, and the initial strains are very likely to undergo chromosomal changes. In short, a competitive growth experiment in HU will likely produce a karyotypically heterogeneous population, which makes it difficult to attribute fitness differences to a particular karyotype.

Figure 35. Percentage of fluorescent SC5314 vs. non-fluorescent *C. albicans* (YFT1 or YFT2) after competitive growth assays for 24 hours (average of three biological replicates).

3.5. Role of RNR genes in conferring HU resistance

3.5.1. Requirement of individual *RNR* genes on Chromosome 2 for trisomy 2-mediated HU resistance

Having now ascertained that the adaptation to HU was due to an additional Chromosome 2, we next wanted to find out which genes on chromosome 2 might be responsible for providing this increased resistance. We started first with the known drug target of HU, which is the enzyme ribonucleotide reductase, required for producing nucleotides that are used in DNA synthesis during S-phase of the cell cycle. A search for RNR genes in *C. albicans* led us to three *RNR* genes (*RNR1*, *RNR21* and *RNR22*), which are present on Chromosome 2 in *C. albicans*, providing us with a clear lead on our next steps. We hypothesised that additional copies of *RNR* genes would buffer the effect of *RNR* inhibition by HU, providing cells with a higher tolerance for the drug.

We set out to study exactly which *RNR* gene(s) was necessary and sufficient for providing increased resistance to HU. To study for necessity, we generated single deletion strains under different karyotypic backgrounds: one in the diploid strain

SC5314, and the second set in a trisomic 2 background (YFT1), using the SAT-Flipping strategy described by Sasse and Morschhaeuser [162]. In the diploid strain, we were able to generate only heterozygous deletions of *RNR21* and *RNR22*, but not *RNR1*. Attempts to do so with *RNR1* resulted in strains that had no deletions after “flipping” the cassette out, despite successful integration of the cassette after transformation (data not shown), suggesting that *RNR1* is severely haploinsufficient. Deletion of *RNR1* was only successful in the trisomic 2 background. Single deletions of *RNR21* and *RNR22* in a trisomic 2 background were also successfully generated. In the case of the trisomic 2 strain transformations, we did not activate the flippase enzyme to achieve a clean deletion, as we did with deletions in SC5314. This was because the resulting strains appeared to lose the allele with the deletion when verified with PCR (data not shown).

We tested a pair of sibling strains (YFT1 and YFT2) along with our heterozygous deletion strains for *RNR21* and *RNR22* in liquid growth assays, as well as assessed their level of filamentation under HU IC₅₀ treatment. We did not detect significantly different growth rates in these strains compared to SC5314 in the absence of HU (Figure 36A). At the HU IC₅₀, YFT1 grew better than SC5314 and its euploid sibling, consistent with our spotting assay results (Figure 36B). YFT1 also demonstrated lower filamentation levels than all the other tested strains, possibly corresponding to reduced replication stress from HU (Figure 37). The strains carrying a heterozygous deletion of *RNR21* and *RNR22* grew slightly poorer compared to SC5314 at the IC₅₀ of HU, but this difference was not statistically significant. This could be due to the relatively short duration of the growth assay (7 – 8 hours) and growth differences might not be apparent within this window of time.

Figure 36. Growth rates of the different heterozygous deletion mutants in liquid YPD medium. A. Growth rate measured over 7 – 8 hours in YPD, in absence of HU. B. Growth in YPD at HU IC₅₀ 7 – 8 hours post-treatment (unpaired t-test, two-tailed). Results are the average of three biological replicates.

Figure 37. HU-induced filamentation 6 hours post-treatment (one-way ANOVA, Sidak multiple comparisons test).

Therefore we tested the above strains along with the trisomic 2 deletion series using the spotting assay, with different concentrations of HU. This assay provided the possibility of detecting growth differences that become apparent over longer periods of time (24 – 48 hours after spotting), compared to liquid growth assays.

In the absence of HU, all strains grew robustly, although the trisomic 2 strain YFT1 showed a slightly lower growth rate compared to the rest of the strains (Figure 38).

Figure 38. Spotting assays with *RNR* deletion mutants. Top: YPD plates containing increasing concentrations of HU. Images are representative of all three biological replicates. Bottom: Quantification of spot intensity relative to SC5314 at different concentrations of HU. Data analysed using one-way ANOVA with Sidak-Holm multiple comparisons test; average of three biological replicates.

As can be observed both by visual inspection and quantification of each strain's growth under different concentrations of HU, the *RNR2*^{1+/-} mutant demonstrated an extremely strong sensitivity to HU, even at the IC₃₅, in which SC5314 still maintained a fair growth rate, although lower than YFT1 (Figure 38). At the IC₅₀, growth of *RNR2*^{1+/-} was virtually absent. These results were as expected, as *RNR21* encodes for the small subunit of the RNR complex, which contains the tyrosyl radical for ribonucleotide reduction, the direct chemical target of HU. We expected the heterozygous knockout mutant of *RNR22* to also demonstrate the same sensitivity as the *RNR2*^{1+/-} mutant, since *RNR22* is a paralogue of *RNR21* (sequence identity of 76%; see Appendix for the multiple sequence alignment result of *RNR21* and *RNR22*), but curiously this was not the case, and the *RNR22*^{+/-} mutant showed the same sensitivity to HU as SC5314 under the conditions we tested.

We were also able to assess the effect of *RNR* gene copy reduction in trisomic 2 strains under HU treatment. At the IC₅₀, the *RNR1*^{+/+/-} mutant was sensitive to HU, to a similar degree as SC5314 and YFT2 (the euploid sibling of YFT1), showing that all three copies of *RNR1* are required for trisomy 2-mediated HU resistance. Like the diploid *RNR2*^{1+/-} mutant, the *RNR2*^{1+/-/+/-} mutant was also sensitive to HU, but not more so than the *RNR22*^{+/+/-} mutant and the diploid strains. These results taken together indicate that *RNR1* and *RNR21* are both individually required for trisomy 2-mediated HU resistance, and hint at an equal contribution of *RNR1* and *RNR21* towards this resistance. This is to be expected, since the functional RNR complex needs both the regulatory and catalytic subunits in the correct stoichiometric levels.

3.5.2. Sufficiency of individual *RNR* genes on Chromosome 2 for trisomy 2-mediated HU resistance

In order to study the sufficiency of each *RNR* gene for trisomy 2-mediated HU resistance, we decided to generate overexpression mutants for each of the *RNR* genes we had deleted earlier. The results of our study of the requirement of each *RNR* gene towards HU resistance predicted that no single *RNR* gene

overexpression would be sufficient for HU resistance, and so we also included plans to generate a double overexpression mutant, specifically for *RNR1* and *RNR21*. Towards this end we requested assistance from the lab of Christoph d'Enfert at the Institut Pasteur, who kindly provided us with the transactivation cassettes which would enable conditional overexpression of each *RNR* gene under presence of tetracycline (shown in Figure 15 in the Materials and Methods).

The first overexpression mutant we successfully generated (by PCR verification) was for *RNR1*, and so pilot studies for this overexpression system were performed using this mutant. We successfully constructed a strain for *RNR1* overexpression, here named and assessed their capacity for overexpression at 0.2 and 0.4 $\mu\text{g/mL}$ anhydrotetracycline (ATC) in YPD liquid medium, at 30 °C, which were much lower concentrations of ATC compared to those used by Chauvel *et al.* for overexpression (2.5 $\mu\text{g/mL}$ ATC) [164]. Significant *RNR1* overexpression, as measured by qPCR was could be achieved already at 0.4 $\mu\text{g/mL}$ ATC, showing that the Tet_{ON} system we were using for overexpression was functional and robust for *RNR1* (Figure 39).

Figure 39. Left: Gene expression levels of *RNR1* in the Clp-*RNR1* mutant with increasing concentrations of ATC, in YPD at 6 hours post-treatment, 30°C (One-

way ANOVA with Holm-Sidak's multiple comparisons test, average of two biological replicates). Right: Growth rate relative to untreated cultures.

We also tried to generate a conditional expression mutant for *RNR21* which we tested for overexpression in presence of ATC. However we were unable to produce any consistent overexpression for *RNR21* (Figure 40), despite using doses that would have produced a strong overexpression of *RNR1*. As such, we were unable to test for the sufficiency of *RNR21* in trisomy 2-mediated HU resistance in subsequent tests.

Figure 40. Gene expression levels of *RNR21* for the Clp-*RNR21* strain in the absence and presence of ATC (Mann-Whitney test, two-tailed).

Figure 41. Testing an *RNR1* overexpression strain in the absence or presence of 2.5 µg/mL ATC, under absence or presence of HU (average of two biological

replicates). SN148 is the parental strain, while pNIMX is directly derived from SN148, containing the Tet transactivator, but not the gene under P_{TET} control.

As seen from Figure 41, *RNR1* overexpression did not rescue our strain under HU treatment, unlike that of the trisomic 2 YFT1 strain, showing that *RNR1* overexpression alone is not sufficient for HU resistance.

3.6. Concomitant resistance of trisomic 2 strains to caspofungin *in vitro* and *in vivo*

Caspofungin (CSP) is a first-line antifungal drug used in the treatment of systemic candidiasis. Work by other colleagues in the lab revealed that CSP-resistant strains of *C. albicans* displayed trisomy 2 as well (Figure 42). This serendipitous finding led us to examine if HU-evolved trisomic 2 strains also displayed such resistance to CSP.

Figure 42. CSP-generated trisomic 2 strains are resistant to CSP compared to their euploid counterparts (unpublished data by Giang Le).

Figure 43. Percentage of surviving colonies on YPD agar containing 0.08 mg/L CSP, relative to total number of colonies plated on YPD agar (one-way ANOVA with Tukey multiple comparisons test). Data points in different colours indicate ploidy of strains (black: euploid, red: trisomy 2, green: non-trisomy 2 aneuploidy).

HU-evolved trisomic 2 strains did show a significant increase in survivors under CSP treatment (Figure 43). We also did the same test on a few strains which underwent 14 passages in HU (same strains as in Figure 34) and had much stronger resistance to HU, to see if HU resistance alone was predictive of CSP resistance. We found that these strains had poor survival on CSP, similar to that of the wild-type SC5314. This result strongly suggests that trisomy 2, and not HU resistance, is required for CSP resistance.

Given the association of trisomy 2 with both caspofungin and HU resistance, we next wanted to examine if CSP-generated trisomic 2 strains were also resistant to HU, in the same degree as HU-generated trisomic 2 strains. We found that these CSP-derived strains also grew much better than SC5314 at the IC_{50} of HU, just like HU-generated trisomic 2 strains (Figure 44). These results show that trisomy 2 on its own can provide simultaneous resistance to both HU and CSP, and that these resistances are genetically based, and not mediated by pre-exposure to a particular drug.

Figure 44. CSP-derived trisomic 2 strains show increased HU resistance compared to SC5314, as with HU-generated trisomic 2 strains, when tested with spotting assays. Images are representative of three biological replicates. Quantification results are the average of three biological replicates (data analysed by one-way ANOVA with Holm-Sidak multiple comparisons test, **: $p < 0.005$).

We next wanted to find out if CSP resistance was mediated by the same molecular mechanism as HU resistance. To test this hypothesis, we used trisomic 2 strains with deletions of *RNR1* and *RNR21*, and we found that these strains retained similar levels of resistance as that of the parent trisomic 2 strain (Figure 45). From these results we conclude that Chromosome 2-mediated CSP resistance occurs through RNR-independent mechanisms, and it is very likely that Chromosome 2-mediated HU resistance and Chromosome 2-mediated CSP resistance are underlain by different genetic requirements.

Figure 45. CSP resistance in trisomic 2 strains with deletions of *RNR* genes located on Chromosome 2. HU^R: HU-resistant, HU^S: HU-sensitive. Data analysed using one-way ANOVA with Holm-Sidak's multiple comparisons test (average of three biological replicates).

We next wanted to examine if the association between trisomy 2, HU treatment and CSP resistance had any significant implications for treatment outcome with CSP during systemic *C. albicans* infection. Based on our previous findings, we hypothesised firstly that HU treatment could negatively impact CSP treatment response during systemic *C. albicans* infection by a HU-generated trisomic 2 strain. Secondly, we hypothesised that infection with a HU-generated trisomic 2 strain would be more difficult to treat with CSP (see Figure 13 in the Materials and Methods for the detailed experimental timeline).

Figure 46. CSP treatment prolongs survival time (A) and improves morbidity (weight loss in first 2 weeks post-infection) (B) during systemic infection with SC5314, but HU treatment leads to loss of treatment efficacy (C and D) (survival analysed by log-rank test).

As expected, we found that CSP treatment prolonged survival and reduced morbidity during systemic infection with SC5314, showing that the dose we used had an antifungal effect *in vivo* (Figure 46A and B). As shown previously, systemically infected mice treated with HU had an increased rate of mortality compared to untreated mice (Figure 18A). CSP treatment however was unable to rescue these HU-treated mice in the same manner as it did in untreated mice, both in terms of mortality and morbidity (Figure 46C and D).

Figure 47. CSP treatment does not change outcome of systemic infection with a trisomic 2 strain, both in terms of mortality and morbidity (survival analysed by log-rank test).

