Elucidation of the Role of a Cryptic RNAi Machinery in the Human Pathogenic Yeast *Candida albicans*

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by

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DECLARATION

I do hereby declare that the work described in this thesis titled 'Elucidation of the role of a cryptic RNAi machinery in the human pathogenic yeast *Candida albicans*' is an original piece of work that has been carried out by me under the guidance and supervision of Dr. Kaustuv Sanyal, Associate Professor, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, 560064, India

Aditi Batra

Date:

CERTIFICATE

This is to certify that this thesis titled "Elucidation of the role of a cryptic RNAi machinery in the human pathogenic yeast *Candida albicans*" submitted by Aditi Batra, as part of the project for the Master of Science degree at Jawaharlal Nehru Centre for Advanced Scientific Research, is based on the studies carried out by her under my supervision and guidance

Prof. Kaustuv Sanyal

Date:

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Aditi

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ABBREVIATIONS

Abbreviation	Full form
°C	Degree Celsius
ml	Milli litre
µl	Micro litre
µg	Micro gram
bp	Base pairs
kb	Kilo base
mM	Milli molar
h	Hours
rpm	Revolutions per minute
OD_{600}	Optical density at wavelength of 600nm
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
kDa	Kilo Dalton
MDa	Mega Dalton
M.W.	Molecular Weight

SUMMARY

RNA interference (RNAi) is the phenomenon of degradation of target RNA, mediated by small regulatory RNAs, that results in silencing of chromatin and protection of the cell against invading viruses and endogenous mobile genetic elements. Though the phenomenon was first described in the nematode worm *Caenorhabditis elegans* by Fire and colleagues, the protein machinery required for RNAi is now known to be present across the prokaryotic and eukaryotic lineages with some species having the full complement of the machinery, others possessing only a subset thereof and some even lacking the machinery altogether.

In the taxa in which the machinery is found, one or more pathways of RNAi may be functional. These pathways are distinguished based on the small regulatory RNA involved, and are named as the microRNA, small interfering RNA and PIWI-interacting RNA pathways based on the small RNA they utilize. These pathways carry out one or more of the functions associated with the RNAi machinery. Barring a few exceptions, the protein machinery required for RNAi is conserved across the three pathways. Of the entire complement of RNAi proteins, two are unambiguously recognized to be present at the heart of silencing process. These two proteins, Dicer and Argonaute, are required for RNAi in most eukaryotic organisms. Dicer performs the function of generation of the small regulatory RNAs while Argonaute is involved in target recognition using these small RNAs and in downstream effector functions. Originally known for its role in chromatin silencing and cellular defence, novel functions of the RNAi machinery in genome maintenance, transcription and processing of RNA species have been discovered, highlighting its functional diversity.

The fungi are a major lineage of the Eukarya with enormous diversity in ecology, morphology and lifecycles. As is the case with other eukaryotes, the RNAi machinery found in various fungal taxa is also functionally diverse. The non-canonical functions include quelling, DNA repair and meiotic silencing of unpaired DNA in *Neurospora crassa*, pathogenesis in *Botrytis cinerea* and sex-induced silencing in *Cryptococcus neoformans* to name a few. The diversity in RNAi in the fungal kingdom is not restricted to the functions of the machinery alone but also extends to the domain architecture of the proteins involved. This is exemplified by the non-canonical Dicer of the budding yeasts. This protein has retained only the catalytic RNase III domain and the double stranded RNA binding domain of its

higher eukaryotic counterparts, yet is able to perform the same function of small RNA generation as them, albeit with a slightly different mechanism. Budding yeasts also possess an Argonaute protein, which along with Dicer comprises the RNAi machinery of these yeasts. The budding yeast Argonaute resembles the Argonautes of the higher eukaryotes in domain architecture and presumably functions by the same mechanism.

Candida albicans is the most commonly isolated yeast pathogen of humans. This budding yeast, like some others in its group, possesses the non-canonical Dicer and Argonaute proteins. Even though, endogenous silencing of genes by these proteins has been examined, the results of these studies are inconclusive. Given the diverse array of functions RNAi is known to perform in fungi, it is conceivable that the RNAi machinery of *C. albicans* may be performing additional roles aside from that in gene silencing. An earlier study has explored other possible functions associated with this machinery, but not extensively. The aim of this project is to assess the capability of the RNAi machinery of *C. albicans* to silence genes and to investigate other functions, if any, of the machinery in this organism. Specifically, we were interested to determine whether the machinery is involved in controlling position-effect variegation at the *C. albicans* centromeres, drawing parallels from the closely related fission yeast *Schizosaccharomyces pombe*. This thesis deals with results pertaining functions of the RNAi machinery in *C. albicans* other than that in gene silencing.

During the course of this study, it was discovered that absence of Dicer results in growth retardation of *C. albicans* which was further observed to be function of temperature. This slow growth was determined to arise not due to cell mortality but instead probably due to an increase in the time taken to complete the cell cycle itself. Such a link between cell cycle and RNase III enzymes is not unique and has been shown in other species as well. From analysis of flow cytometry plots it was proposed that this delay is presumably occurring in the S phase of the cycle as the plots of the mutant showed an accumulation of cells in the S-phase as compared to the wild type. To test if the processes of DNA replication or DNA repair, the major processes in the S-phase, were affected in the absence of Dicer, the mutant cells were treated with the replication inhibitor hydroxyurea (HU) and oxidizing agent dimethyl sulfoxide (DMSO). The mutants were found to be a fold more sensitive to DMSO indicating that Dicer has a role to play in protecting the cell against oxidative damage. This role however, cannot yet be associated with repair of damaged DNA as oxidative damage by DMSO is not specific to DNA. It is however possible that such a scenario may exist as Dicer is known to be involved in DNA repair in other species. Upon treatment with HU, the

mutants were found to be resistant to the drug as compared to the wild type. As of now, we are unsure of what is the cause for such a phenotype and can only list few possibilities which could result in HU resistance. Absence of Dicer was also observed to lead to an increase in cellular size.

Deletion of *Argonaute* did not affect growth as was observed with Dicer. We also did not see any of the other phenotypes upon *Argonaute* deletion that were observed for Dicer depletion. Further, there is no other protein with domain architecture similar to Argonaute present in the *C. albicans* genome which could carry out Argonaute's function in its absence thus ruling out the possibility of redundancy. Based on these facts, we believe that in *C. albicans* Dicer and Argonaute may have functionally diverged with respect to the above mentioned phenotypes.

In *S. pombe*, position-effect variegation is exerted on transgenes inserted in the centromeres. Deletion of RNAi genes of *S. pombe* alleviates this position-effect variegation, thus implicating RNAi in controlling the phenomenon. Position-effect variegation is also seen on transgenes inserted in *C. albicans* centromeres. We wanted to ascertain whether, like in the closely related ascomycete *S. pombe*, RNAi also affects this phenomenon at the *C. albicans* centromeres. We have found that in the absence of either Dicer or Argonaute, this phenomenon is unaffected at *C. albicans* centromeres, thus ruling out a function similar to that observed in *S. pombe*.

In conclusion, in this work we have attempted to characterize the functions of the RNA interference proteins of the human fungal pathogen *C. albicans*. We have observed some phenotypes associated with absence of Dicer and based on them speculate that it may be playing a role in the cell cycle. Deletion of *Argonaute* does not confer the same phenotypes as depletion of Dicer; this fact along with other evidences suggests that they may have functionally diverged. Further, we have found that unlike in *S. pombe*, the position-effect variegation observed at *the C. albicans* centromeres is not under the control of the RNAi machinery.

CHAPTER 1:

INTRODUCTION

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1.1 RNA Interference: A historical perspective

The phenomenon of RNA Interference (RNAi), silencing of chromatin by small double stranded RNA, was discovered in the nematode worm *Caenorhabditis elegans* (Fire, Xu et al. 1998). This study was based on the work carried out on characterization of PAR-1, a putative serine threonine kinase, essential for establishing cellular asymmetry during development in *C. elegans* (Guo and Kemphues 1995). In an attempt to degrade the putative PAR-1 mRNA using an antisense approach Guo and Kemphues fortuitously found that the sense RNA was as effective as the anti-sense RNA in supressing gene expression. This finding was paradoxical with the existing notion of antisense mediated degradation wherein the antisense RNA was assumed to act by hybridizing with the target resulting in the formation of a double stranded RNA which could then lead to inhibition of translation or destruction of the message by cellular ribonucleases (Sen and Blau 2006). Fire and colleagues reasoned that the preparations of sense and antisense single stranded RNA could be contaminated with double stranded RNA molecules which would then be the ones responsible for bringing about the silencing (Fire, Xu et al. 1998). To test their hypothesis, the group extensively purified single stranded sense and antisense RNA and tested their effect on silencing alongside that of a deliberately prepared double stranded RNA. The group found that double stranded RNA was at least tenfold more effective in silencing target genes than the single stranded sense or antisense RNA and that the former were found to be effective only if injected into the animals as a mixture, suggesting hybridization of the two to form double stranded RNA in vivo (Fire et al, 1998). This seminal study established double stranded RNA as the trigger for silencing of endogenous genes in a sequence-specific manner and laid down the foundations of the field of RNAi.

Silencing of a homologous target by a double stranded RNA further led to the notion that a number of previously characterized, homology-dependent gene silencing mechanisms might share a common biological root (Hannon 2002). In 1990, Napoli and Jorgensen, in an attempt to generate petunias with a deeper flower colour, made transgenic plants by introducing the transgene responsible for flower pigmentation under an overexpression promoter (Napoli, Lemieux et al. 1990). To their surprise, they obtained flowers either with variegated pigmentation or ones lacking pigment altogether. Introduction of the transgene had thus silenced expression not only from the transgene itself but also from the endogenous copy.

This phenomenon was termed co-suppression (Napoli, Lemieux et al. 1990). It is now known that co-suppression can be produced by highly expressed single copy transgenes and by moderately expressed transgenes which integrate in the genome in complex multicopy arrays (Hannon 2002). A similar phenomenon has also been shown in *Neurospora crassa*, wherein the introduction of a transgene caused 'quelling' of the endogenous gene (Romano and Macino 1992). In parallel to these developments, studies by several laboratories also revealed that plants responded to invading RNA viruses by targeting their genome for degradation and that endogenous genes could be silenced by inclusion of homologous sequence in a viral replicon (Hannon 2002). Thus, in hindsight, it is evident that silencing by double stranded RNA produced by both transgenes and replicating RNA viruses was the commonality in the aforementioned studies.

RNAi has come a long way since its discovery nearly three decades ago. The mechanistic details of the phenomenon have been probed in both the prokaryotic and eukaryotic lineages and molecular players underpinning the process have been uncovered. Aside from its importance as a conserved chromatin silencing mechanism, RNAi has also been optimized as a tool to study gene function due to its capabilities of sequence specific suppression of gene expression. The progress in the field has been staggering and yet more aspects of this intricate system wait to be uncovered.

1.2 Mechanisms/pathways of RNA Interference

The machinery for RNA interference is found both in prokaryotes and eukaryotes though the former possess a much pared version of the machinery (Makarova, Wolf et al. 2009). Within the eukaryotes the machinery is widespread and varied with some organisms having the complete set with multiple paralogues of the constituent proteins, some with only a subset of the full machinery and others lacking the machinery all together (Shabalina and Koonin 2008). In those organisms having the machinery or parts thereof, one or more pathways of RNAi are present. Though there may be slight variations amongst organisms in these pathways, they are more or less conserved and are associated with the different functions performed by the machinery. These pathways and the accompanying functions are described in some detail below.

1.2.1 The silencing trigger

The double stranded RNA silencing trigger can be either exogenous or endogenous (Carthew and Sontheimer 2009). Genomes of invading RNA viruses or synthetic double stranded RNA

injected by experimenters constitute exogenous triggers while endogenous triggers include double stranded RNA from transposons, centromeres, repetitive sequences, natural sense-antisense pairs or hairpin RNA transcribed from the genome. The small RNAs derived from the latter are called microRNAs or miRNAs while those derived from the former three constitute small interfering RNAs or siRNAs. A third pathway, involving PIWI-interacting (piRNAs) has been reported in animals and is known to function in the germline (Carthew and Sontheimer 2009).

1.2.2 MicroRNAs

Micro RNAs are present in both the animal and plant lineages of the Eukarya and have recently been reported in the fungal kingdom as well (Villalobos-Escobedo, Herrera-Estrella et al. 2016). In plants, miRNAs are produced from individual transcription units located within intergenic regions while in animals the location of miRNAs is more diverse. In the latter, multiple miRNAs can be produced as part of a single transcription unit or may be even present in the intronic regions of protein coding transcripts aside from their production from individual transcription units (Figure 1A) (Shabalina and Koonin 2008, Carthew and Sontheimer 2009). The initial processing of miRNAs in both plants and animals requires some extra steps compared to the siRNA pathway but this pathway converges with the siRNA pathway in the cytoplasm of the cell (Wilson and Doudna 2013). In animals, the RNA produced from the transcription of the genomic locus harbouring the miRNA is called the primary miRNA (pri-miRNA). This transcript contains a hairpin shaped secondary structure. The junction of the stem of this hairpin and flanking single stranded RNA is recognized by a double stranded RNA binding protein called DiGeorge syndrome critical region gene 8 (DGCR8). Binding of this protein to the junction helps to position a class II ribonuclease III (RNase III) family protein Drosha to cleave the hairpin RNA ~11bp away from this junction, thus releasing the hairpin structure now called the pre-miRNA (Figure 1 B). Together DGCR8 and Drosha form a complex known as the Microprocessor complex. The pre-miRNA is then exported out of the nucleus into the cytoplasm where it forms the substrate for cleavage by another RNase III enzyme, Dicer. A class III enzyme of the RNase III family, Dicer recognizes double stranded RNA and cuts them into smaller fragments 20-30 nucleotides in length (depending upon the species). These products of Dicer have a 2 nucleotide overhang at their 3’ terminus and a phosphate group at their 5’ terminus. Dicer's action on pre-miRNA liberates the terminal loop leading to the formation of a mature miRNA (Figure 2) (Wilson and Doudna 2013). Plants do not possess a homologue of Drosha, instead

the generation of the pre-miRNA and the mature miRNA are both carried out by a Dicer homologue, DCL-1 in the nucleus (Carthew and Sontheimer 2009).