Mice infected with the trisomic 2 strain YFT8 (derived after 14 passages in HU IC_{50} and showing higher resistance to HU and CSP; see Figures 34 and 43) showed a delayed mortality rate compared to those infected with SC5314 (Figures 46A and 47A; median survival with SC5314: 9 days, median survival with YFT8: 19.5 days). This could be indicative of reduced fitness during systemic infection because of aneuploidy, as discussed earlier. However, despite the apparently lower fitness of YFT8 *in vivo* compared to SC5314, CSP treatment produced no significant improvement in terms of mortality (Figure 47A, $p = 0.3558$) or morbidity (Figure 47B). In the case of HU-treated mice, no improvement in mortality was seen with CSP treatment as well (Figure 47C). Improvement in morbidity was observed (Figure 47D), but this only occurred on day 8 post-infection after CSP treatment ceased, and not during the one-week CSP

treatment regime, whereas an almost immediate improvement was seen in SC5314-infected mice (Figure 46B). These results with YFT8 indicate a reduced efficacy of CSP against a trisomic 2 strain. Our combined results from this set of *in vivo* experiments lead us to conclude that: 1) HU abolishes the beneficial effects of CSP during systemic candidiasis, and 2) karyotypic variants of *C. albicans* which harbour trisomy 2 and could be selected for by HU treatment, are also less sensitive to CSP treatment. These results imply that HU treatment during systemic infection may lead to little or no beneficial effect from CSP treatment, and that alternative antifungal drugs should be considered in such circumstances.

4. Discussion

In summary, we confirm that HU is typical of classical chemotherapeutic compounds in its immunosuppressing properties. Even more interestingly, we have also found that HU treatment is capable of generating aneuploidy *in vitro* and *in vivo*, thereby enhancing genomic variation in the population; that HU selects for a particular aneuploidy trisomy 2 under *in vitro* conditions, because it provides a fitness advantage during HU treatment. This fitness advantage is mediated by an increase in copy number of *RNR1* and *RNR21*, located on Chromosome 2. Furthermore, trisomy 2 also provides resistance to CSP, a first-line antifungal drug used in treatment of systemic candidiasis, leading to reduced efficacy of CSP treatment in our mouse model of systemic *C. albicans* infection.

In its immunosuppressive effects, HU reduced the absolute number of neutrophils and monocytes in the blood in a dose-dependent manner, but left the relative proportion of these cells unchanged. However our results also made it clear that HU treatment alone – and any immunosuppressive effects following it – did not precipitate disseminated disease. HU-treated mice that were benignly colonised with *C. albicans* did not show any spontaneous deaths, indicating that disseminated *C. albicans* infection requires more than just immunosuppression.

Dissemination from the GI tract possibly requires a significant breach of the epithelial barrier, which does not appear to be caused by HU [104].

On the other hand, in our systemic infection studies, *C. albicans* is directly injected into the bloodstream, thus bypassing the need to break through the epithelial barrier. In this system, we observed that HU treatment does cause a detrimental effect on survival in a dose-dependent manner, which correlates with immunosuppression. This result appears to support the long-standing view that systemic *C. albicans* infection and its outcome in cancer patients is determined mostly by immunosuppression. However, we also took into account our measurement of fungal burden in the kidneys 2 days after infection, which showed that the level of CFUs at different HU doses were similar – despite differing degrees of immunosuppression. Specifically, at 250 mg/kg HU, the reduction in monocytes was mild and not statistically significant, while at 1000 mg/kg HU the reduction in monocytes is significant. The similarity in fungal burden arising from 250 and 1000 mg/kg HU treatment, in spite of the difference in extent of immunosuppression casts some doubt over the paradigm that chemotherapy-induced immunosuppression is the primary determinant of systemic *C. albicans* infection risk and its outcome, and also suggests that other factors may be at play.

Before excluding immunosuppression as the sole contributing factor to disease, we also studied the level of kidney neutrophils and monocytes, since they are more likely to be the main determinants of kidney fungal burden, and their levels may not necessarily correlate with the level of circulating neutrophils and monocytes. In the kidneys, neutrophil levels were not significantly different in untreated and HU-treated mice. However monocytes levels were significantly reduced in both the 250 mg/kg and 1000 mg/kg groups, and to a similar degree, which stands in contrast to our observations of monocytes in the blood. As such, the kidney fungal burden in early stages of the infection does correlate with monocyte levels – in the kidney. Therefore, we conclude that chemotherapy-induced immunosuppression is a strong determinant of kidney fungal burden. But

neither fungal burden nor immunosuppression appears to be a strong determinant of mortality, since mice treated with 250 mg/kg HU had significantly better survival compared to those treated with 1000 mg/kg HU.

One of the limitations of our studies is that we did not investigate other foci of infection during systemic candidiasis, such as the liver or brain. Organ failure in these systems is also a possible contributor towards mortality. Another limitation is that our fungal burden measurements were taken quite early during the infection, with no other timepoints later in infection. It is possible that the dynamics of fungal clearance by the immune system over the later days during infection are different in the various dose groups, and this could have a significant impact on mortality, even if the initial fungal burden is similar. A third consideration is that *C. albicans* in the kidney may display similar levels of growth, but not similar morphological forms. If more *C. albicans* was present in true hyphal form at 1000 mg/kg HU than at 250 mg/kg HU, this could lead to more tissue damage, and more rapid organ failure and mortality. However, we are limited by the lack of histological examination that could address this possibility.

Another line of investigation that could address the lack of correlation between mortality, fungal burden and immunosuppression – but has not been undertaken up till this study – is whether chemotherapy in a mammalian host can induce changes in *C. albicans*. More specifically, changes which might lead to improved adaptation to the host environment, or evasion of immune defences, both of which could lead to increased virulence or antifungal resistance. Studying this possibility was the main aim of this thesis, and towards this end, we characterised karyotypic changes arising in HU-treated mice, under conditions of systemic disease, as well as benign colonisation. We included the latter, as we believe that it would be even more relevant, given the state with which *C. albicans* exists in humans and the normal route of HU administration. As stated before, *C. albicans* is a natural coloniser of the human gastrointestinal tract. Furthermore, in systemic infection most cases appear to originate from endogenous sources of *C. albicans*, hence changes in *C. albicans* that reside in the GI tract may very well impact

disease arising from these colonisers later on [171]. HU is also administered primarily through the oral route. Given these two circumstances, it is not implausible that in a patient treated with HU, resident *C. albicans* encounters biologically meaningful concentrations of HU in the stomach, an organ also colonised by *C. albicans*.

During systemic infection, we did not observe an increased karyotypic variation in *C. albicans* in the kidneys of HU-treated mice. Since plasma concentrations of HU in humans so low that they are not expected to affect *C. albicans* growth based on our *in vitro* dose-response experiments, it is likely that *C. albicans* in the kidneys does not encounter HU in a biologically relevant concentration, and thus we do not observe increased incidence of aneuploidy [65].

On the other hand, we observed a significantly increased karyotypic variation in the stomachs of HU-treated mice compared to untreated mice, after a relatively short time of 11 days. Additionally, the effect of HU on *C. albicans* also appeared to manifest as significantly lower colonisation levels in the stomach compared to the caecum. Since HU slows cell division, the resident microflora (such as *C. albicans*) exposed to the drug could also be affected in terms of growth. Since chemotherapy is often given over long periods of time (e.g. several weeks), it is possible that even more profound effects on the karyotypes of *C. albicans* may be found in cancer patients undergoing chemotherapy. Since the karyotypic variants of *C. albicans* are generated in the stomach, they may be able to seed lower parts of the GI tract, even if they are not generated *in situ*. Dissemination of *C. albicans* that occurs from the lower parts of the GI tract could also involve such karyotypic variants, whose response to host defences and antifungal treatment may differ from the relatively benign original colonisers, possibly impacting the outcome of systemic infection [171].

It is important to consider that the selective pressures in different parts of the GI tract may affect the stability of karyotypes existing in a local population, and that karyotypic variation generated in the stomach may easily be lost as soon as *C. albicans* migrates away from the stomach. To address this, we also karyotyped *C.*

albicans in the caecum. Our results show that increased karyotypic variation was present only in the stomach, and not in the caecum, which is much lower in the GI tract. *C. albicans* derived from the caecum of both untreated and HU-treated mice produced only trisomy 7. HU is rapidly absorbed, and hence may not make it to lower parts of the GI tract in biologically meaningful concentrations [62,65]. Ultimately whether karyotypic variants generated in the stomach persist in the lower GI tract is dependent on the local environment, which could differ greatly between a healthy host and a host with cancer. Even though we do not see increased karyotypic variation in the caecum of HU-treated mice, this could be due to the local environment within a non-cancerous host.

Following this theme, we also wish to highlight that in our colonisation model, we are unable to account for the changes in GI microbiota that might occur due to chemotherapy, and consequently its contribution to the environment that *C. albicans* resides in. *C. albicans* is not a natural coloniser of the murine GI tract, and colonisation must needs be established by depleting the natural GI microbiota through pre-treatment of mice with an antibiotic cocktail [104,172]. This is not a trivial alteration as the gut microbiota is known to be a pleiotropic regulator of *C. albicans* colonisation and infection [173]. There have been comparatively few studies on the effect of chemotherapy on the gut microbiota, but studies in humans and animal models show that chemotherapy reduces the total number of gut bacteria and changes the composition of the gut microbiota [174-176]. Exactly which populations are affected by chemotherapy seems to be a matter of contention. Zwielehner *et al.* found greater changes in the groups Clostridium XIVa, while van Vliet *et al.* reported the greatest changes in bifidobacteria and *Bacteroides* species [175,177]. This disparity could be due to both studies being limited in sample size, the heterogeneity in therapy and pre-existing medical conditions in the study populations, as well as differences in methodology. These findings are suggestive of chemotherapy's effect on the gut microbiome, but more systematic and comprehensive approaches would be required to obtain more definitive answers. In highlighting these studies, we simply wish to emphasise that the microbiota plays an important role in the

regulation of *C. albicans* growth *in vivo*. As such, it likely also plays a selective role for *C. albicans*, and its absence in our studies may have led to a lack of selection for particular karyotypic variants, contributing to the homogenous karyotypic landscape in the caecum.

Having discussed the downsides of antibiotic pre-treatment in our experimental model, we also wish to show that it can be helpful, in that the absence of the microbiota in large part simplifies the system. This enables us to study certain factors in isolation, specifically that of chemotherapy's effect on the mammalian host (well-defined) and the colonising *C. albicans* (poorly defined). This allows us to better assess the individual contribution of these two factors towards *in vivo* karyotypic variation in *C. albicans*.

Generating aneuploidy in *C. albicans* is also possible with other chemotherapeutic drugs besides HU. We screened a range of drugs with different mechanisms of action *in vitro*, and of the drugs we screened, the antimetabolites (methotrexate and 5-fluorouracil, in addition to HU) appeared to be the most potent in terms of impact on *C. albicans* growth and morphology. Cisplatin, an alkylating agent (chemically reacts with DNA to form crosslinks, which interfere with DNA replication) was also another drug that we found to affect *C. albicans* growth and morphology. All three drugs also generated some aneuploidies.

Other chemotherapeutic drugs on the other hand (doxorubicin, topotecan, cyclophosphamide and paclitaxel), did not appear to cause any short-term changes in *C. albicans* growth and morphology. Both doxorubicin and topotecan target the DNA topoisomerases, which is required for relieving structural stress on DNA strands during replication to allow replication machinery to proceed [178,179]. Paclitaxel binds to β -tubulin, a major component of microtubules, stabilising the structure, which interferes with its normal functioning, as microtubules need to be both easy to construct and deconstruct to fulfill their functions [129,130]. The absence of effect in *C. albicans* suggests that despite the highly important functions that DNA topoisomerases and β -tubulin fulfil, and

the strong evolutionary conservation that generally accompanies this property, there are still sufficient differences to render *C. albicans* immune to their effects.

Since the effects of chemotherapeutic drugs are evident from changes in growth, we can also surmise that doxorubicin, topotecan and paclitaxel cause genomic changes in *C. albicans* within an *in vitro* context, at least at the doses we used. In the case of cyclophosphamide, however, we expected it to be active on *C. albicans*, owing to its alkylating properties, which is dependent of the chemical composition of DNA. Since the chemical composition of DNA is virtually the same in all organisms, it was unexpected that cyclophosphamide had no effect on *C. albicans* growth at all. Cyclophosphamide is a prodrug, which is metabolised in mammals (through the cytochrome P450 system) in the liver to phosphoramidate mustard, which is the active form that actually causes DNA crosslinks [180]. The lack of effect in *C. albicans* therefore could be due to the possibility that the enzymes converting cyclophosphamide to its active form is not present in *C. albicans*. It would be interesting to see if cyclophosphamide treatment in mammalian hosts colonised with *C. albicans* would then be able to affect *C. albicans*, since conversion of the drug to its active form would be possible in this system.

The highlight of these *in vitro* experiments is that drugs altering the short-term growth and morphology of *C. albicans* also generate whole-chromosome aneuploidy. These drugs, as mentioned above, are all known to interfere with DNA replication, whether by forming crosslinks or by blocking the formation of DNA building blocks. Slowed and stalled replication forks arise as a consequence of treatment with antimetabolites and DNA crosslinkers, and such effects are known to result in smaller-scale mutations [72,73,121]. Whole chromosome aneuploidy however is preferentially generated through defects in mitosis, and exactly how antimetabolites and DNA crosslinkers interfere with this is not clear [181]. However some recent work is beginning to shed light on this. A known cause of replication fork interference – similar to that caused by antimetabolites such as HU – is re-replication. Triggering of re-replication in *S. cerevisiae* has

been found to produce Rad52-dependent gene amplification, in addition to aneuploidy arising from re-replication in centromeric regions [182,183].

Components of the origin recognition complex (ORC) are required for pre-replicative complex assembly, which is in turn required for the initiation of replication. Depletion of *ORC1* components has been postulated to cause defects during replication, leading to interruptions of centromeric regions, which may affect kinetochore binding, which in turn impacts chromosome segregation, possibly leading to aneuploidy [184]. However, progression of mitosis in *Orc1*-depleted cells takes place only in cells that also lack components of the spindle assembly checkpoint, specifically Mad2 and Bub1 [184]. Since our studies in which aneuploidy was generated with HU were performed using SC5314 or SC5314-derived strains, which does not contain deletions of any such genes, mediating the formation of aneuploidy through perturbation of *Orc1* does not seem a likely scenario, unless HU simultaneously perturbs Mad2 and Bub1 along with *Orc1*.