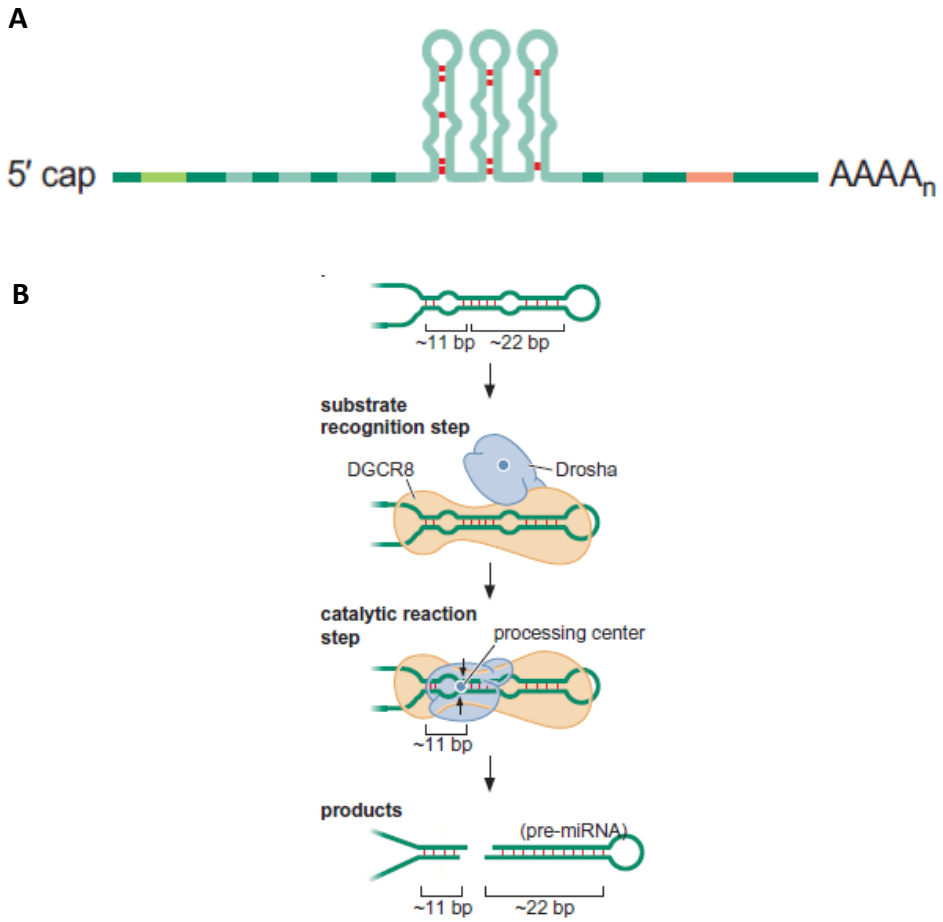


Figure 1: Processing of the pri-miRNA by the Microprocessor complex. (A) In animals miRNAs may be present in diverse genomic locations. Shown here is a cluster of miRNAs present in the introns of an ORF. Plants miRNAs are produced from individual transcription units present in intergenic regions. (B) The pri-miRNA in animals is acted upon by the microprocessor complex which consists of the proteins DGCR8 and Drosha to generate the pre-miRNA which is then exported to the cytoplasm to be processed by Dicer. Details of the process are described in the text. Plants do not possess a homologue of Drosha and instead processing of pri-miRNAs is carried out by a Dicer homologue DCL-1. Image has been taken from Molecular Biology of the Gene, by J.D. Watson, seventh edition.

1.2.3 Small interfering RNAs and the effector complex

Small interfering RNAs (siRNAs) are generated by the action of Dicer on the long double stranded RNA precursors mentioned in subsection 1.2.1. Compared to miRNAs, siRNAs contain either no or very few mismatches in the duplex. Once produced, the siRNA and the miRNA are loaded onto the effector molecule of RNAi- Argonaute (Figure 2). This process

involves double stranded RNA binding proteins which aid in loading (Carthew and Sontheimer 2009). Argonaute is part of a larger complex called the RNA Induced Silencing Complex or RISC (Pratt and MacRae 2009). The exact molecular composition of the RISC is not formally defined. The minimal RISC, sufficient for target recognition and cleavage, was shown to be just an Argonaute protein bound to a small RNA. However, biochemical isolations of the RISC have discovered a variety of ribonucleoproteins ranging from 150KDa to 3MDa with a variety of intermediate sizes (Pratt and MacRae 2009). The Argonaute bound to the small double stranded RNA within the RISC represents the inactive form of the complex. Activation requires ATP hydrolysis and is accompanied by cleavage followed by disassociation of one of the strands-the passenger strand of the double stranded RNA molecule (Figure 2). The remaining strand, the guide strand is responsible for target recognition presumably via Watson-Crick base pairing (Hannon 2002). The mechanism by which the guide strand is distinguished from the passenger strand still remains undetermined (Wilson and Doudna 2013).

1.2.4 Fate of the targeted RNA

As mentioned above, the active RISC seeks out the target RNA through Watson Crick base pairing with the guide strand of the miRNA or the siRNA loaded in Argonaute. Upon finding the target there can be different fates possible depending upon the degree of complementarity between the guide and the target (Carthew and Sontheimer 2009). Perfect complementarity, as is often the case with siRNAs, can lead to endonucleolytic cleavage of the target by Argonaute. This generates fragments of the target RNA which can now be acted upon by cellular exonucleases to complete the degradative process (Carthew and Sontheimer 2009). In cases of imperfect complementarity between guide and target- as is found with miRNA guides, or when Argonaute lacks endonucleolytic activity, translation of the message is inhibited followed by deadenylation and degradation of the message (Figure 2) (Wilson and Doudna 2013).

1.2.5 PIWI interacting RNAs

As will be discussed in section 1.4.2, the eukaryotic Argonautes constitute a superfamily which is subdivided into three phylogenetic clades (Pratt and MacRae 2009). One of these clades, the PIWI proteins (named after Drosophila PIWI, for **P**-element-**i**nduced **wi**mpy testes), associate with small RNAs in the animal germline and bring about silencing of transposons (Iwasaki, Siomi et al. 2015). The aforementioned small RNAs, termed as PIWI-

interacting RNAs or piRNAs are 24-31nt long and have a mechanism of biogenesis different from that of miRNAs or siRNAs. Their biogenesis primarily occurs by a process known as the 'ping-pong cycle'. piRNA precursors are single stranded RNA transcripts expressed from intergenic loci called piRNA clusters. These clusters harbour a large number, and various types of transposon sequences. The single stranded precursor produced from them is processed in a Dicer-independent manner to generate piRNAs which have a strong 5' uridine bias (Iwasaki, Siomi et al. 2015). These short single stranded RNAs are then loaded onto the PIWI family proteins aubergine (AUB) and PIWI, which use them as guides to target mRNA of active transposons for cleavage in the cytoplasm (Castel and Martienssen 2013). The products of this cleavage reaction constitute 'sense' piRNAs that are loaded onto the PIWI family member AGO3. This then acts as an effector molecule to direct the cleavage of the single stranded piRNA precursor to generate more 'antisense' piRNAs, thus completing the ping-pong cycle. Though it is the predominant mechanism of biogenesis of piRNAs, a lesser understood ping-pong independent pathway of piRNA biogenesis operates in the somatic follicle cells surrounding the oocytes in the female germ line (Castel and Martienssen 2013). Some nontransposon genes have also been identified to be regulated by piRNAs (Iwasaki, Siomi et al. 2015).

1.2.6 Silencing at the level of transcription

The pathways mentioned till now bring about silencing post transcription; however, RNAi can also control transcription from genomic loci by bringing about heterochromatinization and DNA methylation (Lippman and Martienssen 2004, Xie and Yu 2015). Heterochromatin formation by RNAi has been best studied in the fission yeast *Schizosaccharomyces pombe*, where it is involved in the regulation of centromeric heterochromatin (Volpe, Kidner et al. 2002). Briefly, heterochromatin formation by RNAi in *S. pombe* involves recruitment of histone methyl transferase Clr4 which methylates histone H3 at lysine 9. This modification leads to the recruitment of downstream proteins which eventually results in chromatin compaction (Holoch and Moazed 2015). Even though, the link between RNAi and heterochromatin was first described in *S. pombe*, direct interactions between the two have now been established in animals, plants and ciliates as well, therefore making the regulation of heterochromatin by RNAi a generic phenomenon (Carthew and Sontheimer 2009).

Plants have served as the model system to study DNA methylation by RNAi. A DNA-dependent RNA polymerase produces transcripts from the genomic loci to be silenced.

siRNAs derived from these transcripts are loaded onto an Argonaute protein which is then guided to the site of transcription where it recruits DNA methyl transferases to catalyse de novo DNA methylation (Xie and Yu 2015). DNA methylation, if occurring at the promoter region of a gene can lead to transcriptional gene silencing. Unlike post transcriptional gene silencing, silencing at the transcriptional level by RNAi is stable and heritable (Hannon 2002).

1.2.7 Signal amplification

RNAi is a very effective mechanism with only a few molecules required for potent silencing. This is because the silencing trigger undergoes amplification by means of an RNA dependent RNA polymerase (RdRP) (Figure 2) (Carthew and Sontheimer 2009). The multiple double stranded RNAs thus produced can give rise to multiple siRNAs which lead to augmentation of the downstream response. Signal amplification is also the reason why silencing is systemic in nematodes and plants even though the trigger may have been localized to one part. Although involved in signal amplification, RdRPs are not an essential part of the machinery as is apparent from their loss in several species (Carthew and Sontheimer 2009).

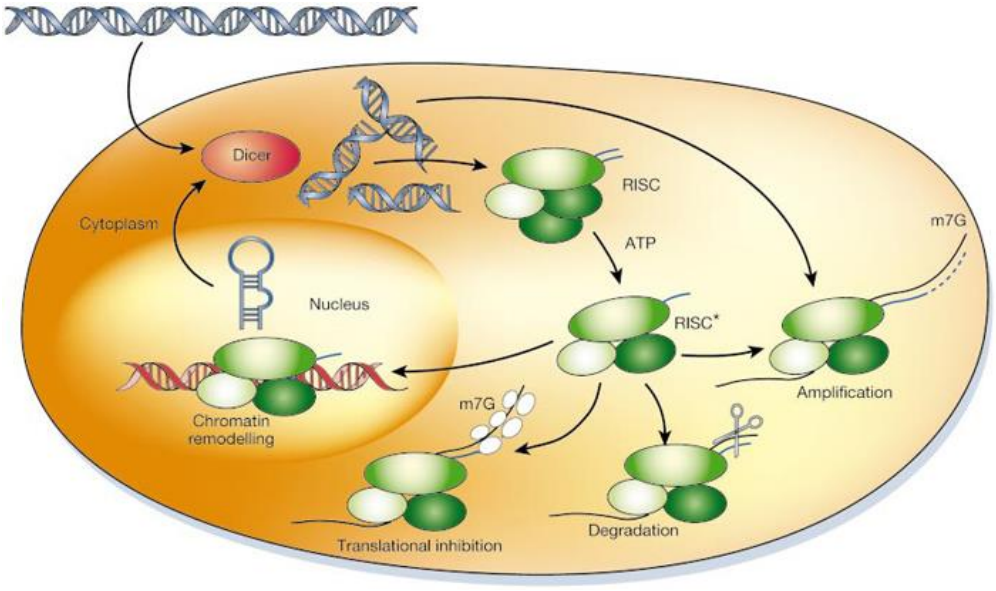


Figure 2: A model for the mechanism of RNA Interference. The double stranded RNA silencing trigger can come from a variety of sources as is described in the text. The trigger is recognized and processed by Dicer into small RNAs of a fixed length depending on the species in question. These double stranded RNAs are then loaded onto the RISC. Activation of the complex takes place by ATP hydrolysis and unwinding of the duplex. The activated complex is guided to the target where any one of the downstream effector functions is possible. Signal amplification occurs by the action of a RNA-dependent RNA polymerase and is responsible for the efficiency of the interference. (Hannon 2002).

1.3 Roles of RNA Interference

RNAi is known to perform a variety of functions in eukaryotes. RNA from the genomes of invading viruses and that transcribed from mobile genetic elements within the genome of the cell trigger RNAi resulting in their degradation (Hannon 2002). This has a protective role for the cell-defending the cell against virions and preventing infection while also guarding against mutations in its own genome by restricting the motion of transposons. RNAi also provides a way of transcriptionally and post transcriptionally regulating gene expression by its action either directly on mRNAs or at genomic loci coding for them through DNA methylation (Carthew and Sontheimer 2009). This regulation further affects the cellular processes in which these genes act. For example, RNAi controls vital cellular processes like cell growth, tissue differentiation and cell proliferation. As a result of this, RNAi dysfunction is linked to cardiovascular disease, neurological disorders and many types of cancer (Wilson and Doudna 2013). Aside from these canonical functions described above, other novel functions of the proteins of RNAi in various eukaryotic organisms have been uncovered. Dicer and Drosha, for instance, have been found to have miRNA independent functions (Burger and Gullerova 2015). These range from their involvement in DNA repair in a variety of organisms, to regulation of transcription at various levels and their role in processing of RNA species such as in pre-mRNA splicing, ribosomal RNA synthesis and processing of non-coding RNA. Some of these functions of this machinery in the fungal kingdom will be dealt with later in this thesis.

1.4 Structural aspects of Dicer and Argonaute

As described in section 1.2, the RNAi machinery includes a host of proteins which act in a coordinated manner to bring about silencing of the target locus or viral defence. Each protein has its role in silencing; however, two proteins are unambiguously recognized to be present at the heart of the process. These two proteins-Dicer and Argonaute-are required for RNAi in most eukaryotic organisms. The former protein performs the function of generation of the small RNAs while the latter protein is involved in target recognition followed by downstream effector functions, respectively. Insights into the mechanism of action of these proteins have been gleaned from studies of their domain architecture and three dimensional structures. The former with respect to its implications on protein function will be discussed in this section.

1.4.1 Domain architecture of Dicers

Dicer belongs to the Ribonuclease III family of proteins (Wilson and Doudna 2013). The RNase III family is subdivided into three classes based on their domain architectures. Class I includes bacterial RNase III enzymes which contain only one catalytic domain (called the RNase III domain) and a double stranded RNA binding domain (dsRBD), which is involved in non-sequence specific double stranded RNA binding. The dsRBD is present C-terminal to the catalytic domain. The RNase III domain can cleave only one strand of the target RNA, thus to cleave both strands, the bacterial enzymes work as homodimers. This family also includes the yeast RNT1 protein. The class II proteins contain two RNase III domains followed by a dsRBD. Drosha belongs to this class of enzymes. Here, the pair of RNase III domains constitute the active site and are present adjacent to each other in the three dimensional structure. Each domain cleaves one strand of RNA, with both the domains working together to cleave the double stranded target. Dicer comes under the umbrella of class III proteins which like class II proteins contain two RNase III domains and a C-terminal dsRBD. N-terminal to the RNase III domains are the PAZ (from **P**iwi **A**rgonaute and **Z**wille) and helicase domains (Figure 3B). The PAZ domain is responsible for recognizing the 2 nucleotide overhang at the 3' terminus which is characteristic of RNase III cleavage. This helps to position the RNA in the active site of the RNase III domains in such that the cleavage happens at regular intervals to generate small RNAs of a fixed size. The function of the Helicase domain is best understood in *Drosophila* where it is implicated in recognition of siRNAs and miRNAs. It also functions to translocate the protein on the double stranded substrate in an ATP dependent manner. The domain architecture of Dicer proteins just described varies to some extent between organisms; however, despite this its principle dicing function remains conserved (Wilson and Doudna 2013).

1.4.2 Domain architecture of Argonautes

The eukaryotic Argonautes constitute a superfamily which can be classified into three major phylogenetic clades based on amino acid sequence similarities (Pratt and MacRae 2009). The largest clade is named Argonaute after its founding member in *Arabidopsis* and contains proteins which associate with siRNAs and miRNAs. The second clade Piwi is found mostly in animal germline cells where it interacts with piRNAs and functions in the silencing of transposons. A diverse set of nematode specific Argonautes have been grouped into the third clade which is called worm Argonautes or WAGOS (Carthew and Sontheimer 2009, Pratt

and MacRae 2009). The domain architecture of Argonaute is more conserved than that of Dicers. Argonaute has four major domains- the N-terminal domain, the PAZ domain and the MID (for 'middle') and PIWI domains (Figure 3A) (Wilson and Doudna 2013). The function of the PAZ domain is the same as that in Dicers, that is, recognition of the 2 nucleotide overhang at the 3' terminus of substrate RNA. The MID domain on the other hand recognizes the 5' terminus of the small RNA and is responsible for imparting specificity in terms of the 5' nucleotide. The PIWI domain is the catalytic domain and adopts an RNase H like fold and hydrolytically cleaves the target RNA in an RNase H like manner in case of Argonautes possessing endonucleolytic activity. The protein is bilobal with the N-terminal domain and the PAZ domain are present on one lobe while the MID and PIWI domains on the other (Wilson and Doudna 2013).

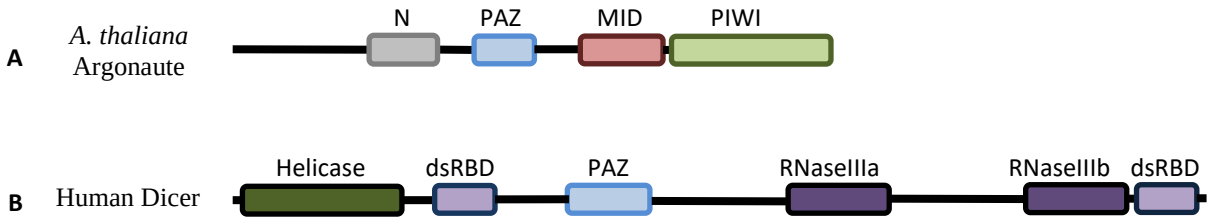


Figure 3: Domain architecture of the two central RNA interference proteins, Argonaute and Dicer. (A) Domain architecture of the *Arabidopsis thaliana* Argonaute protein. Argonaute contains four major functional domains, namely the N-terminal, PAZ, MID and PIWI domains. The protein is bilobal in three dimension with the N-terminal and PAZ domains on one lobe and the MID and PIWI on the other. (B) Domain architecture of human Dicer. Like Argonaute, Dicer too has four functional domains; the Helicase, PAZ, RNase III and dsRBD. The RNase III domain, which is the catalytic domain, is found in two copies, each required to cleave one strand of the silencing trigger. Details pertaining to the function of these domains are mentioned in the text. Adapted from Drinnenberg et al, 2009 and Nakanishi et al, 2012

1.5 RNA Interference in the fungal kingdom

The fungal kingdom is a major lineage of the Eukarya estimated to comprise nearly 5.1 million species with enormous diversity in ecology, morphology and life cycles (Blackwell 2011). Studies of RNAi in fungi have been of historical significance with the phenomenon of quelling in *Neurospora crassa* being one of the first reports of gene silencing by RNAi. Even now, studies in fungi contribute significantly to our understanding of RNAi in eukaryotes; with regulation of centromeric heterochromatin in fission yeast as the best characterized mechanism of heterochromatin formation by RNAi till date (Carthew and Sontheimer 2009).

As is the case with other eukaryotes, novel functions associated with the RNAi proteins have been discovered in the fungal kingdom as well. Despite that, canonical functions of RNAi still exist in fungi. For example, RNAi has been shown to be involved in defence against mycoviruses in *Cryphonectria parasitica* and *Aspergillus nidulans* (Nicolas, Torres-Martinez et al. 2013). It also protects the genome against transposons, as has been demonstrated in *Neurospora crassa*, *Cryptococcus neoformans*, *Mucor circinelloides* and *Magnaporthe oryzae.* Further, micro-RNA like RNAs (milRNAs) and exonic-siRNAs (ex-siRNAs) have been found in *Neurospora crassa* and *Mucor circinelloides*, respectively and were shown to regulate expression of target genes. Small RNAs have also been reported from *Magnaporthe oryzae* and a host of other fungi (Villalobos-Escobedo, Herrera-Estrella et al. 2016). Additionally, transcriptional silencing by RNAi has also been observed in *S. pombe* (Volpe, Kidner et al. 2002), a function which will be discussed in more detail later.

Some of the novel functions of RNAi in fungi have been well characterized whilst new ones are being discovered (Villalobos-Escobedo, Herrera-Estrella et al. 2016). A RNA silencing-related phenomenon called Meiotic Silencing by Unpaired DNA (MSUD) was uncovered in *N. crassa* during studies of the Asm-1 gene (Shiu, Raju et al. 2001). *N. crassa* is haploid throughout its life cycle except for a transient diploid stage formed by fusion of two haploid nuclei of opposite mating type. The zygote thus formed undergoes meiosis followed by post-meiotic mitosis to form eight haploid ascospores. During meiosis sequences may be present on one parental chromosome but not on its homologue leading to the presence of unpaired DNA. This unpaired DNA is sensed which triggers the production of RNA from this DNA. This single stranded RNA is converted into double stranded RNA which brings about small RNA mediated silencing of the locus. MSUD is also observed in the fungus *Giberella zeae* (Shiu, Raju et al. 2001, Dang, Yang et al. 2011, Chang, Zhang et al. 2012).

Apart from MSUD, the RNAi machinery of *N. crassa* is also involved in transgene suppression (Romano and Macino 1992) and DNA damage repair (Lee, Chang et al. 2009). Transgene suppression or 'Quelling' in *N. crassa* is similar to the phenomenon of co-suppression observed in plants. It was discovered when the fungus was transformed with constructs of the *al-1* and *al-3* genes coding for enzymes involved in carotenoid biosynthesis. Carotenoid pigments are responsible for the orange colour of *N. crassa*. The transformants obtained were white or pale orange going to show that introduction of the transgene had silenced expression from both the transgene and the endogenous copy (Romano and Macino 1992). This phenomenon is now known to be controlled by RNAi and the underlying

molecular mechanism has been delineated (Chang, Zhang et al. 2012). Exposure of *N. crassa* cultures to DNA damaging agents leads to the production of small RNAs called qiRNAs. These RNAs map to the ribosomal DNA locus and require the RNAi machinery for their production (Lee, Chang et al. 2009). DNA damage results in cell cycle arrest, a reduction in DNA replication and in protein synthesis. In mutants defective for qiRNA synthesis, DNA damage induced reduction in protein synthesis is abated. Further, mutants for RNAi proteins show increased sensitivity to DNA damaging agents. These results suggest that qiRNAs contribute to DNA damage repair by inhibiting protein synthesis (Lee, Chang et al. 2009, Chang, Zhang et al. 2012).

Cross kingdom RNAi has also been reported in fungi. The phytopathogenic fungus *Botrytis cinerea* uses small RNAs as effector molecules to infect plants (Weiberg, Wang et al. 2013). The *B. cinerea* small RNAs enter the host (*Arabidopsis*) and are loaded onto the host Argonaute protein where they bring about silencing of the host genes responsible for immunity; thus making it easier for *B. cinerea* to infect the host (Weiberg, Wang et al. 2013).

Transgene silencing has also been reported in the human fungal pathogen *C. neoformans* (Wang, Hsueh et al. 2010). A transgene array tandemly integrated in the genome of *C. neoformans* was found to be post transcriptionally silenced during sexual reproduction, a phenomenon termed as sex-induced silencing (SIS). Moreover, small RNAs mapping to the transgene array have been observed in silenced isolates. In RNAi mutant strains, sex-induced silencing and production of these small RNAs is abolished establishing a direct connection between RNAi and the phenomenon (Wang, Hsueh et al. 2010).

1.6 The cryptic RNAi machinery of the budding yeasts

The diversity in RNAi in the fungal kingdom is not restricted to the functions performed by the machinery alone; there is variability in the domain architectures of the proteins involved as well. This is exemplified by the Dicers of the budding yeasts (Drinnenberg, Weinberg et al. 2009). Budding yeasts belong to the subphylum Saccharomycotina within the phylum Ascomycota in the fungal kingdom. Their name stems from their mode of asexual reproduction which happens via budding and from the growth form commonly adopted by them (Alexopoulos 2007). The most notable example from this group is *Saccharomyces cerevisiae*, the common baker's yeast. A model organism for the study of eukaryotic genetics, this yeast lacks all proteins of the RNAi machinery (Drinnenberg, Weinberg et al. 2009). However, closely related budding yeasts have retained the machinery albeit in a modified and

much pared form. *Saccharomyces castellii* (now *Naumovozyma castellii*), *Kluveromyces polysporus* and the human fungal pathogen *Candida albicans* all have an Argonaute homologue, a non-canonical Dicer protein and lack an RNA dependent RNA polymerase (Figure 4) (Drinnenberg, Weinberg et al. 2009). The domain architecture of the Dicer protein is very different from canonical Dicers, as a result of which it went undetected for a very long time; earning the machinery the title 'cryptic'.

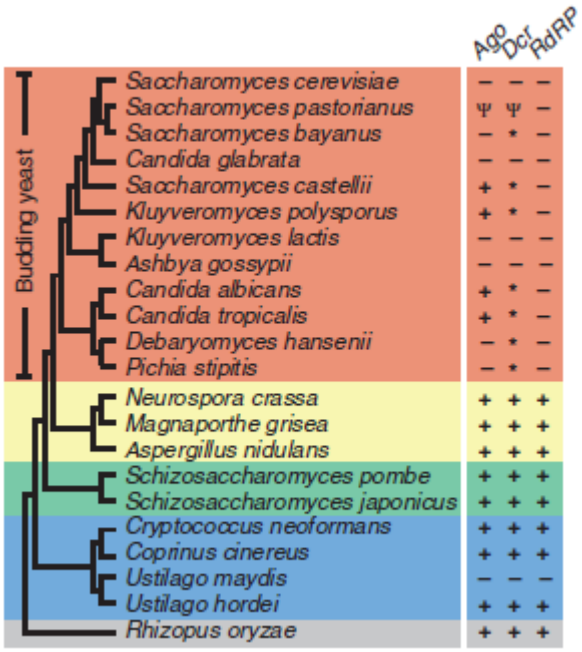


Figure 4: Cladogram showing distribution of RNAi components amongst members of selected sub-phyla of fungi. Basidiomycota (blue), Zygomycota (gray) and Ascomycota, subdivided into Saccharomycotina (orange), Pezizomycotina (yellow), and Taphrinomycotina (green) are shown. The budding yeasts come under the sub-phylum Saccharomycotina. Presence or absence of the RNAi proteins Argonaute (Ago), Dicer (Dcr) and RdRP is indicated on the right. Ψ indicates pseudogenes. *S. castellii*, *K. polysporus*, *C. albicans* and *C. tropicalis* have both Ago and Dcr but lack and RdRP. Some species possess only Dcr but no Ago and others lack all three. (Drinnenberg, Weinberg et al. 2009).

The Argonaute proteins of the budding yeasts resemble those of higher eukaryotes in domain architecture. They contain an N-terminal domain, a PAZ domain and MID and PIWI domains (Figure 5A) (Nakanishi, Weinberg et al. 2012). The Dicers of the budding yeast, however, are a much trimmed version of their higher eukaryotic counterparts. They contain a single RNase III domain, two dsRBDs C-terminal to the catalytic domain and have no helicase or PAZ domains (Figure 5B). Furthermore, sequence-wise the RNase III domain resembles yeast RNT1 RNase III domain more than canonical Dicer RNase III domain (Drinnenberg, Weinberg et al. 2009). Concomitant with a change in domain architecture, the mechanism of action of the yeast Dicer has also changed (Weinberg, Nakanishi et al. 2011). Canonical Dicers bind to one end of their double stranded RNA substrate by recognizing the 2 nucleotide overhang at the 3' terminus using their PAZ domain. This also positions the active site in a manner to dice out small RNAs of a fixed length. Budding yeast Dicers on the other hand have a single RNase III domain and work as homodimers like bacterial RNase III

enzymes to cleave both strands of the target RNA. They bind in the middle of the target RNA and work their way outwards in what has been deemed as the 'inside out mechanism' of budding yeast Dicers. The size of the small RNA produced is determined by the distance between the active sites within the RNase III domains (Weinberg, Nakanishi et al. 2011).