DNA replication stress in *S. cerevisiae*, arising from HU treatment or reduced polymerase δ levels, has been found to cause aneuploidy [185,186]. In the more recent study by Zheng *et al.*, such replication stress also led to the generation of genomic variation, which enabled strains to grow better under replication stress [187]. Even more striking was that more than 60 % of the strains that adapted to replication stress did so through aneuploidy (monosomy of Chromosome XIV). Curiously in both studies, aneuploidy involving chromosome loss was more commonly detected than that of chromosome gain, although the much earlier study by Mayer *et al.* did not contain a means to detect chromosome gain [185]. Exactly why there would be a bias towards chromosome loss was not examined in either study, however Zheng *et al.* postulate that one outcome of replication stress is the incomplete synthesis of one sister chromatid, thus leaving only three copies of a chromosome present at mitosis. This would mean that one of the daughter cells will lack one copy of that chromosome.

Stress-induced mutagenesis may also play a role in the generation of aneuploidy by DNA replication inhibitors. Drugs such as HU lead to the depletion of nucleotide pools during S-phase, triggering the intra-S checkpoint. The checkpoint is an active process requiring the production of many proteins [75]. However checkpoints cannot be activated indefinitely, and yeast cells are eventually able to resume the cell cycle in spite of checkpoint activation (a process referred to as adaptation), which can lead to the exacerbation of DNA damage and mutations in daughter cells [76,188]. Stress may also exerted on cells through cell cycle arrest, possibly through the increased requirement for DNA damage proteins. The longer the replication stress is present, so is the stress exerted on the cell from its increased requirement for protein production. For instance, Hsp90, a molecular chaperone which helps to ensure proper protein folding, is also involved in both the activation of DNA repair pathways and the chromosome segregation machinery [189]. Since the drugs exert replication stress, which activates the repair pathways, we can speculate that they also draw away Hsp90 reserves, which are required for maintaining faithful chromosome segregation.

However, the average plasma concentration of HU does not appear to exert any effects on *C. albicans* in terms of growth, morphology, viability, or karyotypic changes, neither in the short nor long term. In our evolution experiments, we also used a concentration of HU (approximately 0.4 mM) analogous to that of the average plasma concentration in patients. The results of our short-term experiments with HU showed that such changes did not occur within six hours, but we hypothesised that it could take place if exposure to HU was prolonged, as in the course of an evolution experiment. However, even with one week of HU treatment at 0.4 mM, *C. albicans* exhibited no differences in growth, in morphology, or in colony size variation from the untreated population. But even if plasma level concentrations of HU do not affect *C. albicans*, tissue concentrations of HU (which are not known) may be able to do so. If HU preferentially accumulates within certain tissues, the local concentration of HU would be higher than that of the plasma concentration, and may then have an effect on *C.*

albicans. This is in fact a very likely scenario: as mentioned previously, HU is administered orally, hence the stomach is one of the first points of contact between *C. albicans* and HU. Local concentrations within the stomach are likely to be much higher here than in the plasma, according to our theoretical calculations of local HU concentration in the stomach directly after gavage, In such a case, we saw an elevated karyotypic diversity in *C. albicans* isolated from the stomach, as presented earlier.

While HU generated a variety of karyotypes both *in vitro* and *in vivo*, it also produced a recurrent aneuploidy – trisomy of Chromosome 2 *in vitro*. During our evolution experiments, we observed that trisomy 2 also became the dominant karyotype in the population within a week of treatment at the IC₅₀ of HU. Its recurrence was explained through its conferment of a fitness advantage in the presence of HU stress, which is apparent from our spotting assays at the IC₅₀ of HU. In these assays, we also ruled out the possibility that other smaller-scale mutations (such as gene conversion) were responsible for adaptation to HU [185,190]. We did so by using three pairs of isogenic strains derived from trisomic 2 strains arising from short-term treatment with HU, through single passage on YPD agar without HU, and then isolating trisomic 2 and euploid siblings based on colony size. Trisomic 2 strains consistently grew much better than their euploid counterparts. From these results, we were able to identify trisomy 2 as the factor required for HU resistance in these strains, and confirm that increased HU resistance was not due to any other mutations that could have occurred during HU treatment.

We also isolated the molecular components required for HU resistance on Chromosome 2. Chromosome 2 in *C. albicans* carries three of its four *RNR* genes [29]. Amplification of Chromosome 2 results in an increased gene copy number of these genes, and theoretically an increased transcription and translation of its protein products. Hence trisomy 2 confers resistance by simply producing more of the protein that HU inhibits. We successfully generated single deletion mutants for *RNR21* and *RNR22* in both the euploid and trisomic 2 background, and *RNR1* in

the trisomic 2 background only. By assessing their relative resistance to HU, we were able to identify exactly which *RNR* genes on chromosome 2 were actually required for trisomy 2-mediated HU resistance.

RNR1 and *RNR21* are both individually required for HU resistance. This is in keeping with what we know of the active RNR complex, which requires both the large and small subunits for activity. One subunit without the other would not produce an increased level of active protein complex. Having these two genes located on the same chromosome is fortuitous, as it enables a simultaneous increase in copy number of the genes required for making an active protein complex.

However, *RNR22*, which is a paralogue of *RNR21*, appears to be dispensable for HU resistance. This may be a result of *RNR21* being the copy of the small subunit that is preferentially expressed and incorporated into the functional RNR complex. Another possibility is that expression of *RNR21* may be lower than *RNR22*, and so its deletion would produce a more pronounced deficiency. A third possibility is that despite the strong sequence similarity (from which we infer a strong similarity in function), *RNR21* is somehow more sensitive to the effects of HU than *RNR22*, which may point to additional functions that *RNR21* carries out, which *RNR22* does not.

This adaptation involving trisomy 2 arose quite rapidly, considering that these “sibling” strains were derived from trisomic 2 strains originally isolated from HU IC₅₀ treatment after a mere 6 hours. As the doubling time of *C. albicans* is approximately one hour, an IC₅₀ effect during 6 hours would allow for 3 cell divisions at most. Assuming that the HU resistance of trisomic 2 strains enables cell division approximately once every hour (like WT cells in YPD at 37 °C), for trisomy 2 arising from standing genetic variation to reach the frequency that we observed in our short-term acute experiments (2 in 44 clones), trisomy 2 would need to be present at a frequency of

$$(\text{Initial frequency of trisomy 2} \times 8) \times 2^6 = 2/44$$

$$\text{Initial frequency of trisomy 2} = [(2/44)/2^6]/8$$

$$\approx 8.86 \times 10^{-5}$$

This frequency is lower than the rate of spontaneous chromosome loss (2.6×10^{-4} per cell division) [58]. That the adaptation arose within 3 cell divisions suggests that the trisomy arose primarily *de novo*, as selection of trisomy 2 as part of the genomic variation already present in the population would not be detectable at such frequency within only 3 cell divisions.

These results suggest that HU is capable of rapidly inducing aneuploidy in *C. albicans*, and then selecting for the adaptive mutation. That HU induces aneuploidy so quickly suggests that there may also be effects on mitosis as well. However the IC_{50} of HU is a relatively high concentration, far higher than plasma concentrations detected in patients treated with HU. It may be that such effects on mitosis can only be seen with relatively high concentrations, which may not occur in the blood, but may well be present in the stomach. We did not however observe significant occurrence of trisomy 2 in the stomach. This may be because the *in vivo* environment from which we obtained the colonies is not conducive to promoting trisomy 2 in *C. albicans*, or maintaining trisomy 2 strains that do arise. It could also be attributed to the concentration of HU being high enough to induce genomic variation, but not strong enough to exert a selection for trisomy 2.

Trisomy 2 is capable of being a highly effective adaptation to HU, which we infer from its rapid appearance and strong selective advantage. The rapidity of its appearance could be attributed to the fact that three of its four *RNR* genes, encoding the protein products for the RNR complex, are located on chromosome 2. This is in contrast to *S. cerevisiae* which despite its relatively close evolutionary relationship to *C. albicans*, has its four *RNR* genes scattered across the genome instead [85]. The proximity of the individual *RNR* genes to one another thus facilitates the evolution of adaptation by aneuploidy to a stress that targets the product of *RNR* genes in particular.

Besides conferring HU resistance, trisomy 2 also appears to alter filamentation capability. We observed during the long-term evolution experiments that HU-treated populations had high levels of filamentation during the early stages of the experiment (up to day 2 post-treatment), and that the frequency of filamentation appeared to decrease to levels comparable with that of untreated evolution lines grown solely in YPD medium, also correlating with the emergence of trisomy 2. This is in keeping with the observation of filamentation as a response to DNA damage by HU [36,37]. Filamentation may thus be a marker of the degree of replication stress *C. albicans* is under, and the increasing prevalence of trisomy 2 in the population may also have led to reduced replication stress, thus culminating in less filamentation. However, continued observation after one week showed that filamentation began to increase once again, although not to the same levels as seen in the early stages. This contradicts the idea that improved adaptation – as we saw from the results with day 15 strains on spotting assays – leads to a lower DNA damage response.

We hypothesised that the decrease in filamentation is not necessarily due to a reduced DNA damage response, but that certain genes on Chromosome 2 suppress filamentation, and trisomy 2 amplifies this effect through gene dosage alteration. Assays on Spider agar showed that this was not truly the case as HU-evolved strains with trisomy 2 generated wrinkled colonies just as readily as wild-type strains. The reduction in filamentation in the first week is not due to an inability to filament caused by trisomy 2. This is important to consider, since morphological switching is an important virulence factor, and a reduced filamentation ability in response to environmental cues as a result of trisomy 2 may lead to attenuation of a strain. We can also infer from these results that the reduced virulence of a trisomic 2 strain *in vivo* which we observed is not the result of reduced filamentation ability. However, the filamentation pathways activated by Spider agar and HU are different (Spider-induced filamentation relies on the Efg1 pathway, while HU-induced filamentation is most likely mediated through Rad53 and Cdc42) [33,36]. Possibly trisomy 2 inhibits HU-induced filamentation (but not

Spider-induced filamentation), and that loss of trisomy 2 (but not HU resistance), removes this inhibition.

Despite the fact that trisomy 2 grants rapid emergence of HU resistance, it still carries a fitness cost, as we observed in competitive growth experiments with SC5314 in untreated YPD medium. This is also corroborated by the decrease in frequency of trisomy 2 at the end of our two-week evolution experiment. In fact, euploidy became the most common karyotype after 14 daily passages. These euploid strains even grew better than a trisomic 2 strain derived from one-week experiment at the IC_{50} . This shows that there are many ways to achieve HU resistance even without trisomy 2 or aneuploidy. While there were other aneuploidies still retained after 14 passages at the IC_{50} , we did not study if these correlated with HU resistance.

In short, even though trisomy 2 arises rapidly in a HU-treated population, as time progresses, the population gradually loses this aneuploidy, in favour of smaller aneuploidies, and in particular euploidy. This pattern suggests that trisomy 2 is, in the long-term view, merely a temporary holding pattern, enabling cells to at least survive, if not thrive, until another adaptive mutation that does not bear the fitness cost of aneuploidy comes along. Its transience by no means downplays its significance however; cells must be able to survive long enough to adapt, and trisomy 2 is clearly able to buy cells enough time to do so, and could even be a key step towards evolving HU resistance, by being a necessary support for future adaptive mechanisms to take place.

Our results strongly parallel that of similar evolution experiments by Yona *et al.*, in which they detected trisomy of Chromosome 3 in *S. cerevisiae* by exposing cells to heat shock for 450 generations [59]. However, the trisomy was gradually eliminated over subsequent generations, until the evolved population returned to euploidy. They also found that while trisomy 3 provided a fitness benefit in the presence of elevated temperature, such aneuploid clones showed reduced growth in the absence of heat stress, demonstrating that the adaptive aneuploidy comes with a fitness cost. Instead, evolved euploid strains had improved growth

rates regardless of whether heat shock was present, showing how removal of the trisomy enables a refinement of stress adaptation.

We did not observe an emergence of trisomy 2 in the stomach of HU-treated mice. But another factor to take into account in human hosts is the use of antifungal drugs. While HU alone may not be sufficient to select for trisomy 2, we also found another clinically relevant drug capable of selecting for this aneuploidy – CSP, used in treating systemic *C. albicans* infection. Our assays show that trisomic 2 strains have more survivors than other strains in the presence of CSP, regardless of their intrinsic HU resistance. This resistance is apparent not only *in vitro*, but also *in vivo*, as shown by the reduced efficacy of CSP treatment during systemic infection with a HU-generated trisomic 2 strain. A combined selective pressure from both HU and CSP treatment may therefore increase the likelihood of trisomy 2 occurring *in vivo*, and this is a possible concern when considering the choice of antifungal drugs for treatment of systemic candidiasis.

Although multiple drug resistances are commonly detected in *C. albicans* and other microbes, these mechanisms commonly leverage elements that act collectively, such as upregulation of multidrug efflux pumps, which are able to act on several drugs at the same time, or transcription factors related to a specific drug family (e.g. azoles) [191]. Trisomic 2 strains are remarkable for exhibiting multiple resistance mechanisms targeting drugs coming from radically different drug families, represented in our study by HU (an RNR inhibitor) and CSP (a β -D-glucan synthase inhibitor). Our findings demonstrate the pleiotropic effects of aneuploidy, which affects the copy number of multiple genes that are not always functionally related. This enables the generation of phenotypic variation, even variation not selected for by the present environment, but may become relevant in future environments. This “pre-adaptation” of a population potentiates the development of an adaptive strategy towards multiple stresses, enabling much more rapid adaptation and improvement in survival than those achieved by smaller-scale genomic changes.