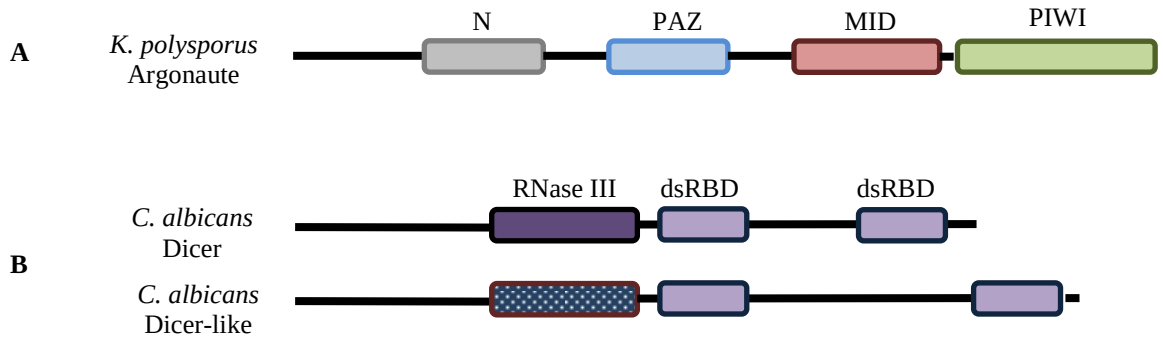


Figure 5: Domain architecture of the RNA interference machinery of the budding yeasts. The budding yeasts possess cryptic RNAi machinery having an Argonaute protein and a non-canonical Dicer protein. (A) The Argonaute of the budding yeasts, represented here by the *K. polysporus* protein has a domain architecture similar to that of higher eukaryotes and probably functions by the same mechanism. (B) The non-canonical Dicer of budding yeasts. The Dicer of budding yeasts is a much pared version of its higher eukaryotic counterparts possessing only an RNAse III domain and two dsRBDs. *C. albicans* has a second Dicer like protein in which residues important for catalytic activity of the RNase III domain have been mutated to residues likely to inactivate RNase activity. Adapted from (Bernstein, Vyas et al. 2012, Nakanishi, Weinberg et al. 2012).

The RNAi machinery has been explored to some extent in all three budding yeasts. Small RNA sequencing has found RNAs 22-23 nucleotides in length bearing chemical features of Dicer cleavage in these species. In *S. castellii*, Dicer and Argonaute have been shown to be capable of silencing a GFP reporter gene. Additionally, introduction of these two *S. castellii* proteins into *S. cerevisiae* has led to the reconstitution a functional RNAi pathway in the baker's yeast (Drinnenberg, Weinberg et al. 2009). Studies of *K. polysporus* Dicer and Argonaute have shed light on structural aspects of these proteins and have been instrumental in deciphering the mechanism of action of the budding yeast (Weinberg, Nakanishi et al. 2011, Nakanishi, Weinberg et al. 2012). In both these species, small RNAs have been observed to map to transposons and subtelomeric repeats, suggesting a role of this machinery in genome defence (Drinnenberg, Weinberg et al. 2009).

Literature on the function of these proteins in *C. albicans* is not as straightforward. *C. albicans* has slightly different machinery-with a Dicer-like protein present in addition to the

Argonaute and non-canonical Dicer proteins (Figure 5B). This Dicer like protein has domain architecture identical as that of the non-canonical Dicer except for the fact that residues important for catalytic activity have been mutated in this protein (Bernstein, Vyas et al. 2012). There have been two studies which have been carried out to assess the function of this machinery in gene silencing in *C. albicans*. The first study demonstrated that a double stranded RNA hairpin trigger could not silence the expression of the target *ADE2* gene despite processing of the hairpin to form small RNAs. Possibility of Argonaute being a pseudogene was also disproved (Staab, White et al. 2011). Later on, however, a second report by another group showed that the *EFG1* gene in *C. albicans* could be silenced by transfecting cells with synthetically manufactured siRNAs (Moazeni, Khoramizadeh et al. 2012). These two studies present contrasting results pertaining to the capability of the *C. albicans* RNAi machinery to silence genes, an issue which still requires to be resolved.

1.7 *Candida albicans*: An opportunistic human fungal pathogen

Taxonomic position:

Domain: Eukarya

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Saccharomycotina

Class: Saccharomycetes

Order: Saccharomycetales

Family: Debaryomycetaceae

Genus: *Candida*

Species: *C. albicans*

Candida albicans, the organism chosen for this study, is the most commonly isolated yeast pathogen from humans. First described in 1839, *C. albicans* is a diploid unicellular yeast commonly found as a commensal in humans and other warm blooded animals (Noble and Johnson 2007). It thrives in diverse niches in its host ranging from skin, gut, oral and vaginal mucosa without causing disease (da Silva Dantas, Lee et al. 2016). The yeast can however proliferate even in healthy people to cause restricted infections of the skin, nails, and mucous membranes. In patients with a compromised immune system, the fungus seizes the opportunity and can cause aggressive infection; attacking virtually any organ and resulting in

the death of as many as 50% of the patients with bloodstream infection (Noble and Johnson 2007).

C. albicans is a polymorphic fungus which can grow as ovoid shaped yeast cells (Figure 6A), as a pseudohypha of elongated and conjoined yeast cells (Figure 6B) or as true hypha with no constrictions at septal junctions (Figure 6C) (Kim and Sudbery 2011, Mayer, Wilson et al. 2013, da Silva Dantas, Lee et al. 2016). When it comes to reproduction, the yeast largely propagates asexually through budding. Though sexual reproduction is not commonly observed in this organism, it has a Mating Type Like locus or MTL with two alleles **a** and **α**. Strains homozygous at this locus-engineered or naturally- switch from the normally observed 'white' form of *C. albicans* to the mating competent 'opaque' form and can undergo mating at a high frequency to form tetraploid cells. These tetraploid cells can undergo concerted chromosome loss to return to a diploid state, thus completing a parasexual cycle (Noble and Johnson 2007).

As a commensal, the pathogen is present in the yeast form. When an infection happens, the cells first proliferate, growing in numbers and spreading in the host (Mayer, Wilson et al. 2013). These yeast cells express cell surface molecules called adhesins which then help mediate the adhesion of the *C. albicans* cells to the host cells. This contact of the pathogen to the host cells triggers yeast to hyphal transition and directed growth of the hypha via thigmotropism. The fungal pathogen now invades the host cell either through induced endocytosis or through active penetration (Mayer, Wilson et al. 2013).

An important virulence factor in pathogenesis by *C. albicans* is the formation of biofilms Costa et al. 2015). These are structures composed of a group of microorganisms belonging to the same or different species adhered to a substratum and encased within a self-produced extracellular matrix. *C. albicans* forms a heterogeneous biofilm composed of the yeast, hyphal and pseudohyphal forms of the pathogen (Figure 6D). The biofilm can be formed either on biotic or abiotic surfaces such as medical devices such as catheters, pacemakers, dentures and prosthetic joints. Formation of a biofilm confers resistance to the pathogen against antifungal drugs and the host immune system and helps disseminate yeast cells to further the infection (Costa et al. 2015, Nobile and Johnson 2015).

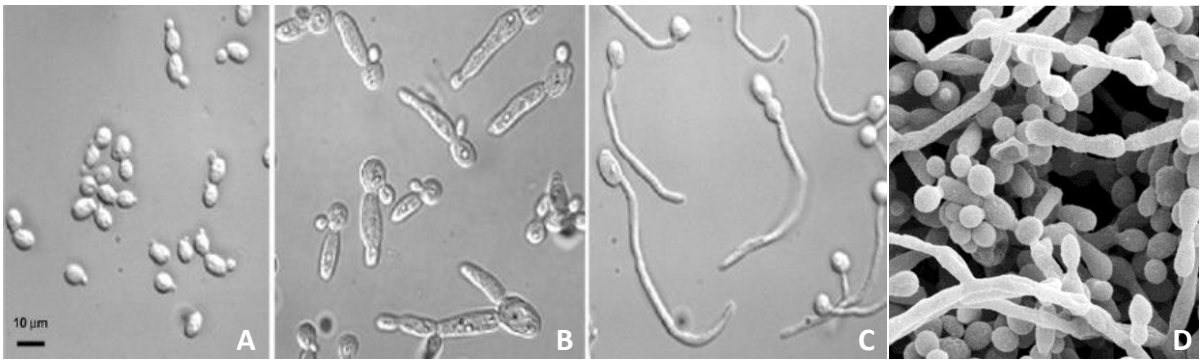


Figure 6: Different morphological states of the polymorphic fungus *C. albicans*. (A) Ovoid shaped cells which constitute the yeast form of the pathogen. This is the most commonly observed form in the laboratory. (B) Psuedohyphal cells, elongated cells joined to each other can be observed. This is considered a form intermediate between yeast and true hyphae. (C) The cells are present as true hyphae, this form is of importance in the virulence of the pathogen. (D) Scanning electron micrograph of a *C. albicans* biofilm. All three morphological states can be observed in the structure. (http://www.usask.ca/biology/kaminskyj/images) (Ramage, Saville et al. 2005)

The haploid genome size of *C. albicans* is around 16 Mb which is distributed amongst eight chromosomes (1-7 and R). The genome has around 12000 ORFs out of which only about 3000 have been verified till date. Furthermore, there exists a translational bias in the genetic code wherein the CTG codon codes for serine rather than leucine leading to the alternative name for the *Candida* clade-the CTG clade (Candida Genome Database). The genome of *C. albicans* displays a high degree of plasticity, which includes genomic changes such as gross chromosomal rearrangements, aneuploidy and loss of heterozygosity (Selmecki, Forche et al. 2010). Such variation in genome organisation helps the fungus to survive in stressful environments, such as, but not limited to, antifungal drug exposure.

There are three wild type isolates of *C. albicans* which are known, namely, SC5314, WO-1 and P37005. These strains are sensitive to the antibiotics nourseothricin and hygromycin B. For the ease of genetic manipulation in the laboratory, derivative strains of these wild type isolates have been constructed by deleting one or more auxotrophic markers (Candida Genome Database). This study has utilized one such derivative strain from SC5314, SN148.

1.8 Rationale and objectives of the study

The phenomenon of RNAi was presumed to be absent from the budding yeasts until the recent discovery of endogenous small RNAs and a non-canonical Dicer protein in them (Drinnenberg, Weinberg et al. 2009). Though attempts have been made in *C. albicans* to assess the role of the RNAi proteins in gene silencing, the results of these studies are inconclusive (Staab, White et al. 2011, Moazeni, Khoramizadeh et al. 2012). The fungi are a

varied group showing diversity in the presence of the RNAi machinery, the architecture of its proteins and the functions performed by them. Given this, it is conceivable that the RNAi machinery of *C. albicans* may be performing additional roles apart from gene silencing. The aim of this study was to assess the capability of the *C. albicans* RNAi machinery to silence genes and to explore other possible functions of this cryptic machinery in this human fungal pathogen. In particular, it was desired to explore a possible role for this machinery in regulating position-effect vareigation at *C. albicans* centromeres drawing parallels from closely related ascomycete fission yeast *S. pombe*.

Centromeres are DNA sequences onto which a protein complex called kinetochore assembles which interacts with microtubules to enable chromosome segregation. They are characterized by the presence of a modified histone variant CENP-A (Roy and Sanyal 2011). *S. pombe* has three centromeres, each one corresponding to a chromosome in the nucleus. The sizes of the centromeres are variable ranging from 35-110 kb. Structurally, each centromere consists of a central core flanked by two kinds of repeated DNA, the innermost (*imr*) and the outermost (*otr*) repeats. The *otr* region is further composed of two types of repeats-the *dh* and *dg* repeats (Figure 7A) (Verdel and Moazed 2005). *Ura4*$^{+}$ transgenes inserted in any of the aforesaid mentioned regions are subject to position-effect variegation (Allshire, Javerzat et al. 1994, Volpe, Kidner et al. 2002). Such strains can grow in both-a medium selecting for expression of *ura4*$^{+}$ (-URA) and in a medium which selects for cells not expressing the *ura4*$^{+}$ gene (5-FOA, for details of positive and negative selection for URA please see chapter 4 section 2.6) indicating silencing of gene expression at centromere in these cells (Figure 7C) which in turn is indicative of heterochromatin (Allshire, Javerzat et al. 1994). The RNAi machinery in *S. pombe* consists of three proteins; an Argonaute, a Dicer and a RdRP. Silencing of the genes located in the *otr* regions is alleviated in RNAi mutants (Figure 7C) implicating the proteins in regulation of the heterochromatin (and thus position-effect variegation) in this region (Volpe, Kidner et al. 2002). The mechanistic details of this regulation have now been worked out (Holoch and Moazed 2015). In a nutshell, transcription by RNA polymerase II produces single stranded RNA from the repeat region which is then acted upon by the RdRP and converted into double stranded RNA. This RNA forms the substrate for Dicer which cleaves the double stranded RNA to produce siRNAs. These siRNAs are loaded onto Argonaute which is present in a complex called the RNA Induced Transcriptional Silencing (RITS) complex. The RITS is then guided to the site of transcription where it recruits Clr4, a histone methyl transferase which methylates histone H3 at lysine 9. This modification is recognized

by the chromodomain containing protein Swi6 which in turn recruits a multienzyme effector complex which mediates heterochromatin formation (Holoch and Moazed 2015).

As described earlier, the genome of *C. albicans* is distributed amongst eight chromosomes each with its own centromere. Out of the eight centromeres one has a structure similar to that of *S. pombe*, consisting of a central CENP-A binding region which is flanked on either side by inverted repeats. The other seven, however, are different in structure having a central CENP-A binding region flanked on either side by ORF free regions instead of repeats (Figure 7B) (Sanyal, Baum et al. 2004). The centromeres though present on different chromosomes are clustered together in space (Sanyal and Carbon 2002). A *URA3* transgene when placed in the CENP-A binding region of *C. albicans* centromeres is subject to position-effect variegation as is observed in *S. pombe* (Figure 7D) (Thakur and Sanyal 2013). From these observations, we hypothesized that the cryptic RNAi machinery of *C. albicans* may be playing a role in regulating position-effect variegation at the centromeres and set out to test this hypothesis.

Studies to determine the role of the RNAi machinery in gene silencing in *C. albicans* are still ongoing and this thesis will describe results pertaining to the second objective instead.

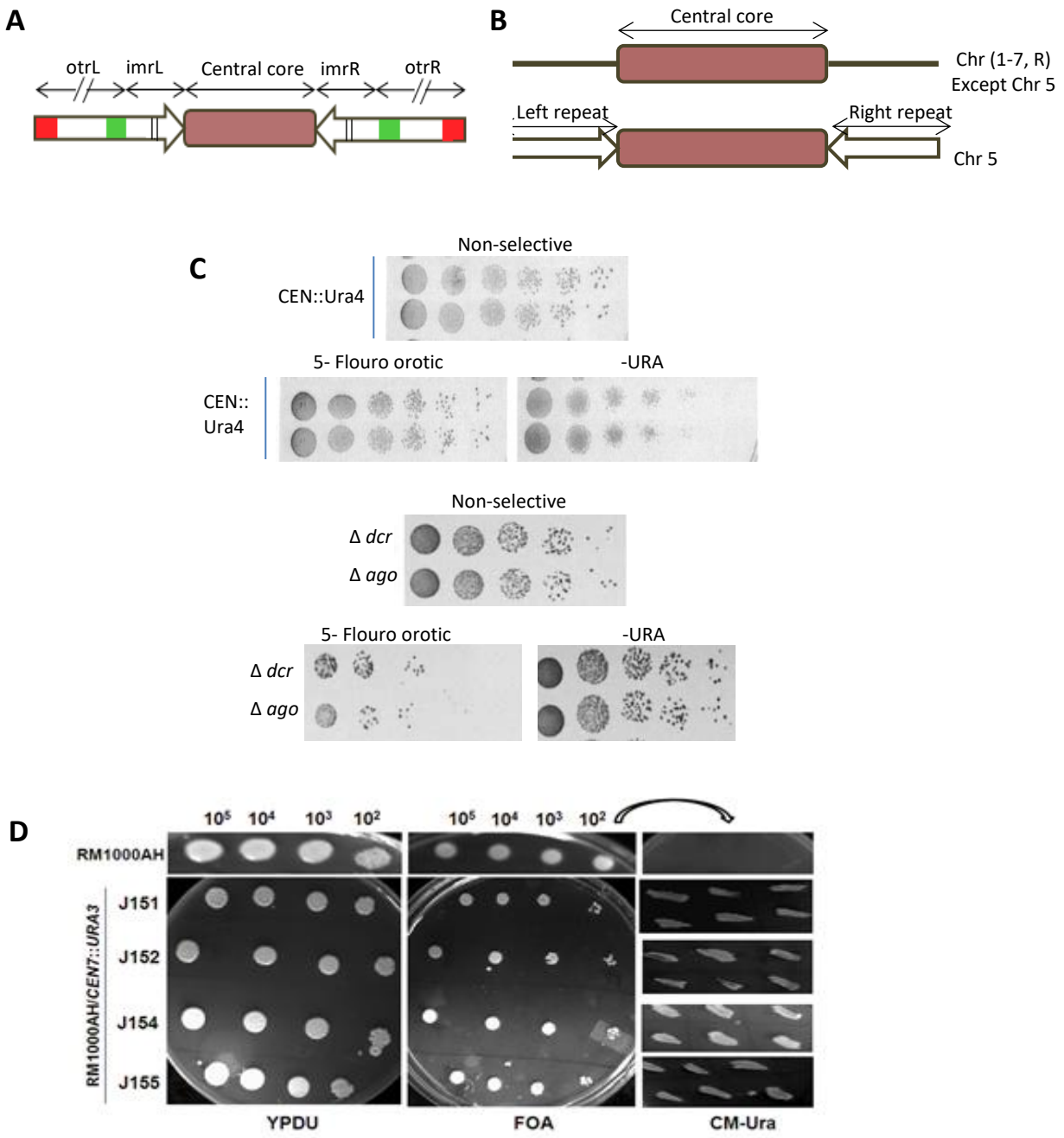


Figure 7: Position-effect variegation at the *S. pombe* and *C. albicans* centromeres. (A) Structure of the *S. pombe* centromeres. The centromeres are composed of a central core region flanked by two kinds of repeated sequences, the innermost (*imr*) and the outermost (*otr*) repeats. The former are further subdivided into the dg and dh repeats. (B) The centromeres of *C. albicans* fall into two categories; one with a structure similar to that of *S. pombe* and another where the centromere consists of a central core flanked by ORF free regions. (C) An *ura4*$^{+}$ gene inserted in any of the *S. pombe* centromeres results in position-effect variegation, a phenotype which is abolished in the absence of RNAi components. (D) An *URA3* gene inserted in the central core of *C. albicans* centromeres also results in position-effect variegation. This silencing is reversible as colonies obtained on medium selecting for silencing of *URA3* can also grow on medium selecting for cells expressing *URA3*. (Allshire, Javerzat et al. 1994, Volpe, Schramke et al. 2003, Thakur and Sanyal 2013).

CHAPTER 2:

RESULTS

CHAPTER 2: RESULTS

To probe the functions of Dicer and Argonaute in *Candida albicans* the approach adopted was to study the consequences of their loss of function. The chapter on results obtained is divided into two parts: Section A which deals with the studies performed on Dicer and Section B that deals with studies on Argonaute.

SECTION A

2.1. *Dicer* conditional mutant shows retarded growth under non-permissive conditions

2.1.1 The *Dicer* gene in *Candida albicans*

The genome of *C. albicans* has two RNase III-containing proteins; a non-canonical Dicer (*DCR1*, Orf 19.3796) and a paralogue called Candida Dicer-like (*CDL*, Orf 19.3773) in which key RNase III active site residues have been mutated to residues likely to inactivate RNase activity (Bernstein, Vyas et al. 2012). The gene of interest to this study, *DCR1*, is 1836 bp long (CaCh4 coordinates 1,037,422-1,039,283), coding for a 68.8 kDa protein containing 611 amino acids. It has three domains, the RNase III and two dsRBDs as is depicted in Figure 5 (B). As mentioned before, it lacks the helicase and PAZ domains found in the Dicers of higher eukaryotes with its RNase III amino acid sequence being more similar to RNT1 RNase III sequence than to any previously identified Dicer RNase III domain (Drinnenberg, Weinberg et al. 2009).

2.1.2 Construction of a conditional mutant of *DCR1*

In order to study loss of function of Dicer, a deletion cassette was constructed (pAB1) in which upstream (US) and downstream (DS) sequences flanking the *DCR1* ORF were cloned into the plasmid pSFS2a (Reuß, Vik et al. 2004). The *Kpn*I/*Sac*I fragment of this plasmid was transformed into the wild type strain SN148 to obtain the strain CaAB001 in which one copy of the gene was replaced by the *CaSAT1* marker. The marker was then recycled by growing the strains in YPMU as mentioned in Materials and Methods to obtain strain CaAB002.

To make a homozygous deletion mutant of *DCR1*, strain CaAB002 was transformed with the *Kpn*I/*Sac*I fragment of pAB1; however, despite multiple attempts a null mutant could not be obtained. Previously as well, another group had attempted to construct a null mutant of *DCR1* in *C. albicans* but could not succeed unless a third copy was integrated at an ectopic locus (Bernstein, Vyas et al. 2012). This raised the possibility that the *DCR1* gene may be essential

for viability in *C. albicans.* To test this hypothesis, the remaining copy of the gene in strain CaAB002 was placed under control of the promoter of the *MET3* gene (Figure 8A). *MET3* codes for an enzyme involved in sulphur metabolism and expression from its promoter can be regulated by the amino acids methionine and cysteine (Care, Trevethick et al. 1999). In addition, the *MET3* promoter driven Dcr1 was expressed as a V5-tagged fusion protein. The V5 tag is derived from a small epitope (Pk) found on the P and V proteins of the paramyxovirus of simian virus 5 (SV5) and is commonly used to tag proteins in yeast genetics (For details of strain construction refer Materials and Methods section 1.2). The strains were confirmed by PCR amplification and Southern hybridization (Figure 8B) (see Materials and Methods tables M1 and M2). The protein levels upon promoter shutdown by addition of methionine and cysteine to the medium were determined by western blotting (Figure 8C). Though a reduction in the amount of protein was observed with time, this decrease was gradual and a considerable amount of protein was detected even after twelve hours of growth in non-permissive medium. Further, the observed size of the fusion protein was between 75 and 100kDA even though the actual size is around 70 kDa.

2.1.3 Growth kinetics of *DCR1* conditional mutant

A considerable amount of protein was detected at the end of twelve hours of growth in non-permissive medium. Consequently, the mutant was grown in non-permissive medium longer to completely deplete the protein. The experimental design followed is depicted in Figure 9A and described in detail in Materials and Methods section 5.1. No protein was detected through western blotting after 24 h of growth in non-permissive medium (Figure 9B) and retardation in growth of the mutant was observed after 36 h of growth in non-permissive medium. To quantify this retardation, a growth curve of the conditional mutant with relevant controls was constructed after having grown the mutant in non-permissive medium for 48 h (Figure 9C). As is evident from the graph, the mutant grown in non-permissive medium showed stunted growth compared to the mutant grown in permissive medium and the wild type and heterozygous deletion mutant grown in non-permissive medium. This trend was observed in all four transformants used though the degree of reduction varied between transformants.

Forty eight hours post promoter shut down is taken as the state in which the conditional mutant mimics the null mutant and all liquid culture assays described hereon have been carried out at that time point.

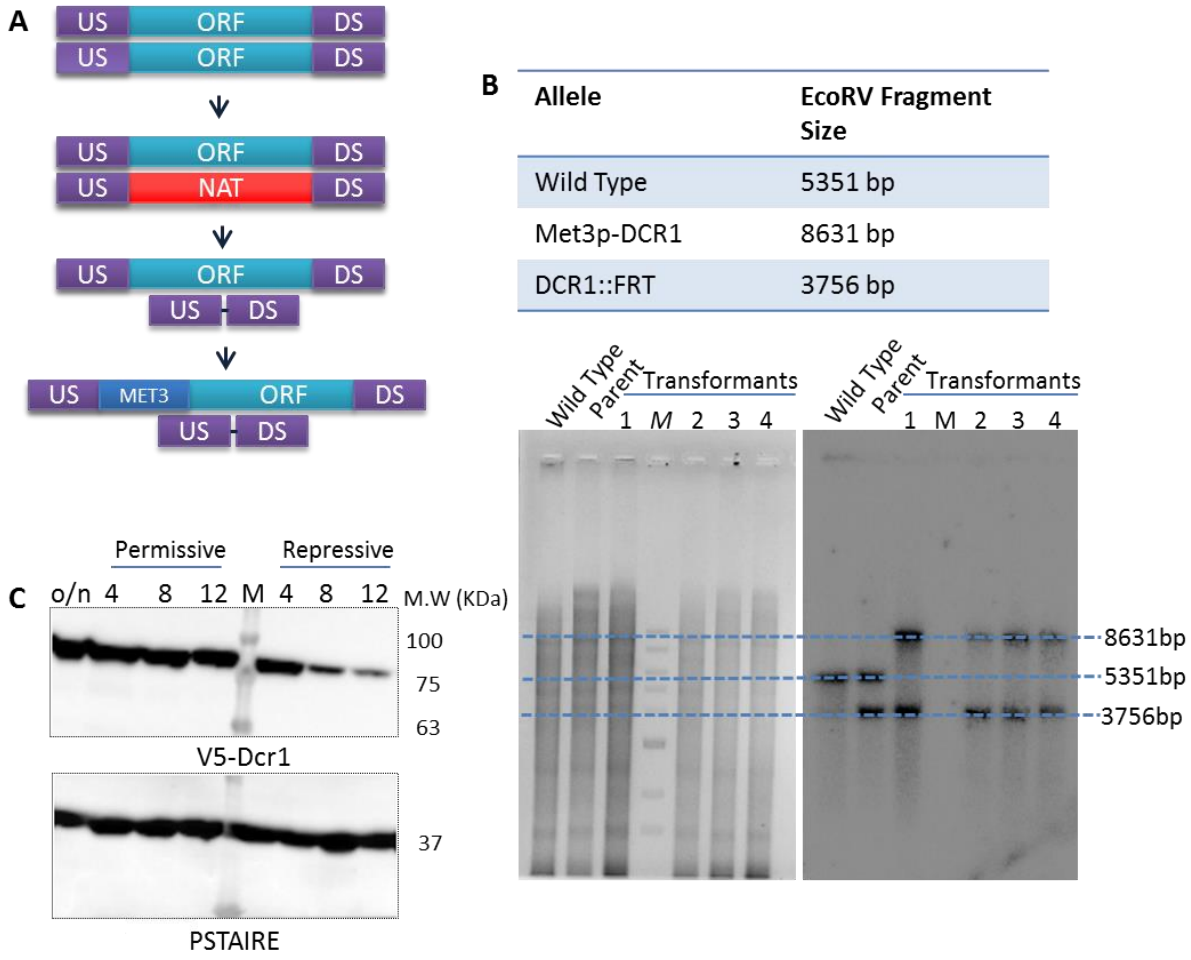


Figure 8: Construction of the conditional mutant of *DCR1*. (A) Flow chart depicting construction of the *DCR1* conditional mutant. *C. albicans* is a diploid organism with each gene present in two copies. The first copy of *DCR1* was deleted by replacement with the *CaSAT1* marker (*NAT*) through homologous recombination using pAB1. The marker was then recycled and the remaining copy was put under the control of the *MET3* promoter. (B) Confirmation of the conditional mutant strains using Southern blotting. The expected band sizes corresponding to each allele are tabulated. A total of four transformants were confirmed by Southern blotting. The transformant lanes have the bands corresponding to the first copy deletion and *MET3* allele but not the wild type allele. Loading order is marked above the lanes, M: NEB 1kb ladder. (C) Western blotting to show reduction in protein levels upon promoter shutdown. Overnight cultures of the conditional mutant were re-inoculated into permissive and non-permissive medium the next day and cells were collected for western blotting every four hours. Dcr1 was detected using anti-V5 antibodies. PSTAIRE served as the loading control. o/n: overnight culture, loading order and protein marker sizes are marked.