5. Conclusion

Cancer patients are at increased risk of opportunistic microbial infections, of which a leading cause is *C.albicans*. Chemotherapy-induced immunosuppression and epithelial barrier disruption – both effects impinging solely on the human host – are recognised as the primary causes of this increased risk of infection. In this work we investigated whether chemotherapy's sphere of influence extends beyond the human host and reaches the pathogen itself. We have found that various chemotherapeutic drugs are capable of generating genomic changes in *C. albicans* via aneuploidy, which we attribute to the genotoxic nature of chemotherapy and the highly conserved nature of their targets among eukaryotes. The focus of our work has been on HU, an RNR inhibitor impeding nucleotide synthesis, owing to the broad karyotypic variation it generates both *in vitro* and *in vivo*. Such variation can play a role in promoting adaptation to various host environments. In fact we have found that HU-generated trisomic 2 strains are resistant to not only HU, but also to CSP, a first-line antifungal drug used for treating systemic candidiasis. Our findings suggest that certain chemotherapeutic regimens may not be compatible with certain antifungal regimens, and that alternative antifungal drugs may need to be considered in HU-treated cancer patients with systemic candidiasis.

While our scope has been limited to that of *C. albicans*, other members of the human microbiota – some of which also cause opportunistic infections – could also be affected in a similar fashion to *C. albicans*. It may be worthwhile to study how such effects also impact other aspects of health, given the microbiota's involvement in a vast array of functions that maintain health, such as the control of fungal growth and mediating immune responses.

The simultaneous HU and CSP resistance conferred by trisomy 2 demonstrates the adaptive advantage aneuploidy can offer, compared to smaller or more targeted adaptive mutations. While aneuploidy comes with a fitness cost, it also changes the copy number of many genes at once – even genes that are not functionally related – enabling adaptation to multiple unrelated stresses. Such

adaptation requires much less time than those accomplished by smaller and more precise mutations evolved towards a specific stress.

Overall our work demonstrates that chemotherapy has more far-ranging effects than initially expected. Chemotherapy has the potential to genomically alter members of the resident human microbiota, and such variation may have clinical implications for the outcome and treatment of opportunistic microbial infections in cancer patients. Our findings may suggest new avenues for studying the prevention and treatment of opportunistic fungal infections.

6. References

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7. Appendix

upRNR1 F	TTTTTTGGTACCGAAGCTCTAAATCTCATACAGGC
upRNR1 R	TTTTTTGGGCCC AAGAAAAAGATCAACGAAATGG
doRNR1 F	TTTTTTCCGCGGGTTCTTTTCTGAGCGATGTTGTC
doRNR1 R	TTTTTTGAGCTCCAGTGTTAATTGGCTTTCTTT
upRNR1 F1	CGTTTAACGTAACCTAGTTCAATG
doRNR1 R1	TTCTATATTGCAAAAAGTGAGGG
RNR1 rtF	CAAAAAGTGCTGGAGGAATC
RNR1 rtR	CAACAGCAGCATGGTAGATAGTC
upRNR21 F	TTTTTTGGTACCTTACATCTGCTTGAAATAATACAC
upRNR21 R	TTTTTTGGGCCCATAATAAAGAAGAAAAGAAGAGAG
doRNR21 F	TTTTTTCCGCGGGTTGGGTGTTATTCCTTTTACTT
doRNR21 R	TTTTTTGAGCTCTCCGCCCAATCTGTATGT
upRNR21 F1	CACCTTAGCACAAAATGAGTTAC
doRNR21 R1	CAACTCTTCATTGATTTTTAGGA
RNR21 rtF	CGTCGTTATGTGATGTTCCC
RNR21 rtR	TCAAGTTACAGTTCATACCCAAC
upRNR22 F	TTTTTTGGTACCTCCCATCACTTGTTTCTTTATC
upRNR22 R	TTTTTTGGGCCCCGGTGCTTTGTTTTGTTGTTG
doRNR22 F	TTTTTTCCGCGGCAGACGCTTTGGTGGATTTC
doRNR22 R	TTTTTTGAGCTCGTGTTTGAATTGTGGGGTTTTT
upRNR22 F1	CAAATCCCCAATTCTAACTG
doRNR22 R1	ATTATAAGGGAAGTGAGTGGTGG
RNR22 rtF	CGTTAGCCTCATTCTGGACTG
RNR22 rtR	TCAACAGCCTCGGTGATG
pSFS2_F1_1	CAGATCCACTAGTTCTAGAGC
pSFS2_F1_2	CACGTATAAACTAGACCTC
pSFS2_R1_1	CAAACCAATCATTATTGGATCCG
pSFS2_R1_2	GTATAGGAACTTCCTCGAGG
FLP rtR	TGTGGCTATTTCCCTTATCTGC
SAT rtF	AATTCGGTGATCCCTGAGC
ClpUL	ATACTACTGAAATTTCTGACTTTC
ClpUR	ATTACTATTTACAATCAAAGGTGGTC
NIM1_verif	TTTACGGGTGTTAAACCTTCGAT
ADH1_verif	ACAAGCTCATTGAGTGACGAAAAG

Table 9. List of primers and primer sequences used in generating RNR deletion and overexpression strains. ClpUL, ClpUR, NIM1_verif and ADH1_verif were obtained from Chauvel *et al.* [164].

Gene	Orientation	Primer sequence	Genome position		Amplicon length (bp)	Slope
			start	end		
RNR1	forward	TACCATGCTGCTGTTGAAGC	640,303	640,284	87	-3.183
	reverse	AACCTTGAGAGGCTGGAGAA	640,216	640,235		
RNR21	forward	CCATCTCCAGAGATTGTCGAA	603,783	603,763	147	-3.362
	reverse	ACAACAATCTATCAGCGACGAA	603,636	603,657		
RNR22	forward	AAATCATCACCGAGGCTGTT	1,546,874	1,546,893	92	-3.116
	reverse	GGCACATCAAGTCACAATTCA	1,546,966	1,546,946		

Table 10. List of primers and sequences used for *RNR* gene expression assays.

Strain name	Karyotype	Origin
YFT1	2N, +2	Isogenic sibling of YFT2; parental strain is a trisomic 2 strain derived after 6 hours' treatment at HU IC ₅₀
YFT2	2N	Isogenic sibling of YFT1; parental strain is a trisomic 2 strain derived after 6 hours' treatment at HU IC ₅₀
YFT3	2N, +2	Isogenic sibling of YFT4; parental strain is a trisomic 2 strain derived after 6 hours' treatment at HU IC ₅₀
YFT4	2N	Isogenic sibling of YFT3; parental strain is a trisomic 2 strain derived after 6 hours' treatment at HU IC ₅₀
YFT5	2N, +2	Isogenic sibling of YFT6; parental strain is a trisomic 2 strain derived after 6 hours' treatment at HU IC ₉₀
YFT6	2N	Isogenic sibling of YFT5; parental strain is a trisomic 2 strain derived after 6 hours' treatment at HU IC ₉₀
YFT7	2N, +R	Obtained after 14 passages at HU IC ₅₀
YFT8	2N, +2	Obtained after 14 passages at HU IC ₅₀
YFT9	2N, +4	Obtained after 14 passages at HU IC ₅₀
YFT10	2N, +7	Obtained after 14 passages at HU IC ₅₀
YFT11	2N	Obtained after 14 passages at HU IC ₅₀
YFT12	2N	Obtained after 14 passages at HU IC ₅₀
YFT14	2N, +2	Obtained after 7 passages at HU IC ₅₀
SN148	2N	Parental strain of pNIMX; Arg ⁻ Leu ⁻ His ⁻ Ura ⁻ ; Noble and Johnson 2005
pNIMX	2N	Parental strain of Clp-RNR1 and Clp-RNR21 carrying the P _{TET} transactivator and SAT1; Arg ⁻ Leu ⁻ His ⁻ Ura ⁻
Clp-RNR1	2N	Derived from pNIMX; carries the P _{TET} transactivator, SAT1, and RNR1 under control of pTET; Arg ⁻ Leu ⁻ His ⁻ Ura ⁺
Clp-RNR21	2N	Derived from pNIMX; carries the P _{TET} transactivator, SAT1, and RNR21 under control of pTET; Arg ⁻ Leu ⁻ His ⁻ Ura ⁺
RNR21 ^{+/-}	2N	Heterozygous deletion of RNR21; parental strain is SC5314
RNR22 ^{+/-}	2N	Heterozygous deletion of RNR22; parental strain is SC5314
RNR1 ^{+/+/-}	2N, +2	Single deletion of RNR1; parental strain is YFT1
RNR21 ^{+/+/-}	2N, +2	Single deletion of RNR21; parental strain is YFT1
RNR22 ^{+/+/-}	2N, +2	Single deletion of RNR22; parental strain is YFT1

Table 11. List of strains used in our study.

Chromosome	Arm	Region	Assay name	Intergenic region used	Forward primer name	Forward primer sequence	Forward primer length	Reverse primer name	Reverse primer sequence	Reverse primer length	start	end	length	slope
1	left	centromeric	CaChr1Cb	second	Ca21Chr1CbF	TTGTACTTAGCGGTACCTG	21	Ca21Chr1CbR	GAAGAAGTGGAGGAAAGGG	21	1,553,190	1,553,303	113	-3.308
1	left	telomeric	CaChr1Ltz	last	Ca21Chr1LtzF	GGTCACTCTCAGACGTTCC	20	Ca21Chr1LtzR	TTATTCAGGTAGATTGTTGG	22	33,713	33,850	137	-3.287
1	right	centromeric	CaChr1RCb2	second	Ca21Chr1RCb2F	CAATTCCTTGAGGCAATCGG	20	Ca21Chr1RCb2R	TATAAATCTGGGAAACGAGCTACC	23	1,576,565	1,576,645	80	-3.336
1	right	telomeric	CaChr1Rtz	last	Ca21Chr1RtzF	CATGTAATGCCACAGTTTGTTG	22	Ca21Chr1RtzR	ACATCGGATACGAGCAACAG	21	3,150,774	3,150,904	130	-3.393
2	left	centromeric	CaChr2Cg	seventh	Ca21Chr2CgF	CTTCCTGGCAATCGTTTGG	20	Ca21Chr2CgR	TGTGGATAGTTTCAAGTTCTAGAGG	26	1,888,892	1,889,019	127	-3.399
2	left	telomeric	CaChr2Ltz	last	Ca21Chr2LtzF	ATCGACATCACTAATCCCG	20	Ca21Chr2LtzR	CATTGTGTGGCTCGCTGGT	19	32,855	33,000	145	-3.379
2	right	centromeric	CaChr2RC	third	Ca21Chr2RCF	CGTTTCTGGTTGGTTTGGTGC	21	Ca21Chr2RCR	GCCTCGCTGTATTAACCTGTTG	23	1,945,989	1,946,088	99	-3.408
2	right	telomeric	CaChr2Rtx	third from last	Ca21Chr2RtxF	CGTCAACTACACAAATGGCCC	21	Ca21Chr2RtxR	AGAGTCAACTTCCCGCAAC	20	2,180,968	2,181,080	112	-3.392
3	left	centromeric	CaChr3Cc	third	Ca21Chr3CcF	TCCTCCACCACTCTCTCTC	21	Ca21Chr3CcR	TTTGCTATATGTTCTCTGGTCTC	25	818,626	818,707	81	-3.36
3	left	telomeric	CaChr3Ltz	last	Ca21Chr3LtzF	AGAATTTGTCGGGTAGTTC	21	Ca21Chr3LtzR	TGCCACTGCCATTACCAAG	18	44,448	44,577	129	-3.353
3	right	centromeric	CaChr3RCd	fourth	Ca21Chr3RCdF	TACGTGGCCGCTAAGAAA	19	Ca21Chr3RCdR	AGTAAGGAGGTTGGCTTG	19	840,740	840,888	148	-3.402
3	right	telomeric	CaChr3Rtz	last	Ca21Chr3RtzF	GGTTCTCTCGGTAAAGCAAC	21	Ca21Chr3RtzR	TGGTATTACAGAACTTGACTCG	23	1,758,805	1,758,887	82	-3.343
4	left	centromeric	CaChr4Ce	fifth	Ca21Chr4CeF	TTTGGCTCACTGGGAAGC	19	Ca21Chr4CeR	CATTAGTCGAGCGCATCAATGTC	22	976,007	976,101	94	-3.385
4	left	telomeric	CaChr4Ltz	fourth from last	Ca21Chr4LtzF	CTGTAAAGCCAGTTGACAC	22	Ca21Chr4LtzR	GATGTAGACAATGTGCAAGC	21	67,476	67,605	129	-3.327
4	right	centromeric	CaChr4RC	fifth	Ca21Chr4RCF	TTTGGATGAACGATTTTGGCG	21	Ca21Chr4RCR	TGGTGTCTGTACTCGATGTG	20	1,022,176	1,022,293	117	-3.327
4	right	telomeric	CaChr4Rtz	last	Ca21Chr4RtzF	TGGAGATATAGGTTCTAGGTTG	25	Ca21Chr4RtzR	ACCAATATCTCCAAACTCTCG	23	1,585,471	1,586,579	108	-3.366
5	left	centromeric	CaChr5Cg	seventh	Ca21Chr5CgF	GTTTGAATAAGAGGGGTGG	21	Ca21Chr5CgR	TCTATTTGGTGAACCTAGCGAG	22	440,095	440,208	113	-3.306
5	left	telomeric	CaChr5Ltz	third from last	Ca21Chr5LtzF	TGTGTATCGGTTGGGTGAG	20	Ca21Chr5LtzR	GTTGAAACAGATTATAGGAATATGCTCC	28	40,704	40,835	131	-3.323
5	right	centromeric	CaChr5RCd	fourth	Ca21Chr5RCdF	GTTCAATGCACAGTGTATAGC	23	Ca21Chr5RCdR	CGTTTACAAAGTCTTTCCGGG	21	487,467	487,607	140	-3.434
5	right	telomeric	CaChr5Rtz	second from last	Ca21Chr5RtzF	GAAATATACCTCTCAAGCCG	22	Ca21Chr5RtzR	CTCAATTAACCTCAACCCCTC	21	1,136,258	1,136,365	107	-3.385
6	left	centromeric	CaChr6Cc	third	Ca21Chr6CcF	CTGTCTGTGATAAAGTGGTAAAG	25	Ca21Chr6CcR	AAATCCTCACTGACCAAG	21	962,520	962,600	80	-3.325
6	left	telomeric	CaChr6Ltz	second from last	Ca21Chr6LtzF	TGCCATACGACGTAATCTC	20	Ca21Chr6LtzR	GTTATAAACCTTAACCTGTAGCGG	25	36,731	36,869	138	-3.278
6	right	centromeric	CaChr6RC	third	Ca21Chr6RCF	TCTTTTACTCTTCCCTCAAGG	22	Ca21Chr6RCR	ACACGATTACACTGCCAAGAA	21	996,599	996,726	127	-3.327
6	right	telomeric	CaChr6Rtz	last	Ca21Chr6RtzF	CTTTGTGTAGCATTTTGCTCT	22	Ca21Chr6RtzR	AAAGTAAGTTGTGTCCGAGC	20	997,087	997,173	106	-3.343
7	left	centromeric	CaChr7Lch	eighth	Ca21Chr7LchF	TGGGAGTATAGGAAGTCCGTTTC	23	Ca21Chr7LchR	CGTTCGTGTTTGTGTGCGAG	20	393,765	393,860	95	-3.348
7	left	telomeric	CaChr7Ltz	last	Ca21Chr7LtzF	CCTCAGCTGAATGTTGCTG	21	Ca21Chr7LtzR	TGTGGAGACATAGCAATCAAG	22	40,457	40,572	115	-3.355
7	right	centromeric	CaChr7RC	fifth	Ca21Chr7RCF	AAA TGGGAAACCGAGTGTGT	19	Ca21Chr7RCR	GGGTACTATTAATGTGTCACCAAG	25	441,887	441,969	82	-3.323
7	right	telomeric	CaChr7Rtz	last	Ca21Chr7RtzF	CCAAGCACCACTCAAAACAAAG	21	Ca21Chr7RtzR	ACTAAATTCCTCCGCTCATCG	19	916,044	916,168	144	-3.355
R	left	centromeric	CaChr8Cg	seventh	Ca21Chr8CgF	GAGAGTTGGGAAATGAGCTGT	21	Ca21Chr8CgR	CAACAACGACACAAACAGCC	20	1,704,928	1,705,004	76	-3.337
R	left	telomeric	CaChr8Ltz	fourth from last	Ca21Chr8LtzF	TTACTCCCACTACTCTCACTGTTG	24	Ca21Chr8LtzR	GCAACGACACTACCAAC	20	45,392	45,485	93	-3.363
R	right	centromeric	CaChr8RC	sixth	Ca21Chr8RCF	CGAGGGAGAGAGGGAAGAA	20	Ca21Chr8RCR	AGATCGAAATACCGTATAGTGTG	24	1,786,558	1,786,695	137	-3.421
R	right	telomeric	CaChr8Rtz	last	Ca21Chr8RtzF	GAAATGGTGCCTGTAAAGC	21	Ca21Chr8RtzR	CAGTCAATGGTATCCGCCTC	20	2,245,613	2,245,742	129	-3.336