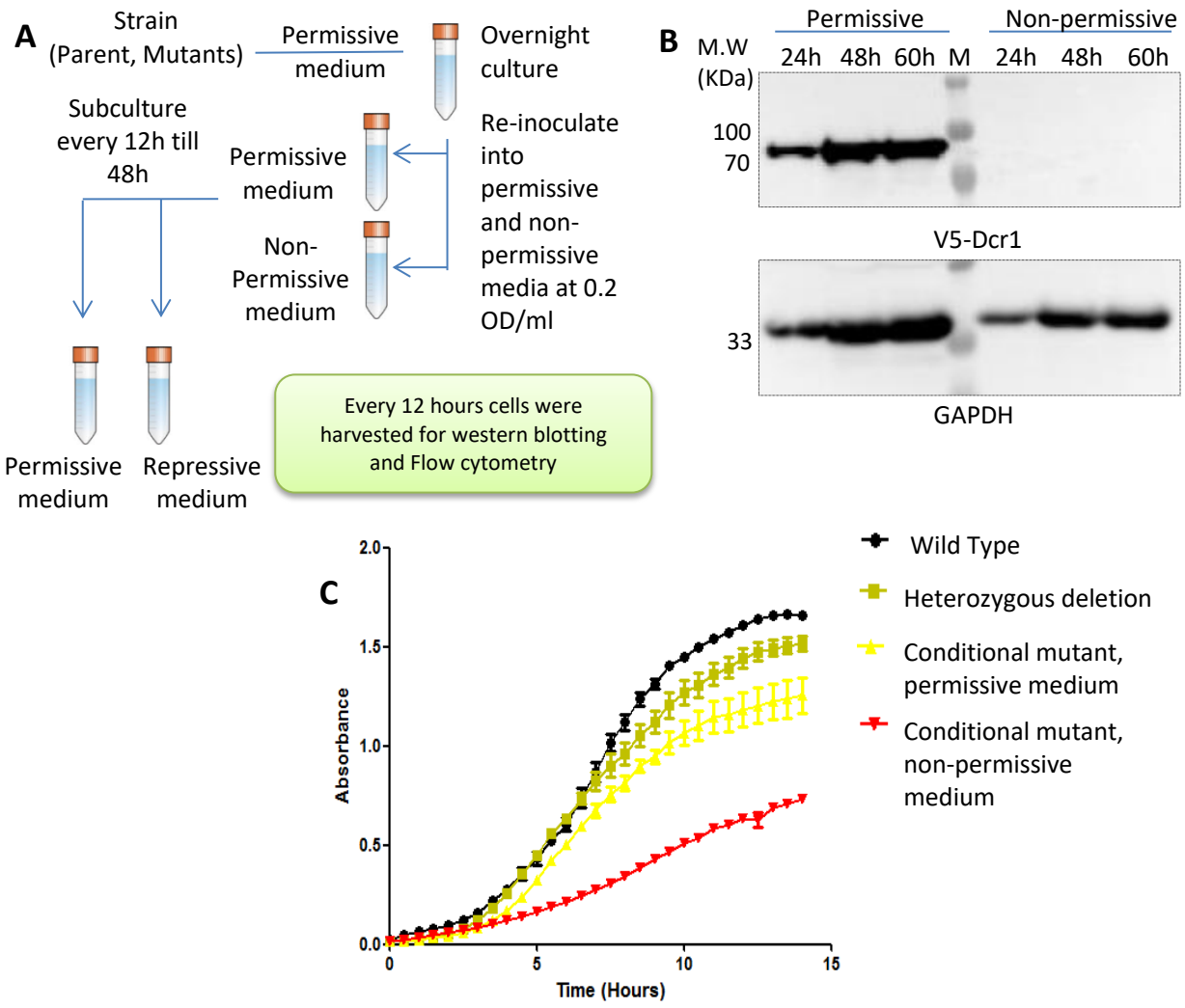


Figure 9: The growth of *DCR1* conditional mutants is retarded under non-permissive conditions. (A) Overnight cultures of wild type SN148, heterozygous *DCR1* deletion mutants and conditional mutants in complete medium were taken and secondary cultures of each strain in permissive and non-permissive media having a starting $O.D_{600}$ of 0.2 were established. These were allowed to grow for 12 h before sub-culturing cells from permissive to permissive and non-permissive to non-permissive media respectively, again at a starting $O.D_{600}$ of 0.2. This was done till 48 h. At every sub-culture, cells were harvested for analysis of protein levels by western blotting and cell cycle by flow cytometry. (B) Western blot analysis of protein levels during growth of conditional mutant in permissive and non-permissive medium. Dcr1 can be detected in lysates of conditional mutants grown in permissive medium and is observed to remain more or less constant over time; whereas, it is undetectable from 24 hours onwards in lysates of conditional mutants grown in non-permissive medium. Loading order and molecular weights are marked on the blot. Dcr1 was detected using anti-V5 antibodies. GAPDH served as loading control. (C) Growth kinetics of conditional mutant in permissive and non-permissive medium compared to wild type and heterozygous controls. Conditional mutants were propagated in permissive and non-permissive media for 48 h along with wild type and heterozygous deletion controls propagated in non-permissive medium for the same time. These cultures were then re-inoculated into corresponding media and a growth curve plotted. Thus, the curve represents growth kinetics when Dcr1 has been completely depleted in the conditional mutant. This curve was plotted using a single transformant of the conditional mutant. There is a 35% increase in the time taken to complete the cell cycle in the case of conditional mutant grown in non-permissive conditions compared to those grown in permissive conditions. The other three transformants analysed also show a reduction in growth though the degree of reduction is variable.

2.2. The defects in growth are temperature dependent

The growth of the *DCR1* conditional mutant was assayed at two different temperatures, 30°C and 37°C. The former is the optimum growth temperature for *C. albicans* and is commonly used to culture the yeast in laboratory conditions while the latter is the body temperature of its human host. The *DCR1/dcr1* heterozygous deletion and conditional mutant (*MET3p-DCR1/dcr1*) strains were streaked on permissive and non-permissive media plates and one set was incubated at 30°C while the other at 37°C. To ensure depletion of the protein, the strains were re-streaked from permissive to permissive and non-permissive to non-permissive media plates and incubated at 30°C and 37°C as before for 24-36 h after which the plates were photographed (Figure 10). It was observed that, as compared to mutant strains grown at 30°C, the ones grown at 37°C showed a greater retardation in growth as was apparent from the much smaller colony sizes of the mutant compared to the heterozygous deletion control at 37°C than at 30°C. Thus, the *DCR1* conditional mutant is temperature sensitive with a greater retardation of growth observed at 37°C compared to 30°C.

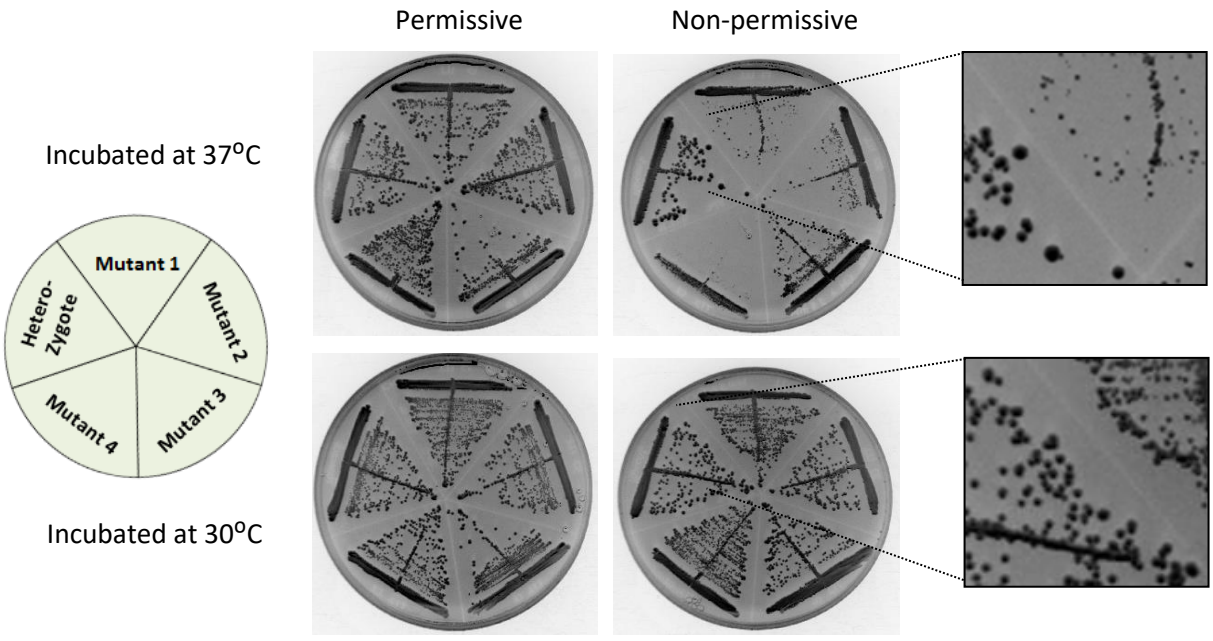


Figure 10: The growth retardation phenotype of *DCR1* conditional mutants is temperature sensitive. The colony sizes obtained after streaking strains on plates are a measure of their growth rates, with a small colony diameter indicating a slower growth rate. As can be observed from the insets, colony sizes of *DCR1* conditional mutants are much smaller than the heterozygous deletion control when strains are incubated at 37°C as compared to when they are grown at 30°C. Schematic of the identity of each strain on the plate is shown on the left.

2.3. Slower growth of the *DCR1* mutant is due to an increase in cell cycle length and not due to large scale cell death

The slow growth phenotype of the *DCR1* mutant could be the result of a cell death, an increase in cell cycle length or both. To test for these possibilities we looked at cell viability and ploidy as assessed through flow cytometry after Dcr1 depletion. The results pertaining to these experiments are described in this section.

2.3.1 Dcr1 depletion does not lead to any significant loss in cell viability

The viability of cells after depletion of Dcr1 was assayed by spotting serial dilutions of the wild type, heterozygous deletion mutant and conditional mutant cultures after growth in non-permissive medium for 48 h on permissive medium plates and incubating one set at 30°C and another at 37°C for 36 h. Dead cells present in the culture would not divide and form colonies on the plate. If there was a population of dead cells in the mutant cultures over and above what was found in wild type then this increased cell death could be attributed to Dcr1 depletion. It was observed that the *DCR1* conditional mutants grew comparably to the heterozygous *DCR1/dcr1* deletion mutants and the wild type at both the temperatures, indicating no significant loss in viability upon Dcr1 depletion at either (Figure 11). Thus, no large scale cell death is associated with depletion of Dcr1; small scale changes in cell numbers, however, cannot be determined from this assay and may still be happening and contributing to slow growth of the mutant.

2.3.2 Flow cytometry reveals cells accumulated in S phase upon Dcr1 depletion

To study the effects of Dcr1 depletion on the cell cycle flow cytometry was carried out *DCR1* conditional mutant cultures grown in non-permissive medium for 48 h along with relevant controls were taken and cells were collected for flow cytometry analysis. Upon flow cytometry it was observed that the wild type and permissive controls showed a profile in which most cells had 2C genome content and a small proportion had 4C genome content. Compared to this, in the mutant the peak corresponding to 2C genome content was reduced and relatively more cells were observed to be accumulated in between the 2C and 4C peaks. Further, the 4C peak was slightly broader and cells with the tail of the distribution extending beyond 4C. This phenotype was seen both at 30°C and 37°C (Figure 12).

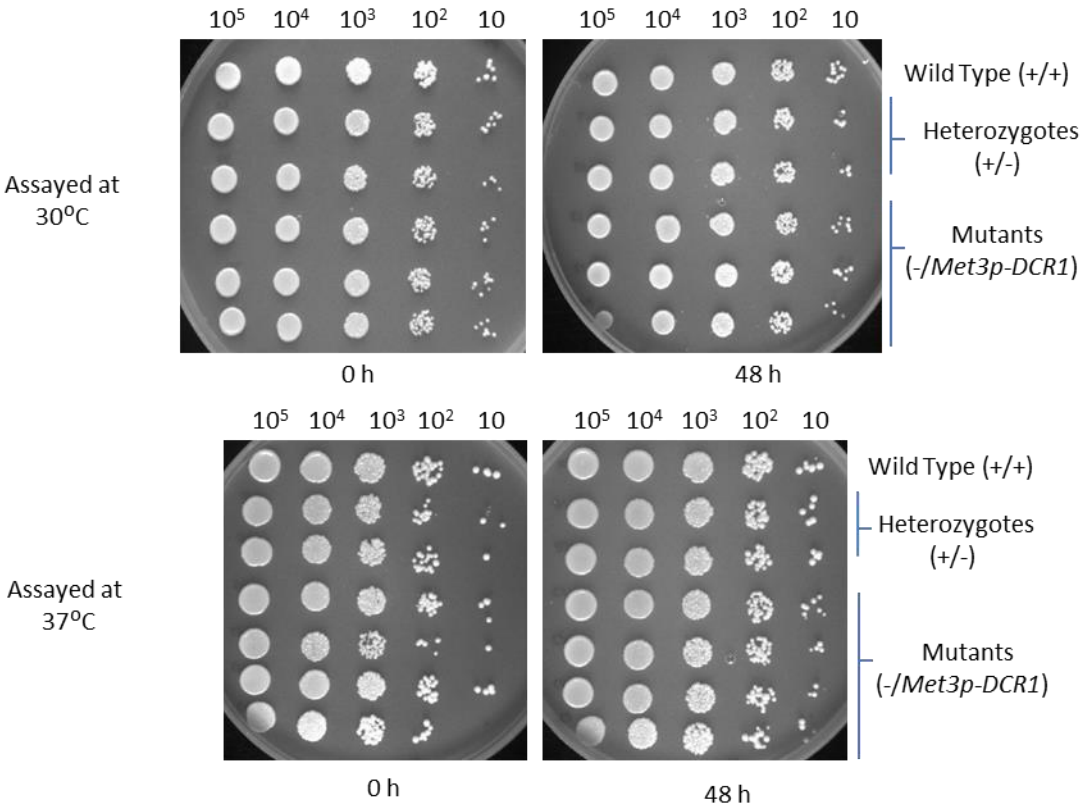


Figure 11: No significant loss in viability is detected upon Dcr1 depletion. Cell viability after Dcr1 depletion was assayed by spotting cultures of wild type, heterozygous deletion mutant and conditional mutants grown in non-permissive medium for 48 h onto permissive medium plates. It is observed that conditional mutants grow comparably to wild type and heterozygous mutants after Dcr1 depletion at both 30°C and 37°C. Number of cells spotted is mentioned at the top of each column, genotype of the spotted strain is given on the right. The assay was carried out with four transformants of the conditional mutant and two of the heterozygous deletion mutant.