Table 12. List of primers used in the karyotyping qPCR for *C. albicans*.

Figure 48. Viability of chemotherapy-treated *C. albicans* after 6 hours of treatment, measured by plating on YPD agar and incubating overnight at 37°C (analysed by one-way ANOVA with Dunnett's multiple comparisons test).

Figure 49. Viability of chemotherapy-treated *C. albicans* after 24 hours of treatment, measured by PI staining.

Figure 50. Microscope images of *C. albicans* incubated in DMEM containing 10% foetal calf serum at 37°C in 5% CO₂.

Figure 51. Competitive fitness of various GI-evolved *C. albicans* strains in the gut, compared to a fluorescently-labelled SC5314 (unpublished data by Gloria Tso).

Colony #	-HU		+HU IC ₅₀		
	pH 4	pH 2	pH 6.6	pH 4	pH 2
1	2N	2N, +1	2N	2N, (+1 RT, -5 RT)	2N
2	2N	2N	2N	2N, +1	2N
3	2N	2N, +4	2N	2N, +7	2N
4	2N	2N	2N	2N	2N
5	2N	2N	2N	2N	2N
6	2N	2N	2N	2N	2N
7	2N	2N	2N	2N	2N
8	2N	2N	2N	2N	2N
9	2N	2N	2N	2N	2N
10	2N	2N	2N	2N	2N
11	2N	2N	2N	2N	2N
12	2N	2N	2N	2N	2N

Table 13. Karyotypes of *C. albicans* after 6 hours of exposure to HU at various pH levels.

Alignment for CaRNR21 vs CaRNR22:

C2_07570W_A|RNR22 COORDS:Ca22chr2A_C_albicans_SC5314:1545974-1547158W
(1185 nucleotides)

Sequence ID: Query_211103 Length: 1185 Number of Matches: 4

Range 1: 142 to 1132

Alignment statistics for match #1

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	699 bits(774)	0.0	754/993(76%)	4/993(0%)	Plus/Plus	
Query	199		AAAAAACATCAAGACTTTTGGCTAAACACAAAGTCCACAGACACAAATTGAAGCAATTA			258
Sbjct	142		AAAAGACACAAAGAGTTTGTAGCCAAACATGTGGTTAACAGACACAAGTTAAAAACAAGAA			201
Query	259		GAAGCTGAAGAACCATTATTGGTTGAAAACAA-ACGTCGTTATGTGATGTTCCCAATCAG			317
Sbjct	202		GAGTCCAACGAGCCATTGCTTACACCAGACAAGACG-CGTCATACCATATATCCGATAAA			260
Query	318		ATACCATGAAATTTGGAACCTTTTACAAGAAGGCTGAAGCTTCCTTCTGGACTGCTGAAGA			377
Sbjct	261		GTATCCCAGATTATGGCAGTTCTATAAAAAGTCGTTAGCCTCATTCTGGACTGCTGAAGA			320
Query	378		AATTGATTTGAGTAAAGATTGGATGACTGGAACAACAAATTAAATGAAAATGAAAGATA			437
Sbjct	321		GCTTGACTTGAGTAAAGATTGGATGATTGGAATAATAAGATGAACGAAAACGAAAGATT			380
Query	438		TTTTATTTCTAGAGTATTGGCATTTTTTGCCGCTCCGATGGTATTGTTGGTGAAAATTT			497
Sbjct	381		CTTTATCTCTCGAGTTTGGCATTCTTTGCTGCATCGGATGGTATTGTTAATGAGAACTT			440
Query	498		AATTGAAAATTTCTCAACTGAAGTTCAACTTCCTGAAGCCAAATCATTCTATGGTTTTCA			557
Sbjct	441		GGTGGAAAATTTCTGTGCAGAAAGTGCAAATACCCGAGGCTAACTGGTTTACAAGTTTCA			500
Query	558		AATCATGATGGAAAACATTCATTCAGAAACCTATTCTTTATTGATTGAAACATATATCAA			617
Sbjct	501		GATCATGATGGAAAACATTCATTCAGAGACTTATTTCGTTGTTAATAGAAAACATATTTCAA			560
Query	618		AGATCCCCAAGAGGCTGATTACTTGTTCAATGCCATTGCCAATATTCCATGTATTCAAAA			677
Sbjct	561		AGACCCCGAAGAAGCAGACTTTTGTTTAATGCTATTGACAATATCCCATTATATAAGAAA			620
Query	678		GAAAGCTGACTGGGCTATCAAATGGATTCAAGATGATGAAGCCTTATATGGTGAAAGATT			737
Sbjct	621		AAAAGCCGATTGGGCAATCAGATGGATCCAGAGCGAGGATGCTTTGTATGCTGAGAGATT			680
Query	738		AGTAGCTTTTGCTGCTGTTGAAGGTATCTTCTCAGTGGTTCATTTGCTTCTATTTTCTG			797
Sbjct	681		GGTTGCATTTGCTGCTGTTGAAGGTATATTTTTCAGTGGCTCGTTTGACGAATTTTCTG			740
Query	798		GTTGAAAAAGAGAGGTTTGATGCCAGGCTTGACTTTCTCAAATGAATTGATTGTAGAGA			857

Sbjct	741	GTTAAAGAAAACGGGGTTTTGATGCCGGGGTTGACATTTTCCAATGAATTAATTTGTTCGAGA	800
Query	858	TGAAGGTTTACACACCGACTTTGCCTGTTTGTTATTTGCTCATTTGCAGAATAGACCATC 	917
Sbjct	801	CGAAGGGATTACACAGATTATGCCTGTTTATTATTTTCATATTTAAAAAATAAACCTTC	860
Query	918	TCCAGAGATTGTTCGAAAGAATTATTACTGAAGCTGTTGATGTTGAAAAAGAATACTTTAC 	977
Sbjct	861	TTCAGAAATAATTGAGAAAATCATCACCGAGGCTGTTGATATCGAGAAAGAATATTTTCAG	920
Query	978	TGATGCTCTTCCAGTGTCAATTGTTGGGTATGAAGCTGTAACCTGATGTGTCAATACATTGA 	1037
Sbjct	921	CGATGCGCTTCCAGTATCCTTATTGGGAATGAATTGTGACTTGATGTGCCAGTATGTTGA	980
Query	1038	ATTCGTCGCTGATAGATTGTTGTTGGCTTTAGGTAACAAGAAAGTA-TACAATGTTACTA 	1096
Sbjct	981	ATTTGTTGCTGATAGATTACTAGTAGCTTTTGGAAATAA-AAAGTACTACAATGTTACCA	1039
Query	1097	ATCCATTCGATTTTCATGGAAAACATTTCTTGGCTGGTAAGACCAATTTCTTTGAAAAAA 	1156
Sbjct	1040	ACCCATTTGATTTTATGGAATATTTCTTGGCTGGTAAACTAATTTCTTTGAAAAGA	1099
Query	1157	GAGTTTCAGACTATCAAAAAGCTGGTGTTATGG 1189 	
Sbjct	1100	GAGTCTCTGATTATCAAAAAGGCTGGTGATGATGG 1132	

Alignment for CaRNR1 vs ScRNR1 (YER070W) SGDID:S000000872,
chrV:298950..301616

Sequence ID: Query_12510 Length: 47710 Number of Matches: 16
Range 1: 1 to 2285

Alignment statistics for match #1					
Score	Expect	Identities	Gaps	Strand	
1514 bits(1678)	0.0	1722/2295(75%)	23/2295(1%)	Plus/Plus	
Query 1		ATGTATGTTTATAAGAGAGATGGCCGTAAAGAGCCAGTACGTTTCGACAAAATCACTGCC			60
Sbjct 1		ATGTACGTTTATAAAAGAGACGGTCGTAAAGAACCTGTCCAATTCGATAAGATTACCGCT			60
Query 61		AGAGTTCAAAGATTATGTTACGGTTTGAATCCAAACCACGTTGAACCAGTTGCTATTACC			120
Sbjct 61		CGTATATCACGCTTATGCTATGGTTTAGATCCAAAACATATCGACGCCGTTAAGGTCACC			120
Query 121		CAAAAAGTTATATCAGGTGTTTACCAGGGGGTTACTACTATTGAGTTAGACAACTTGGCT			180
Sbjct 121		CAACGTATCATTTCTGGTGTCTATGAAGGTGTCACAACAATCGAACTAGACAACTTAGCC			180
Query 181		GCAGAAAT-TGCTGCTACAATGACAACAATTCACCCAGATTACGCTGTCTTAGCCGCTAG			239
Sbjct 181		GCTGAAACATGC-GCTTATATGACTACTGTTTCATCCAGATTACGCCACCCTAGCGGCCAG			239
Query 240		AATTGCCGTATCAAATTTACATAAGCAAACCACCAAACAGTATTCCAAAGT-GTCTAAGG			298
Sbjct 240		AATTGCCATTTCTAATTTACATAAAACAAACCACAAAACAATTTTCTAAGGTTGTCGAAG-			298
Query 299		ATTTATATGAATACATTAATCCTAAGACTGGGTTACACTCTCCTATGATTTCCAAGGAAA			358
Sbjct 299		ATCTTTATAGATACGTCAATGCTGCTACTGGTAAGCCCGCTCCCATGATCTCTGATGATG			358
Query 359		CCTACGACATCATTATGGAACACGAAGATGAATTAACTCAGCCATTGTTTACGACAGAG			418
Sbjct 359		TCTACAATATTTGTCATGGAAAAACAAGGATAAATTGAACTCCGCAATTGCTCTATGACAGAG			418
Query 419		ATTTTAACTACAATTATTTTGGGTTCAAGACTTTGGAAAGATCATATTTGTTACGTATCA			478
Sbjct 419		ATTTTCAGTACAGTTATTTTGGTTTAAACTTTGGAACGTTCTTATTTACTAAGAATCA			478
Query 479		ACGGTAAGGTTGCTGAAAGACCACAACATTTGATCATGAGGGTTGCTGTCGGTATTACAG			538
Sbjct 479		ACGGTCAAGTGGCCGAACGTCCACAACATTTAATTATGAGAGTCGCACTAGGCATCCACG			538
Query 539		GTAATGATATACCAAGGGTCATT--GAAACCTATAACTTGATGTCTCAAAGATTCTTCAC			596
Sbjct 539		GTAGAGATAT--CGAGGCTGCTTTAGAAACGTATAACTTGATGTCTCTAAAATATTTTAC			596
Query 597		CCATGGTTCTCCTTGTTTATTTAACGCTGGTACACCAAGACCACAAATGTCCTCATGTTT			656
Sbjct 597		TCACGCCTCTCCAACGTTGTTCAATGCCGGTACTCCAAAACCTCAAATGTCCTCTTGTTT			656
Query 657		CTTGCTTGCTATGAAGGATGATTCTATTGAAGGTATTTACGACACTTTGAAATCGTGTGC			716