This experiment has been performed with four different transformants over independent trials at 48h and 72h (in some cases) and a similar trend is observed across transformants with the phenotype being more prominent at the later time point.

Since each stage of the cell cycle corresponds to a particular ploidy, progression through cell cycle can be tracked by measuring ploidy of cells through flow cytometry. If the cell cycle is delayed at one or more stages, then one would expect the ploidy corresponding to the stage to be overrepresented in the flow cytometry plot. From this plot one can interpret that there is a delay in the S-phase as ploidies intermediate 2C and 4C are observed. This could be because either the processes in S phase-DNA replication and/or DNA repair are taking longer or,

depletion of Dcr1 is leading activation of the intra-S phase checkpoint leading to a slower cell cycle.

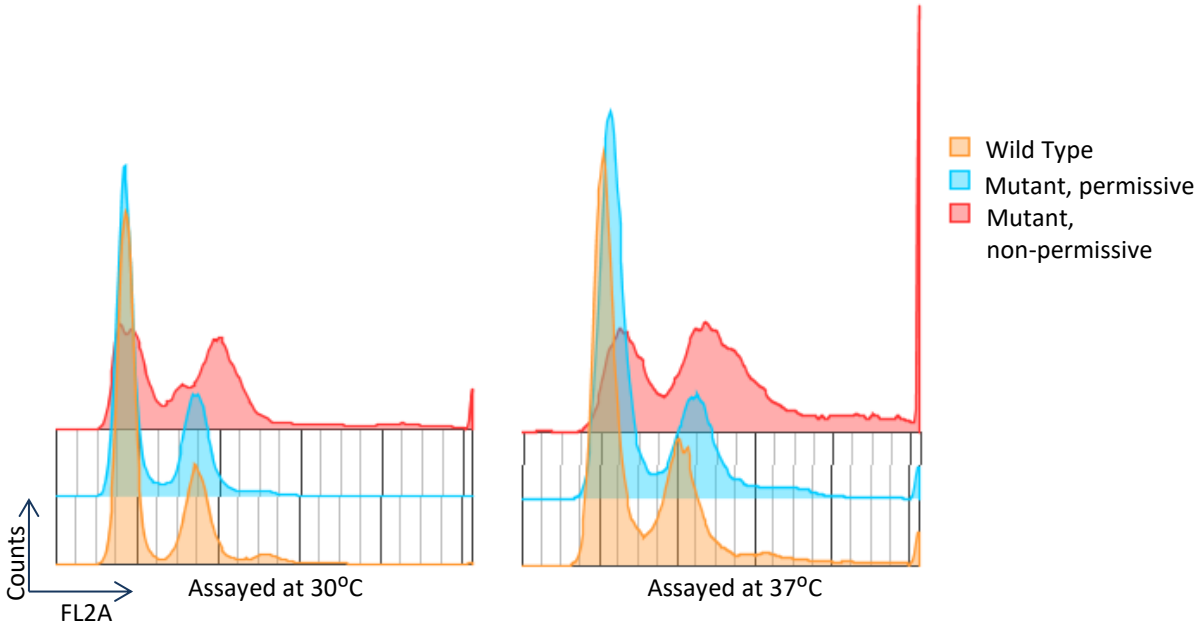
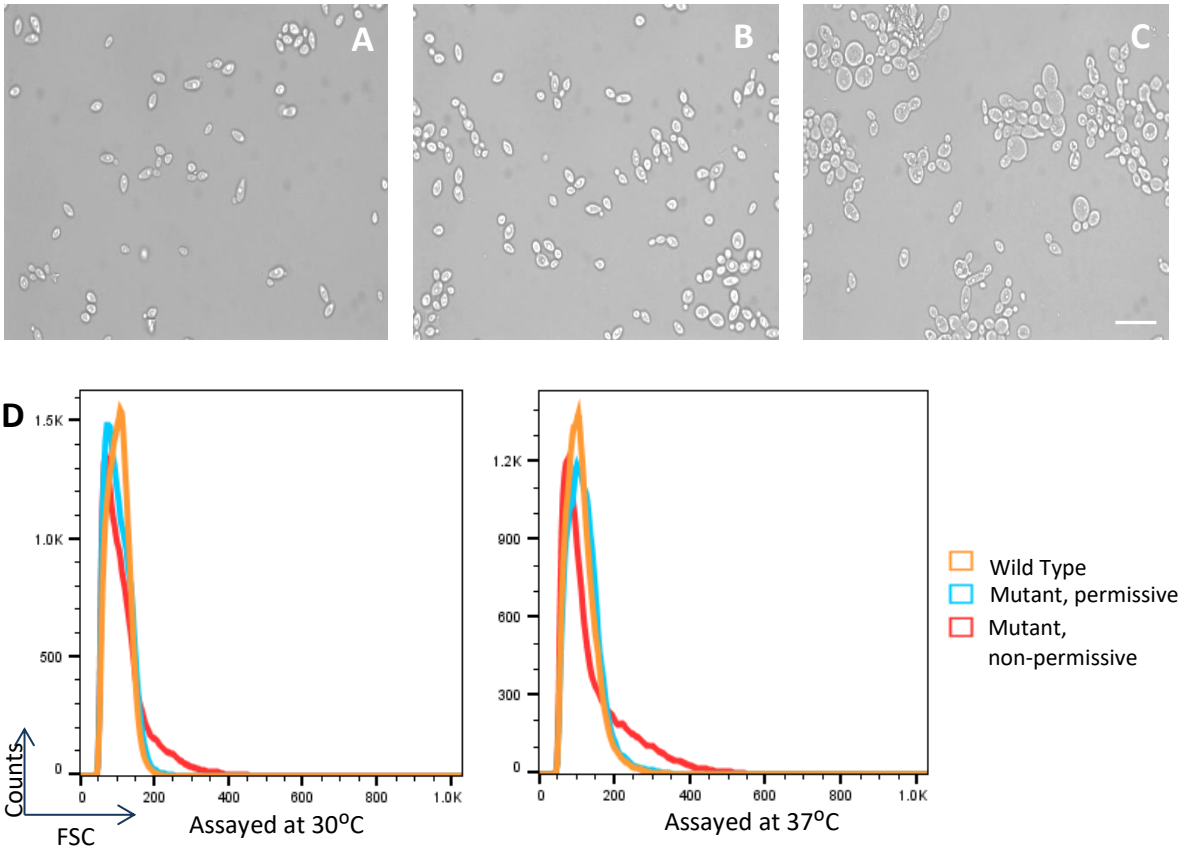


Figure 12: Dcr1 depletion leads to accumulation of cells in S phase. To assess whether the slow growth phenotype of *DCR1* conditional mutants was due to defects in the cell cycle flow cytometry was carried out. As can be seen from the profiles, conditional mutants propagated in permissive medium resemble the wild type with most cells having 2C genome content. Conditional mutants grown in non-permissive conditions however have a profile very different from that of the controls with a much reduced 2C peak and cells with genome content between 2C and 4C observed. This phenotype is seen at both the temperatures tested, 30°C and 37°C.

2.4. *DCR1* conditional mutants have increased cell size

Microscopic observations of cells in which Dcr1 had been depleted revealed that some cells of the population had sizes larger than wild type (Figure 13A, B &C). To quantitate the number of cells displaying this phenotype, forward scatter was measured during flow cytometry and was plotted against cell count to obtain a distribution of cell sizes. This was done for both - cells grown at 30°C and those grown at 37°C (Figure 13D). It was observed that at both temperatures the mutant strain grown in non-permissive medium had a greater percentage of cells in the large size class as compared to the wild type and permissive medium controls. Further, this phenotype of increased cell size was seen to be more prominent at 37°C than at 30°C (Figure 13E).



E

Temperature / Strain / Size	30°C		37°C	
	0-200	200-1000	0-200	200-1000
Wild Type	99.67	0.35	96.27	3.73
Mutant, permissive	99.23	0.79	95.25	4.75
Mutant, Non-permissive	89.70	10.40	78.78	21.22

Values mentioned are in the form of percentage

Figure 13: Cell size increases upon depletion of Dcr1. (A, B &C) A comparison of cell size between wild type, *DCR1* conditional mutants grown in permissive medium and in non-permissive medium, respectively, at 37°C. As can be seen in (C), in non-permissive medium, some cells have cell size larger than wild type. Pseudohyphae formation is also observed. (D) Plot of forward scatter versus frequency to visualize distribution of cell size in the mutant and control populations. Size was measured in mutants grown at 30°C and 37°C. (E) Quantitation of cell size of each strain from the histograms. The percentage of large sized cells (in the class 200-1000) is greater in the mutant grown in non-permissive medium as compared to mutant grown in permissive medium and wild type grown in non-permissive medium. The phenotype is more prominent at 37°C than at 30°C. Bar = 20µm

2.5. Sensitivity to hydroxyurea and dimethyl sulfoxide is altered in *DCR1* conditional mutants

From section A3.2 it was speculated that depletion of Dcr1 probably leads to an increase in cell cycle length which is responsible for the slow growth phenotype of the mutant. It was proposed that this increase is occurring due to a delay in the S phase of the cell cycle. To test whether this was due problems in DNA replication and/or DNA repair, we decided to treat the *DCR1* conditional mutants with drugs affecting these processes adversely. If there are problems in a certain process in the cell, then treating with drugs which are known to affect the process negatively acts as an insult resulting in a more severe phenotype which was otherwise mild or undetected. Hydroxyurea (HU) is a known inhibitor of DNA replication. It acts by negatively affecting ribonucleotide reductase which is involved in the synthesis of deoxyribonucleic acid triphosphates (dNTPs). Without the necessary levels of dNTPs, the S phase checkpoint gets activated leading to replicative arrest (Koc et al, 2004). Dimethyl sulfoxide (DMSO) is a commonly used organic solvent and a potent oxidizing agent. Though not specific to DNA, generation of oxidative radicals by DMSO can lead to DNA damage.

The *DCR1* conditional mutant and the heterozygous deletion mutant were grown in non-permissive medium for 48 h and serial dilutions of each were spotted onto plates containing the requisite drug in non-permissive medium. The plates were incubated at 30°C for a span of 36 h before being photographed (Figure 14). It was observed that the mutants were a fold more sensitive to DMSO at the concentration used and very resistant to HU. Sensitivity to DMSO indicates that whatever process Dicer is involved in is sensitive to oxidative damage. It may or may not be DNA repair since DMSO is not specific to DNA. HU resistance, on the other hand, is an unexpected phenotype. Generally, if proteins involved in replication are mutated then sensitivity to the compound is obtained.

2.6. Position-effect variegation at *C. albicans* centromeres is not affected upon depletion of Dcr1

As mentioned in chapter 1, the position-effect variegation observed at *S. pombe* centromeres is alleviated when the proteins of the RNAi machinery are deleted thus implicating RNAi in regulating the process (Volpe, Kidner et al. 2002). One of the objectives of this study was to assess whether a similar regulation exists in *C. albicans.* To this aim, a *URA3* transgene was

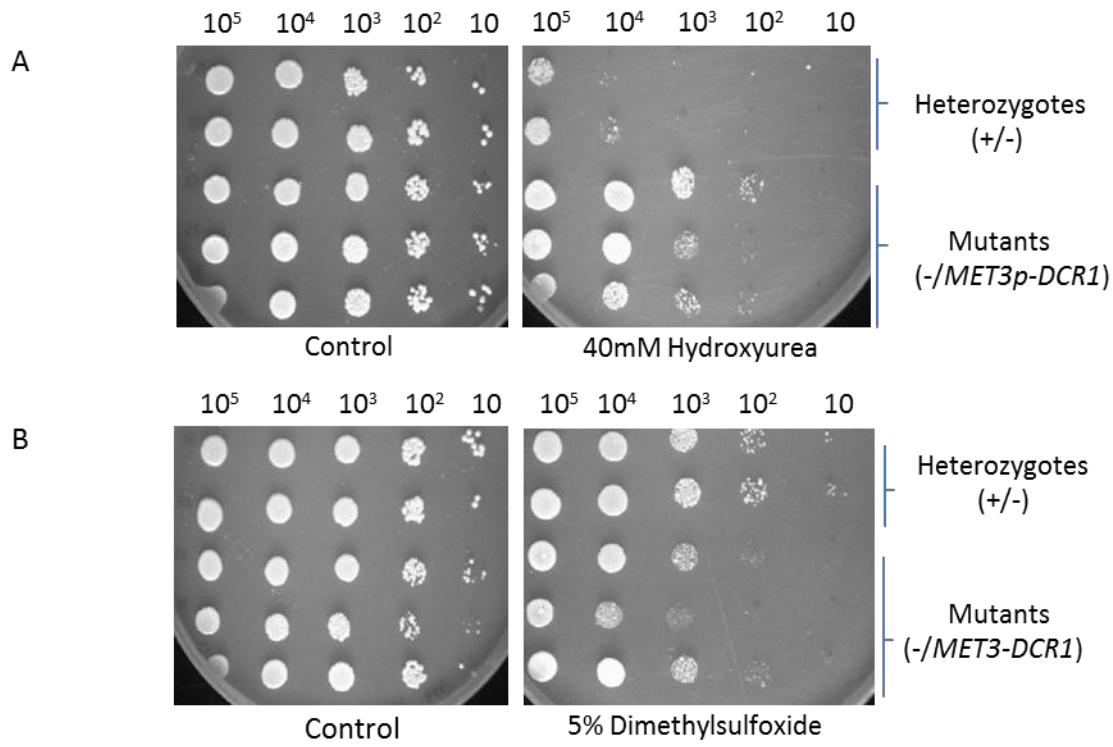


Figure 14: *DCR1* conditional mutants show altered sensitivities to HU and DMSO. The sensitivities of *DCR1* conditional mutants to HU and DMSO were assayed to test for a potential role in DNA replication and/or DNA repair. (A) The mutants were found to be very resistant to HU as was evident from their ability to grow at a dilution thousand times more than the control on the drug plate. (B) Sensitivity to DMSO was observed in *DCR1* conditional mutants compared to controls. For both the drugs, sensitivity was tested for three transformants with similar results being obtained for each. Number of cells spotted is mentioned at the top of each column, genotype of the spotted strain is given on the right.

integrated into the centromere 7 of the *DCR1* conditional mutant and the effect of Dcr1 depletion on position-effect variegation studied.