Sbjct	657	CTTGGTTGCCATGAAGGAGGACTCTATCGAGGGGATTTACGACACCTTGAAGGAATGTGC	716
Query	717	TTTGATCTCAAAAAGTGCTGGAGGAATCGGTTTACACATCCACAACATTCGTTCTACCGG	776
Sbjct	717	TTTGATTTCCAAAAGTGCTGGTGGTATTGGTCTACATATCCATAACATTCGTTCAACTGG	776
Query	777	TGCTTACATTGCTGGTACCAATGGTACTTCTAATGGTATTATTCCAATGGTAAGAGTATT	836
Sbjct	777	TTCTTACATTGCTGGTACAAACGGTACTTCTAACGGTTTAATTCCTATGATTCGTGTTTT	836
Query	837	CAATAACACTGCACGTTATGTGACCAAGGTGGTAACAAGAGACCTGGTGCCTTTGCCTT	896
Sbjct	837	CAATAACACTGCCCCGTTATGTTGACCAGGGTGGTAATAAAAGACCTGGTGCCTTTGCCTT	896
Query	897	GTACTTAGAACCATGGCACAGCGACATTTTTGATTTTCATTGATATTAGAAAGAATCACGG	956
Sbjct	897	TTACCTGGAACCATGGCATGCTGATATATTTGATTTTATTGATATTAGGAAGAACCACGG	956
Query	957	TAAAGAAGAAATCAGAGCCAGAGATTTGTTCCCAGCTTTGTGGATTCCAGATTTGTTTCAT	1016
Sbjct	957	TAAAGAGGAAATTCGTGCAAGAGATTTGTTCCCTGCTCTATGGATTCTGATCTTTTCAT	1016
Query	1017	GAAAAGAGTTGAACAAAATGGTGACTGGACTTTATTCTCACCAA-ATGAGGCCCCAGGCT	1075
Sbjct	1017	GAAGCGTGTCGAAGAAAATGGGACCTGGACATTATTCTCTCCAACATCA-GCTCCTGGTT	1075
Query	1076	TGGCTGATGTTTATGGTGACGAATTCGAAGAATTATACACCAAATACGAAAAAGAAAACC	1135
Sbjct	1076	TAAGCGATTGTTACGGTGACGAGTTTGAGGCTCTATATACCCGCTACGAGAAAGAAGGTC	1135
Query	1136	GTGGTAGACAGACCATCAAAGCTCAAAAATTGTGGTATGCTATTTTGGGAGCCCAAAGTG	1195
Sbjct	1136	GTGGTA---AGACTATCAAAGCCCAAAAATTATGGTATTCCATTTTGGAAAGCTCAAAGTG	1192
Query	1196	AAACAGGTACCCCATTTATGTTATATAAAGATTTCATGTAACAACAAATCCAACCAAAAAGA	1255
Sbjct	1193	AAACTGGTACACCTTTCGTTGTTTACAAGGATGCTTGTAACAGAAAAATCTAATCAAAAAA	1252
Query	1256	ACTTGGGTATTATCAAATCTTCCAACCTGTGTTGTGAAATTGTTGAATATTCTGCTCCAG	1315
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Query	1316	ATGAAGTTGCTGTTTGTAACCTGGCTTCCATTGCCTTGCCATCATTTGTTGAAA-----	1369
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Sbjct	1373	AGGATGGTAAGACTTCCACATACAACCTTCAAAAAATTACATGAAATTGCTAAAGTTGTTA	1432
Query	1430	CCCGTAACTTGAACAGAGTTATTGACCGTAACCATTACCCAGTCCCAGAAGCTGAAAGAT	1489
Sbjct	1433	CTCGTAATTTAAACAGAGTCATTGATCGTAATTACTACCCGTGTTGAAGAAGCGAGAAAAAT	1492
Query	1490	CAAACATGAGACACAGACCAATTGCTTTGGGTGTTCAAGGTTTGGCTGATGCCTTTATGG	1549
Sbjct	1493	CCAATATGAGACATAGACCAATTGCTTTGGGTGTTCAAGGTCTCGCTGACACTTTCATGC	1552

Query	1550	AATTGAGATTACCATTTGACTCTCAAGAAGCTAGAGAATTGAACATTCAAATTTTGTGAGA	1609
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Query	1610	CTATCTACCATGCTGCTGTTGAAGCTTCAATTGAATTGGCTAAAAGAAGGTGCCTACG	1669
Sbjct	1613	CTATTTATCATGCCTCCATGGAAGCTTCTTGTTGAAGTAGCTCAGAAGGACGGTCCATACG	1672
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Sbjct	1673	AAACTTTCCAAGGATCTCCTGCTTCTCAAGGTATACTACAGTTTGATATGTGGGACCAAA	1732
Query	1729	AAACCAACTGAATTATGGGATTGGGATACATTAAAAACAAGATTTGGCCAAACATGGTATG	1788
Sbjct	1733	AACCCTACGGCA-TGTGGGATTGGGACACCTTAAGAAAAAGATATCATGAAGCATGGTGTT	1791
Query	1789	AGAAACTCCTTGTTGGTTGCACCAATGCCTACTGCTTCCACATCACAAATTTTGGGTAAAC	1848
Sbjct	1792	AGAAATTCCTTGACCATGGCACCAATGCCTACTGCATCCACATCCCAAATATTGGGTATAT	1851
Query	1849	AATGAATGTTTTGAACCATACACTTCTAACATTTACTCTAGAAGAGTATTAGCTGGAGAA	1908
Sbjct	1852	AATGAATGTTTCGAACCAAGTCACTTCCAATATGTACTCCCGTCGTGCTTATCCGGTGAA	1911
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Sbjct	1912	TTCCAGGTTGTGAACCCTTACTTACTGCGTGACTTGGTTGATTTAGGTATTTGGGATGAG	1971
Query	1969	GCTATGAAA-AGTAGTATTATTGCTAACAATGGTTCTATCCAAGCCTTACCAAACATCCC	2027
Sbjct	1972	GGTATGAAACAGTA-TCTGATTACACAAAATGGCTCCATTCAAGGCTTACCAAACGTTCC	2030
Query	2028	TGATGAAATCAAGGCATTGTACAAAACCTGTCTGGGAAATCTCACAAAAACATATTATCGA	2087
Sbjct	2031	ACAAGAATTGAAGGACTTATACAAAGACTGTTTGGGAAATTTACAAAAAGACTATCATTA	2090
Query	2088	CATGGCTGCTGATAGAGCAGCATTTATTGATCAATCTCAATCATTAACATTACATCAA	2147
Sbjct	2091	CATGGCAGCCGATCGTTCTGTCTATATTGATCAATCTCATTCCTTGAATTTGTTCTTACG	2150
Query	2148	AGATCCAACAATGGGTAAATTAACCAGTATGCACTTCTACGGTTGGAAGAAAGGTTTAAA	2207
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Query	2208	GACTGGTATGTACTACTTAAGAACACAAGCTGCCAGTGCTGCTATTCAATTTACCATTGA	2267
Sbjct	2211	GACCGGTATGTACTATTTGAGAACCAAGCTGCATCTGCTGCAATTCAATTTACTATTGA	2270
Query	2268	TCAAAAGATTGCTGA 2282	
Sbjct	2271	TCAGAAGATTGCGGA 2285	

Alignment for CaRNR1 vs ScRNR3 (YIL066C)

Sequence ID: Query_137807 Length: 2610 Number of Matches: 4

Range 1: 1 to 2285

Alignment statistics for match #1

	Score	Expect	Identities	Gaps	Strand	
	1362 bits(1510)	0.0	1682/2292(73%)	17/2292(0%)	Plus/Plus	
Query	1		ATGTATGTTTATAAGAGAGATGGCCGTAAAGAGCCAGTACGTTTCGACAAAATCACTGCC			60
Sbjct	1		ATGTACGTTATTAAAAAGAGACGGCCGCAAAGAGCCCGTTCAATTTCGATAAAAATTACCTCC			60
Query	61		AGAGTTCAAAGATTATGTTACGGTTTGAATCCAAACCACGTTGAACCAGTTGCTATTACC			120
Sbjct	61		CGTATCACCCGTTTGTGCATACGGTTTAGACCCAAACCGTATTGATGCTGTTAAGGTAACC			120
Query	121		CAAAAAGTTATATCAGGTGTTTACCAGGGGGTTACTACTATTGAGTTAGACAACTTGGCT			180
Sbjct	121		CAACGTATTATTTCTGGTGTGTACTCCGGTGTACTACCGTTGAGCTGGACAATCTTGCA			180
Query	181		GCAGAAATTGCTGC-TACAAATGACAACAATTCACCCAGATTACGCTGTCTTAGCCGCTAG			239
Sbjct	181		GCTGAAACATGTGCATACA-TGACCACTGTGCACCCGTATTATGCCACTCTAGCCGCTAG			239
Query	240		AATTGCCGTATCAAATTTACATAAGCAAACCACCAAACAGTATTCCAAAGTGCTAAGGA			299
Sbjct	240		AATCGCCATCTCTAACTTACATAAGCAAACCACAAAGCAATTCTCCAAAGTTATTGAGGA			299
Query	300		TTTATATGAATACATTAATCCTAAGACTGGGTACACTCTCCTATGATTTCCAAGGAAAC			359
Sbjct	300		TTTACACGACTGGATTAAACCCAGCTACTGGAAAGCATGCTCCTATGATTTTCGGACGAAAT			359
Query	360		CTACGACATCATTATGGAACACGAAGATGAATTAAACTCAGCCATTGTTTACGACAGAGA			419
Sbjct	360		TTACAACATTGTCATGGAAAAACAAAGATACTTTGAACTCGGCCATCGTGTACGATAGGGA			419
Query	420		TTTAACTACAATTATTTTGGGTTCAGACTTTGGAAAGATCATATTTGTTACGTATCAA			479
Sbjct	420		TTTCCAGTATACGTATTTTCGGATTCAAGACACTGGAGCGTTCGTACTTGCTAAGACTGAA			479
Query	480		CGGTAAGGTTGCTGAAAGACCACAACATTTGATCATGAGGGTTGCTGTCGGTATTCACGG			539
Sbjct	480		CGGTGAAGTGGCAGAACGTCCTCAGCATTTGGTAATGCGTGTGGCGCTAGGTATCCATGG			539
Query	540		TAATGATATACCAAGGGTCATTGAAACCTATAACTTGATGTCTCAAAGATTCTTCACCCA			599
Sbjct	540		TAGCGATATCGAATCTGTGCTGAAGACTTATAATTTGATGTCTTAAGATACTTCACTCA			599
Query	600		TGGTTCTCCTTGTTTATTTAACGCTGGTACACCAAGACCACAAATGTCCTCATGTTTCTT			659
Sbjct	600		CGCTTCCCCAACTTTATTCAACGCTGGTACGCCACATCCTCAAATGTCTTCATGTTTCTT			659
Query	660		GCTTGCTATGAAGGATGATTCTATTGAAGGTATTTACGACACTTTGAAATCGTGTGCTTT			719
Sbjct	660		AATTGCCATGAAGGATGACTCTATCGAAGGTATTTATGATACTTTGAAAGAATGTGCTAT			719

Query	720	GATCTCAAAAAGTGCTGGAGGAATCGGTTTACACATCCACAACATTCGTTCTACCGGTGC	779
Sbjct	720	GATTTCCAAAACCTGCAGGTGGTGTGGTCTTCATATCAACAACATCCGTTCCACAGGTTC	779
Query	780	TTACATTGCTGGTACCAATGGTACTTCTAATGGTATTATTCCAATGGTAAGAGTATTCAA	839
Sbjct	780	TTATATCGCTGGTACCAACGGTACTTCAAACGGGTTGATTCCATGATTTCGTGTTTTCAA	839
Query	840	TAACACTGCACGTTATGTCGACCAAGGTGGTAACAAGAGACCTGGTGCCTTTGCCTTGTA	899
Sbjct	840	TAATACTGCCCGTTATGTGGACCAGGTGGTAACAAGAGACCTGGTGCCTTCGCCCTTTT	899
Query	900	CTTAGAACCATGGCACAGCGACATTTTTTGATTTCATTGATATTAGAAAAGATCACGGTAA	959
Sbjct	900	CTTGGAGCCATGGCATGCAGATATCTTCGACTTTGTCGATATCAGAAAAACACATGGTAA	959
Query	960	AGAAGAAATCAGAGCCAGAGATTTGTTCCAGCTTTGTGGATTCCAGATTTGTTTCATGAA	1019
Sbjct	960	GGAAGAAATTCGTGCAAGAGATTTGTCCCTGCTCTATGGATCCCTGATCTTTTCATGAA	1019
Query	1020	AAGAGTTGAACAAAATGGTGACTGGACTTTTATTCTCACCAAATGAGGCCCCAGGCTTGGC	1079
Sbjct	1020	ACGTGTTCAAGAGGATGGGCCTTGGACTTTGTTTTCGCCCAGTGCTGCCCCAGGTTTAGA	1079
Query	1080	TGATGTTTATGGTGACGAATTCGAAGAATTATACACCAAATACGAAAAAGAAAACCGTGG	1139
Sbjct	1080	TGATGTGTGGGGTGATGAATTTGAAGAACTATATACTCGTTACGAAAGAGAAGGTCGTGG	1139
Query	1140	TAGACAGACCATCAAAGCTCAAAAATTGTGGTATGCTATTTTGGGAGCCCAAACCTGAAAC	1199
Sbjct	1140	TAAA---ACAATTAAAGCCCAAAAGTTGTGGTATGCCATTTTGCAAGCACAGACAGAAAC	1196
Query	1200	AGGTACCCCATTTATGTTATATAAAGATTCATGTAACAACAAATCCAACCAAAAAGAACTT	1259
Sbjct	1197	AGGTACACCTTTTCATGGTTTATAAGGACGCATGTAACAGGAAGACAAAACCAACAGAACTT	1256
Query	1260	GGGTATTATCAAATCTTCCAACCTGTGTTGTGAAATTGTTGAATATTCTGCTCCAGATGA	1319
Sbjct	1257	AGGTACTATCAAATCATCTAATTTATGTTGTGAAATCGTCGAATATTCTCCCCGGATGA	1316
Query	1320	AGTTGCTGTTTGTAACCTGGCTTCCATTGCCTTGCCATCATTTGTTGA-----AAATGA	1373
Sbjct	1317	AACTGCAGTTTGTAATTTAGCTTCTATTGCCCTACCAGCATTCGTTGAGGTTTCAGAAGA	1376
Query	1374	TGAAAAAAGTACTTGGTACAACCTTGACAAATTACATCAGGTCATAAGGTTGTCACCCG	1433
Sbjct	1377	TGGTAAACTGCAAGCTATAATTTGAGAGATTACACGAGATTGCTAAAGTCATTACTCA	1436
Query	1434	TAACCTGAACAGAGTTATTGACCGTAACCATTACCCAGTCCCAGAAGCTGAAAGATCAAA	1493
Sbjct	1437	CAACTGAACAGAGTTATCGACCGTAATTACTATCCAGTTCCCAGGCTAGAAAATTCAAA	1496
Query	1494	CATGAGACACAGACCAATTGCTTTGGGTGTTCAAGGTTTGGCTGATGCCTTTATGGAATT	1553
Sbjct	1497	TATGAAGCATAGACCTATTGCTCTTGGTGTCCAGGGTTTGGCCGATACTTATATGATGTT	1556

Query	1554	GAGATTACCATTTGACTCTCAAGAAGCTAGAGAATTGAACATTCAAATTTTTGAGACTAT	1613
Sbjct	1557	GCGTCTACCCTTTGAATCGGAAGAAGCTCAAACCTCTAAACAAACAAATCTTCGAAACTAT	1616
Query	1614	CTACCATGCTGCTGTTGAAGCTTCAATTGAATTGGCTAAAGAAGAAGGTGCCTACGAAAC	1673
Sbjct	1617	TTACCATGCTACTCTTGAAGCCTCCTGTGAATTGGCCCCAAAAAGAAGGTAAATATTCTAC	1676
Query	1674	CTATCCAGGTTCTCCAGCCTCTCAAGGTTTATTACAATTTGATTTGTGGAACAGAAAACC	1733
Sbjct	1677	TTTTGAAGGTTCTCCAGCTTCTAAGGGTATTTTACAATTCGATATGTGGAACGCTAAACC	1736
Query	1734	AACTGAATTATGGGATTGGGATACATTAACAAGATTGGCCAAACATGGTATGAGAAA	1793
Sbjct	1737	ATTTGGCATGTGGGATTGGGAAACCTTAAGAAAGGACATTGTTAAACATGGGTAAAGAAA	1796
Query	1794	CTCCTTGTTGGTTGCACCAATGCCTACTGCTTCCACATCACAAAATTTGGGTAACAATGA	1853
Sbjct	1797	CTCTTTGACTATGGCACCAATGCCAACCGCCTCAACTCCCAAATCTTGTTATAATGA	1856
Query	1854	ATGTTTTGAACCATACACTTCTAACATTTACTCTAGAAGAGTATTAGCTGGAGAATTCCA	1913
Sbjct	1857	ATGCTTCGAACCAGTGACCTCAAACATGTACTCTCGTCGTCTGCTGGTGAATTCCA	1916
Query	1914	AATTGTCAATCCATATTTATTGAAGGACTTGGTTGATTTGGGTGTCTGGAACGACGCTAT	1973
Sbjct	1917	AGTTGTTAATCCATATTTACTACGTGATTTAGTCGACCTGGGTATTTGGGATGATAGTAT	1976
Query	1974	GAAA-AGTAGTATTATTGCTAACAATGGTTCTATCCAAGCCTTACCAAACATCCCTGATG	2032
Sbjct	1977	GAAACAATA-TCTAATTACACAAAATGGTTCTATTCAAGGCTTACCAAATGTGCCACAAG	2035
Query	2033	AAATCAAGGCATTGTACAAAACGTCTGGGAAATCTCACAAAAACATATTATCGACATGG	2092
Sbjct	2036	AATTGAAGGAATTATACAAAACCGTCTGGGAAATCTCTCAAAAGACCATTATCAATATGG	2095
Query	2093	CTGCTGATAGAGCAGCATTT--ATTGATCAATCTCAATCATTAACATTCACATCAAAGA	2150
Sbjct	2096	CTGCTGATCGTGC--CATCTACATCGATCAGTCTCATTCCTTGAATCTTTTCTTGCAAGC	2153
Query	2151	TCCAACAATGGGTAAATTAACCAGTATGCACTTCTACGGTTGGAAGAAAGGTTTAAAGAC	2210
Sbjct	2154	ACCATCAATGGGTAAAGATTACTAGTATGCATTTCTACGGTTGGAAGAAAGGTTTAAAAAC	2213
Query	2211	TGGTATGTACTACTTAAGAACACAAGCTGCCAGTGCTGCTATTCAATTTACCATTGATCA	2270
Sbjct	2214	TGGTATGTACTACTTAAGAACGCAAGCCGCCTCCGCTGCTATTCAATTTACCATTGATCA	2273
Query	2271	AAAGATTGCTGA 2282	
Sbjct	2274	AGAGGTTGCCGA 2285	

Alignment of CaRNR1 vs HsRNR large subunit:

CaRNR1 vs ENA|AF107045|AF107045.1 Homo sapiens ribonucleotide reductase M1 subunit (RRM1) gene, complete cds

Sequence ID: Query_167394 Length: 45043 Number of Matches: 14

Range 1: 40048 to 40229

Alignment statistics for match #1

	Score	Expect	Identities	Gaps	Strand	
	120 bits(132)	2e-28	138/183(75%)	2/183(1%)	Plus/Plus	
Query	2015		TACCAAACATCCCTGATGAAATCAAGGCATTGTACAAAACGTCTGGGAAATCTCACAAA			2074
Sbjct	40048		TACCAGAAATTCCTGATGACCTGAAGCAACTTTATAAAACGTGTGTGGGAAATCTCTCAGA			
	40107					
Query	2075		AACATATTATCGACATGGCTGCTGATAGAGCAGCATTTATTGATCAATCTCAATCATTAA			2134
Sbjct	40108		AAACTGTTCTCAAGATGGCAGCTGAGAGAGGTGCTTTCATTGATCAAAGCCAATCTTTGA			
	40167					
Query	2135		ACATTACATCAAAGATCC-AACAATGGGTAAATTAACCAGTATGCACTTCTACGGTTGG			2193
Sbjct	40168		ACATCCACATTGCTGAGCCTAACTAT-GGCAAAC TACTAGTATGCACTTCTACGGCTGG			
	40226					
Query	2194		AAG			2196
Sbjct	40227		AAG			40229

Query	950	CTGTTGATGTTGAAAAAGAATACTTTACTGATGCTCTTCCAGTGTCATTGTTGGGTATGA	1009
Sbjct	908	CTGTGGAAATTGAACAAAGATACTTCTTGGACGCCTTACCAGTTGCTTTGCTAGGTATGA	967
Query	1010	ACTGTAACTTGATGTGTCAATACATTGAATTCGTCGCTGATAGATTGTTGTTGGCTTTAG	1069
Sbjct	968	ACGCTGACTTAATGAACCAATACGTTGAGTTCGTCGCCGACAGACTGTTGGTTGCTTTTCG	1027
Query	1070	GTAACAAGAAAGTATACAATGTTACTAATCCATTCGATTTTCATGGAAAACATTTCTTGG	1129
Sbjct	1028	GTAACAAGAAATACTACAAGGTCGAAAACCCCTTCGATTTTCATGGAAAACATCTCCTTGG	1087
Query	1130	CTGGTAAGACCAATTTCTTTGAAAAAGAGTTTCAGACTATCAAAAAGCTGGTGTTATGG	1189
Sbjct	1088	CCGGTAAGACCAACTTCTTCGAAAAGAGAGTTTCTGACTACCAAAAAGGCTGGTGTTATGT	1147
Query	1190	CCAAGACCGAAAACAAAGAAGCCGATGCTTTTACCTTTGATGAAGATTTCTAA	1242
Sbjct	1148	CCAAGTCGACTAAGCAAGAAGCCGGTGCTTTCACCTTCAACGAAGACTTTTAA	1200

Alignment for CaRNR21 vs ScRNR4 (YGR180C SGDID:S000003412,
chrVII:855264..856301)

Sequence ID: Query_170359 Length: 1038 Number of Matches: 5
Range 1: 82 to 1038

Alignment statistics for match #1

	Score	Expect	Identities	Gaps	Strand	
	188 bits(208)	2e-51	643/979(66%)	35/979(3%)	Plus/Plus	
Query	277		TTGGTTGAAAACAAACGTCGTTATGTGATGTTCCCAATCAGATACCATGAAATTTGGAAC			336
Sbjct	82		TTGATGGAAAACAGCCGTAGATTCGTGATGTTCCCTATCAAATACCACGAAATCTGGGCT			141
Query	337		TTTTACAAGAAGGCTGAAGCTTCCTTCTGGACTGCTGAAGAAATTGATTTGAGTAAAGAT			396
Sbjct	142		GCTTACAAGAAGGTTGAAGCCTCTTTCTGGACTGCGGAAGAAATCGAATTGGCTAAGGAC			201
Query	397		TTGGATGACTGGAACAACAAATTAAATGAAAATGA-AAGATATTTTATTTCTAGAGTATT			455
Sbjct	202		ACTGAAGATTTC--CAA-AAATTGACTGATGACCAGAAGACCTAC-ATCGGTAACCTGTT			257
Query	456		GGCATTTTTGTCCGCTTCCGATGGTATTGTTGGTGAAAATTTAATTGAAAATTTCTCAAC			515
Sbjct	258		AGCCTTGTCCATTCTTCTGACAACCTTGTCAACAAGTACTTGATCGAAAACCTTTCTGC			317
Query	516		TGAAGTTCAACTTCCTGAAGCCAAATCATTCTATGGTTTTCAAATCATGATGGAAAACAT			575
Sbjct	318		CCAATTGCAAAACCCTGAAGGTAAGAGTTTCTACGGGTTCCAGATTATGATGGAAAACAT			377
Query	576		TCATTTCAGAAACCTATTCTTTATTGATTGAAACATATATCAAAGATCCCCAAGAGGCTGA			635
Sbjct	378		CTACTCTGAAGTTTACTCCATGATGGTTGATGCCTTCTTCAAGGA-CCCTAAGAAC---A			433
Query	636		TTACT-TGTTCAATGCCATTGCCAATATTCCATGTATTCAAA-AGAAAGCTGACTGGGGCT			693
Sbjct	434		TCCCTCTATTCAAGGAAATTGCCAATTTGCC-TGAAGTCAAGCATAAGGCTGCTTTC---			489
Query	694		ATCAA---ATGGATTCAAGATGATGAAGCCTTATATGGTGAAAGATTAGTAGCTTTTGCT			750
Sbjct	490		ATCGAGAGATGGATTTCACACGATGACAGCTTGTATGCTGAAAGACTAGTAGCATTTGCT			549
Query	751		GCTGTTGAAGGTATCTTCTTCAGTGGTTCAATTGCTTCTATTTTCTGGTTGAA--AAAGA			808
Sbjct	550		GCAAAGGAAGGTATTTTCCAAGCTGGTAACTATGCTTCTATGTTCTGGTTGACTGACAAA			609
Query	809		GAGGTTTGATGCCAGGCTTGACTTTTCTCAAATGAATTGATTTGTAGAGATGAAGGTTTAC			868
Sbjct	610		AAGATT--ATGCCAGGTTTAGCAATGGCCAACAGAAACATCTGTAGAGACAGAGGTGCCT			667
Query	869		ACACCGACTTTGCCTGTTTGTATTGCTCATTTGCAGAAT--AGACCATCTCCAGAGAT			926
Sbjct	668		AACTGACTTTTCATGCTTGCTATTTGCCCATTTG-AGAACCAAGCCAAACCCCA-AGAT			725
Query	927		TGTCGAAAGAATTATTACTGAAGCTGTTGATGTTGAAAAAGAATACTTTACTGATGCTCT			986

Sbjct	726	CATTGAAAAAATCATTACCGAAGCCGTGGAAAATTGAAAAGGAATACT--ACTCAAACCTCT	783
Query	987	T--CCAGTGTTCATTGTTGGGTATGAACTGTAACCTTGATGTGTCAA-TACATTGAATTCGT	1043
Sbjct	784	TTGCCAGTAGAAAAATTTGGTATGGATTTGAAGAGCAT-TCACACCTACATAGAATTTGT	842
Query	1044	CGCTGATAGATTGTTGTTGGCTTTAGGTAACAAGAAAGTATACAATGTTACTAATCCATT	1103
Sbjct	843	CGCTGACGGTCTATTACAAGGTTTCGGTAACGAAAAATACTACAACGCCGTCAACCCATT	902
Query	1104	CGATTTTCATGGAAAACATTTCCCTTGGCTGGTAAGACCAATTTCTTTGAAAAAAGAGTTTC	1163
Sbjct	903	CGAATTCATGGAGGATGTCGCTACCGCTGGTAAGACCACCTTCTTTGAAAAGAAGGTTTC	962
Query	1164	AGACTATCAAAAAGCTGGTGTTATGGCCAAGACCGAAAAACAAAGAAGCCGATGCTTTTAC	1223
Sbjct	963	CGACTACCAAAAAGGCCAGTGACATGTCTAAGTCCGCTACC---CCATCCAAGGAAATTA	1019
Query	1224	CTTTGATGAAGATTTCTAA	1242
Sbjct	1020	CTTTGATGATGACTTCTAA	1038

Alignment of CaRNR21 vs HsRNR small subunit (gi|568815596:10122568-10131419
Homo sapiens chromosome 2, GRCh38.p2 Primary Assembly)

Sequence ID: Query_83979 Length: 8852 Number of Matches: 16
Range 1: 2168 to 2280

Alignment statistics for match #1

	Score	Expect	Identities	Gaps	Strand	
	69.8 bits(76)	1e-14	83/113(73%)	0/113(0%)	Plus/Plus	
Query	517		GAAGTTCAACTTCCTGAAGCCAAATCATTCTATGGTTTTCAAATCATGATGGAAAACATT			576
Sbjct	2168		GAAGTTCAGATTACAGAAGCCCGCTGTTTCTATGGCTTCCAAATTGCCATGGAAAACATA			2227
Query	577		CATTCAGAAACCTATTCTTTATTGATTGAAACATATATCAAAGATCCCCAAGA			629
Sbjct	2228		CATTCTGAAATGTATAGTCTTCTTATTGACACTTACATAAAAGATCCCAAAGA			2280

Alignment for CaRNR22 vs ScRNR2 (YJL026W)

Sequence ID: Query_53024 Length: 1200 Number of Matches: 2

Range 1: 312 to 1146

Alignment statistics for match #1

	Score	Expect	Identities	Gaps	Strand	
	482 bits(534)	5e-139	610/836(73%)	2/836(0%)	Plus/Plus	
Query	297		AGCCTCATTCTGGACTGCTGAAGAGCTTGACTTGAGTAAAGATTTGGATGATTGGAATAA			356
Sbjct	312		AGCTTCTTTCTGGACCGCTGAAGAAATTGATTTGTCTAAGGATATCCATGACTGGAACAA			371
Query	357		TAAGATGAACGAAAACGAAAGATTCTTTATCTCTCGAGTTTTGGCATTCTTTGCTGCATC			416
Sbjct	372		CAGAATGAACGAAAACGAGAGATTTTTCATTTCCAGAGTTCTTGCCTTTTTCGCCGCTTC			431
Query	417		GGATGGTATTGTTAATGAGAACTTGGTGGAAAATTTCTGTGCAGAAAGTCAAATACCCGA			476
Sbjct	432		TGACGGTATTGTTAATGAAAACCTGGTTGAAAACCTCTCCACCGAAGTCCAAATTCAGAA			491
Query	477		GGCTAAACTGGTT-TACAAGTTTCAGATCATGATGGAAAACATTCATTCAGAGACTTATT			535
Sbjct	492		GGC-AAAGAGTTTCTACGGTTTCCAAATCATGATTGAAAATATTCACTCTGAAACTTACT			550
Query	536		CGTTGTTAATAGAAACATATTTCAAAGACCCCGAAGAAGCAGACTTTTTGTTTAATGCTA			595
Sbjct	551		CCTTGTGATCGATACTTACATCAAGGACCCTAAAGAAAGTGAATTCTTGTTCATGCCA			610
Query	596		TTGACAATATCCCATTATAAGaaaaaaaGCCGATTGGGCAATCAGATGGATCCAGAGCG			655
Sbjct	611		TTCACACCATCCAGAAATCGGTGAGAAAGGCCGAATGGGCTTTAAGATGGATTCAAGACG			670
Query	656		AGGATGCTTTGTATGCTGAGAGATTGGTTGCATTTGCTGCTGTTGAAGGTATATTTTTCA			715
Sbjct	671		CTGACGCCTTGTGTTGGTGAAAGACTAGTTGCCTTTGCCTCCATTGAAGGTGTCTTTTCT			730
Query	716		GTGGCTCGTTTGCAGCAATTTCTGGTTAAAGAAACGGGGTTTGATGCCGGGGTTGACAT			775
Sbjct	731		CCGGTTCCTTTGCCTCCATTTTCTGGTTGAAAAAGAGAGGTATGATGCCCGGTTTAACCT			790
Query	776		TTTCCAATGAATTAATTTGTCGAGACGAAGGGATTACACAGATTATGCCTGTTTATTAT			835
Sbjct	791		TTTCCAACGAATTGATCTGTAGAGACGAAGGTTTGACACCCGACTTTGCATGCTTGTGT			850
Query	836		TTTCATATTTAAAAAATAAACCTTCTTCAGAAATAATTGAGAAAATCATCACCGAGGCTG			895
Sbjct	851		TCGCCCATTGGAAGAACAAACCAGACCCAGCCATTGTTGAAAAAATTGTCACCGAGGCTG			910
Query	896		TTGATATCGAGAAAGAATATTTACGCGATGCGCTTCCAGTATCCTTATTGGGAATGAATT			955
Sbjct	911		TGGAAATTGAACAAAGATACTTCTTGACGCCTTACCAGTTGCTTTGCTAGGTATGAACG			970
Query	956		GTGACTTGATGTGCCAGTATGTTGAATTTGTTGCTGATAGATTACTAGTAGCTTTTGAA			1015
Sbjct	971		CTGACTTAATGAACCAATACGTTGAGTTCGTCGCCGACAGACTGTTGGTTGCTTTCGGTA			1030

Query	1016	ATAAAAAGTACTACAATGTTACCAACCCATTTGATTTTATGGAAAATATTTTCCTTGGCTG	1075
Sbjct	1031	ACAAGAAATACTACAAGGTCGAAAACCCCTTCGATTTCATGGAAAACATCTCCTTGGCCG	1090
Query	1076	GTAAAACTAATTTCTTTGAAAAGAGAGTCTCTGATTATCAAAAGGCTGGTGTGATG	1131
Sbjct	1091	GTAAGACCAACTTCTTCGAAAAGAGAGTTTCTGACTACCAAAAGGCTGGTGTATG	1146

Alignment for CaRNR22 vs ScRNR4 (YGR180C SGDID:S000003412,
chrVII:855264..856301)

Sequence ID: Query_125465 Length: 1038 Number of Matches: 3
Range 1: 520 to 977

Alignment statistics for match #1

	Score	Expect	Identities	Gaps	Strand	
	80.6 bits(88)	6e-19	299/461(65%)	6/461(1%)	Plus/Plus	
Query	664		TTGTATGCTGAGAGATTGGTTGCATTTGCTGCTGTTGAAGGTATATTTTTCAG-TGGCTC			722
Sbjct	520		TTGTATGCTGAAAGACTAGTAGCATTTGCTGCAAAGGAAGGTAT-TTTCCAAGCTGGTAA			578
Query	723		GTTTGCAGCAATTTTCTGGTTAAA-GAAACGGGGTTTGATGCCGGGGTTGACATTTTCCA			781
Sbjct	579		CTATGCTTCTATGTTCTGGTTGACTGACAAAAAGATT-ATGCCAGGTTTAGCAATGGCCA			637
Query	782		ATGAATTAATTTGTCGAGACGAAGGGATTCACACAGATTATGCCTGTTTATTATTTTCAT			841
Sbjct	638		ACAGAAACATCTGTAGAGACAGAGGTGCCTACACTGACTTTTCATGCTTGCTATTTGCC			697
Query	842		ATTTAAAAAATAAACCTTCTTCAGAAATAATTGAGAAAATCATCACCGAGGCTGTTGATA			901
Sbjct	698		ATTTGAGAACCAAGCCAAACCCCAAGATCATTGAAAAATCATTACCGAAGCCGTGGAAA			757
Query	902		TCGAGAAAGAATATTTTCAGCGATGCGCTTCCAGTATCCTTATTGGGAATGAATTGTGACT			961
Sbjct	758		TTGAAAAGGAATACTACTCAAACCTCTTTGCCAGTAGAAAAATTTGGTATGGATT-TGAAG			816
Query	962		TGATGTGCCAG-TATGTTGAATTTGTTGCTGATAGATTACTAGTAGCTTTTGAAAATAAA			1020
Sbjct	817		AGCATTCACACCTACATAGAATTTGTCGCTGACGGTCTATTACAAGGTTTCGGTAACGAA			876
Query	1021		AAGTACTACAATGTTACCAACCCATTTGATTTTATGGAAAATATTTCCCTTGGCTGGTAAA			1080
Sbjct	877		AAATACTACAACGCCGTCAACCCATTCGAATTCATGGAGGATGTCGCTACCGCTGGTAAG			936
Query	1081		ACTAATTTCTTTGAAAAGAGAGTCTCTGATTATCAAAAAGGC	1121		
Sbjct	937		ACCACCTTCTTTGAAAAGAAGGTTTCCGACTACCAAAAAGGC	977		

Protein sequence alignment of CaNR21 (subjectssequence) vs CaNR22 (query sequence)
Range 1: 37 to 414

Alignment statistics for match #1

	Score	Expect	Method	Identities	Positives	Gaps
	602 bits(1553)	0.0	Compositional matrix adjust.	284/382(74%)	334/382(87%)	4/382(1%)
Query	14	KVTEKSTPIVTKPESDKTVEKASDDSEIDLPEVYKRHKEFLAKHVNRHKLKQEEESNEPL				73
		K+ +++ I ++ + +KA+++ + L K+H++FLAKH V+RHKLKQ E+ EPL				
Sbjct	37	KLKDEAQTIHEVKQAVASTDKATEEKDDSL----KKHQDFLAKHKVHRHKLKQLEAEEPL				92
Query	74	LTPDKTRHTIYPIKYPELWQFYKKSLSASFWTAEELDSLKDLDWNNKMNENERFFISRVL				133
		L +K R+ ++PI+Y E+W FYKK+ ASFWTAEELDSLKDLDWNNK+NENER+FISRVL				
Sbjct	93	LVENKRRYVMFPRIYHEIWNFYKKAESFWTAEELDSLKDLDWNNKLNENERYFISRVL				152
Query	134	AFFAASDGIVNENLVENFCAEVQIPEAKSVYKFQIMMENIHSETYSLLIETYFKDPPEEAD				193
		AFFAASDGIV ENL+ENF EVQ+PEAKS Y FQIMMENIHSETYSLLIETY KDP+EAD				
Sbjct	153	AFFAASDGIVGENLIENFSTEVQLPEAKSFYGFQIMMENIHSETYSLLIETYIKDPQEAD				212
Query	194	FLFNAIDNIPFIRKKADWAIWQSEDALYAERLVAFAAVEGIFFGSGSFAAIFWLKKRGL				253
		+LFNAI NIP I+KKADWAI+WIQ ++ALY ERLVAFAAVEGIFFGSGSFA+IFWLKKRGL				
Sbjct	213	YLFNAIANIPCIQKKADWAIKWIQDDEALYGERLVAFAAVEGIFFGSGSFASIFWLKKRGL				272
Query	254	MPGLTFSNELICRDEGIHTDYACLLFSYLKNKPSSEIIEKIITEAVDIEKEYFSDALPVS				313
		MPGLTFSNELICRDEG+HTD+ACLLF++L+N+PS EI+E+IITEAVD+EKEYF+DALPVS				
Sbjct	273	MPGLTFSNELICRDEGLHTDFACLLFAHLQNRPSPEIVERIITEAVDVEKEYFTDALPVS				332
Query	314	LLGMNCDLMCQYVEFVADRLLVAFGNKKYYNVTNPFDPMENISLAGKTNFFEKRVSQYQK				373
		LLGMNC+LMCQY+EFVADRLL+A GNKK YNVTNPFDPMENISLAGKTNFFEKRVSQYQK				
Sbjct	333	LLGMNCNLMCQYIEFVADRLLLALGNKKVYNVTNPFDPMENISLAGKTNFFEKRVSQYQK				392
Query	374	AGVMEKVENKNEKTGLFDQDF* 395				
		AGVM K ENK FD+DF*				
Sbjct	393	AGVMAKTENKEADAFTFDEDF* 414				

8. Author's Publications

Teoh F, Pavelka N. How chemotherapy increases the risk of systemic candidiasis in cancer patients: current paradigm and future directions. *Pathogens* 2016, 5(1), 6; doi:10.3390/pathogens5010006

9. Poster Presentations

Teoh F, Pavelka N. Hydroxyurea generates aneuploidy and enhances its adaptive potential. Poster session presented at: EMBO Conference on Experimental Approaches to Evolution and Ecology Using Yeast and Other Model Systems. 2016 Oct 19 – 23; Heidelberg, Germany.

Teoh F, Pavelka N. Hydroxyurea generates aneuploidy and enhances its adaptive potential. Poster session presented at: Gordon Research Conference on Molecular Mechanisms in Evolution. 2015 Jun 28 - Jul 3; Easton, MA, United States of America.

Teoh F, Pavelka N. Mechanisms underlying the increased risk of systemic candidiasis in cancer patients. Poster session presented at: 4th Singaporean Immunology PhD Student Retreat. 2014 Aug 29; Singapore.

Teoh F, Pavelka N. Mechanisms underlying the increased risk of systemic candidiasis in cancer patients. Poster session presented at: Gordon Research Conference on Cellular and Molecular Fungal Biology. 2014 Jun 15-20; Holderness, NH, United States of America.

Teoh F, Pavelka N. Mechanisms underlying the increased risk of systemic candidiasis in cancer patients. Poster session presented at: EMBO Workshop for Complex Systems in Immunology. 2013 Dec 2- 4; Singapore.