The *URA3* gene in *C. albicans* codes for orotidine-5'-phosphate decarboxylase which is an enzyme required for the *de novo* synthesis of the pyrimidine nucleotides uridine triphosphate, cytidine triphosphate, and thymidine triphosphate. 5-flouro-orotidic acid (FOA) is a fluorinated derivative of the pyrimidine precursor orotic acid. When cells expressing the *URA3* gene are grown in a medium having FOA, the orotidine-5'-phosphate decarboxylase enzyme catalyses the decarboxylation of FOA to form 5-Flourouracil which is a toxic metabolite. Thus, cells expressing *URA3* can survive in a medium not supplemented externally with uridine but die in a medium containing FOA. In *S. pombe*, a wild type strain having *ura4*$^{+}$ gene (orthologue of *C. albicans URA3*) integrated at the centromere can grow on both - complete medium lacking uridine and complete medium having FOA, indicating

that a subpopulation of cells express *ura4*$^{+}$ while another subpopulation have it silenced - a phenomenon known as position-effect variegation (Allshire, Javerzat et al. 1994).

The *DCR1* conditional mutant strain having *URA3* at the centromere was grown in non-permissive medium along with relevant controls for 48 h and serial dilutions of the cultures were then spotted onto plates lacking uridine, plates having FOA and complete medium plates all having methionine and cysteine to maintain non-permissive conditions. It was observed that the growth of the mutant on FOA plates was comparable to that of the *DCR1/dcr1* heterozygous deletion control (Figure 15), thus ruling out a function of the RNAi machinery similar to that observed in *S. pombe*.

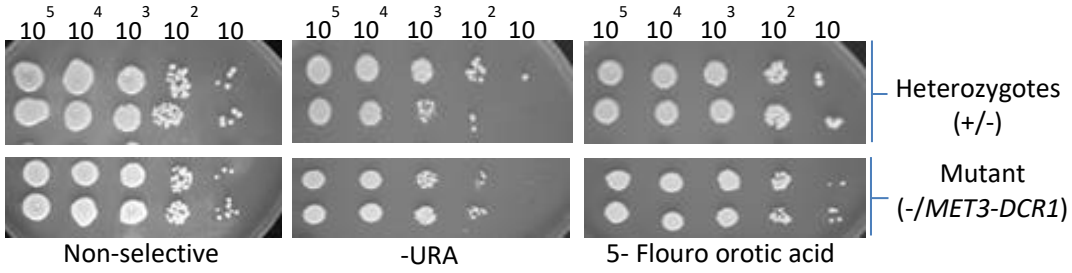


Figure 15: Dcr1 depletion does not perturb position-effect variegation at *C. albicans* centromeres. *DCR1/dcr1* heterozygous deletion mutants and conditional mutants were grown in non-permissive medium for 48 h following which serial dilutions were made from these cultures and spotted on CM, CM-URA and CM+FOA, all having methionine and cysteine added to a final concentration of 2.5mM. The conditional mutants were observed to grow comparable to the heterozygous controls. Genotypes of the strains spotted are mentioned on the right. The study was carried out using three transformants of the *DCR1* conditional mutant; two of them are represented here.

SECTION B

2.7 Argonaute is non-essential for viability in *C. albicans*

2.7.1 The *Argonaute* gene in *Candida albicans*

C. albicans Argonaute is a protein that is encoded by *AGO1* gene (Orf19.2903 CaCh4 coordinates 1,407,066 - 1,409,819). The ORF is 2754 bp long and the protein weighs 104.2 kDa containing 917 amino acids. The domain architecture of *C. albicans* Argonaute is similar to that of higher eukaryotes with the four major domains being the N-terminal, PAZ, MID and PIWI (Nakanishi, Weinberg et al. 2012).

2.7.2 Construction of the *AGO1* null mutant

To study the functions of Argonaute in *C. albicans*, it was desired to create a homozygous deletion mutant of the same. The first copy of the *AGO1* gene was deleted in a manner similar to *DCR1* by cloning upstream (US) and downstream (DS) regions flanking the *AGO1* ORF in the plasmid pSFS2a (Reuss et al, 2004) and using the *Kpn*I/*Sac*I fragment of the resultant plasmid pAB4 to transform the wild type strain SN148. The strain thus obtained had one copy of the *AGO1* gene intact while the other had been replaced by the *CaSAT1* marker conferring the strain resistance to the antibiotic nourseothricin. The *CaSAT1* marker was then recycled by growing the strain in YPMU as described in Materials and Methods to yield the strain CaAB005. To delete the second copy of the *AGO1* gene, CaAB005 was transformed with the *Kpn*I/*Sac*I fragment of pAB4; however, despite multiple attempts a homozygous deletion mutant could not be obtained. A possible reason for this could be that the cassette used for transformation was replacing the already deleted allele as the homology regions are the same. To avoid this, a second plasmid, pAB5 was constructed in which the downstream region (DS) from pAB4 was replaced by a 3' UTR of the *AGO1* ORF. A cassette like pAB5 with an internal homology region would be expected to preferentially target the remaining allele and not the already deleted allele. Transformation of CaAB005 with the *Kpn*I/*Sac*I fragment of pAB5 resulted in transformants which were homozygous deletion mutants of *AGO1* (Figure 16A). These strains were confirmed by PCR amplification and Southern hybridization as described in Materials and Methods (tables M1 and M3) (Figure 16B & C). Since, null mutants of the gene could be isolated, the *AGO1* gene is non-essential for viability in *C. albicans*.

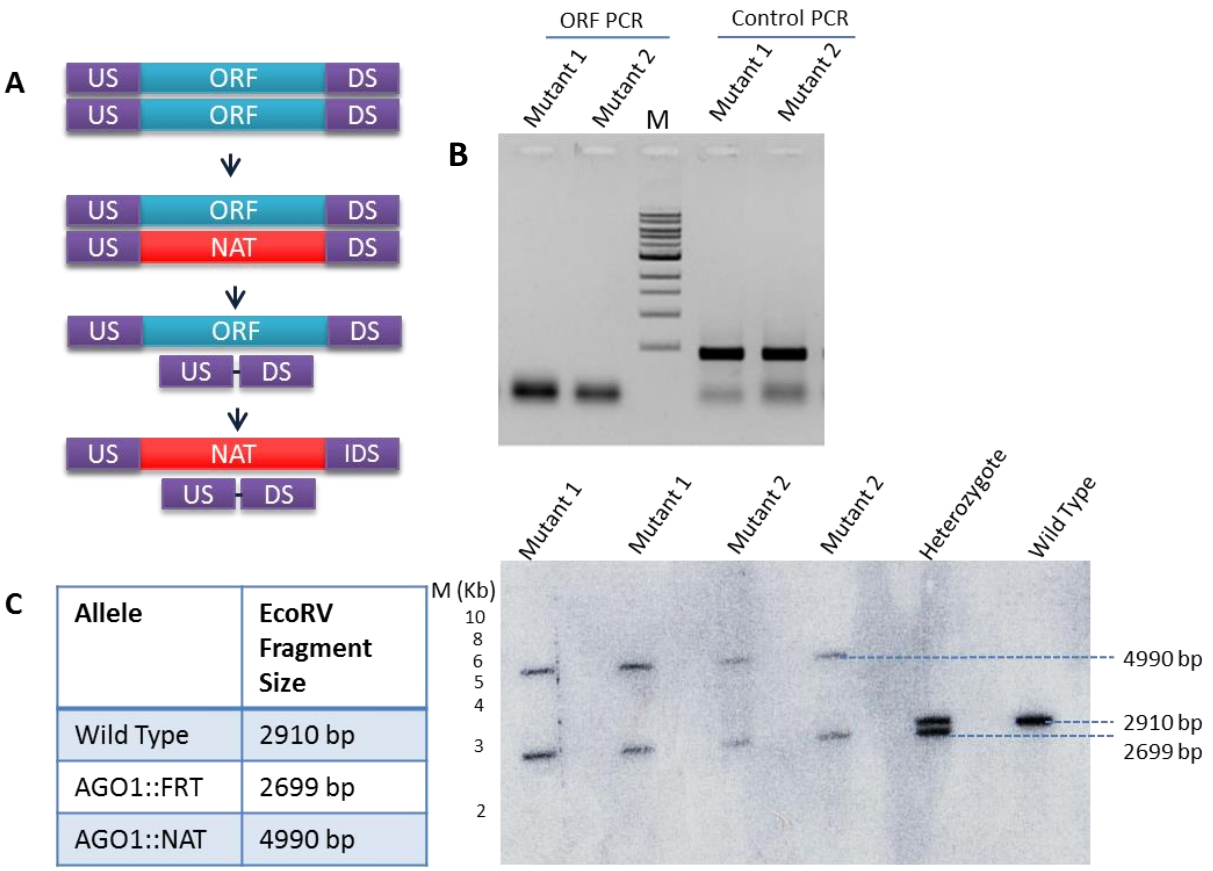


Figure 16: Construction of a null mutant of *AGO1*. (A) Flow chart depicting the steps in the construction of the *AGO1* null mutants. The first copy of the gene was deleted by replacing the ORF with the *CaSAT1* (*NAT*) marker through homologous recombination using plasmid pAB4. The marker was then recycled by growing the strains in YPMU as described in Materials and Methods. The DS of pAB4 was changed to an internal homology region of the *AGO1* ORF yielding plasmid pAB5 using which the second copy of the gene was then deleted. (B) Confirmation of the null mutant strains by PCR amplification. A PCR detecting a region of the *AGO1* ORF does not amplify while a control PCR to check quality of genomic DNA gives an amplicon of the expected size. M: NEB 1kb ladder. (C) Southern blotting to confirm genotypes of the null mutants. The sizes corresponding to each possible allele are tabulated next to which is present the blot. Loading order is mentioned in the blot. As can be seen, lanes having null mutant genomic DNA do not have the allele corresponding to the wild type. Each mutant has been loaded in duplicate. Molecular ladder is marked.

2.8. The growth rate of *AGO1* null mutants is comparable to that of wild type

As a part of characterizing the gene, a growth curve was constructed to assess the growth rate of the mutants with respect to the wild type. Overnight cultures of the wild type, the heterozygous deletion mutant and the null mutants were taken and secondary cultures were established at an initial OD_{600} of 0.1. These were then allowed to grow and OD_{600} was measured every half an hour. OD_{600} was plotted against time to obtain the growth curve

(Figure17A). As can be observed from the graph, the growth rates of the wild type, heterozygous deletion mutant and *ago1/ago1* null mutants are comparable. Thus unlike Dcr1 depletion, *AGO1* deletion does not result in a retardation of growth of the organism.

2.9 *AGO1* deletion does not cause the phenotypes associated with *DCR1* conditional mutants

Dicer and Argonaute are known to work in unison in most organisms to bring about RNA interference. Therefore, it is expected that the downstream process will be affected with a mutation in either. We wanted to determine if this was true in *C. albicans* by testing if the phenotypes associated with Dcr1 depletion were also observed for Argonaute deletion. Results pertaining to these experiments are described in this section.

2.9.1 Growth rates of *ago1* null mutants are not temperature-dependent

The retardation in growth of *DCR1* conditional mutants is dependent on temperature with the phenotype becoming more intense at an elevated temperature, that is, 37°C. To test whether the growth rates of *ago1* mutants are affected by an increase in temperature, the wild type, heterozygous deletion mutant and null mutant strains were streaked on complete medium plates and one set incubated at 30°C and another at 37°C for 24 h after which the plates were photographed (Figure 17B & C). As can be seen from the figure, no significant change is observed in the growth rates of the mutants compared to the wild type with an increase in temperature. Thus, growth rates of *ago1* null mutants, unlike those of *DCR1* conditional mutants are not temperature sensitive.

2.9.2 *ago1* null mutants do not show accumulation of cells in S phase nor have an increased cell size

DCR1 conditional mutants were found to grow slower than wild type and the reason for this slow growth was determined to be an increase in cell cycle length. Flow cytometry analysis of *DCR1* conditional mutants revealed cells accumulated in S phase upon Dcr1 depletion indicating that probably it was at this stage at which the delay was occurring. Though significant reduction in growth rate was not observed in *ago1/ago1* null mutants, flow cytometry analysis was carried out to assess whether an accumulation similar to that in *DCR1* conditional mutants was observed in them. However, it was found that concomitant with a normal growth rate, the flow cytometry profile of the mutants was similar to that of the wild type indicating a normal cell cycle (Figure 18A).

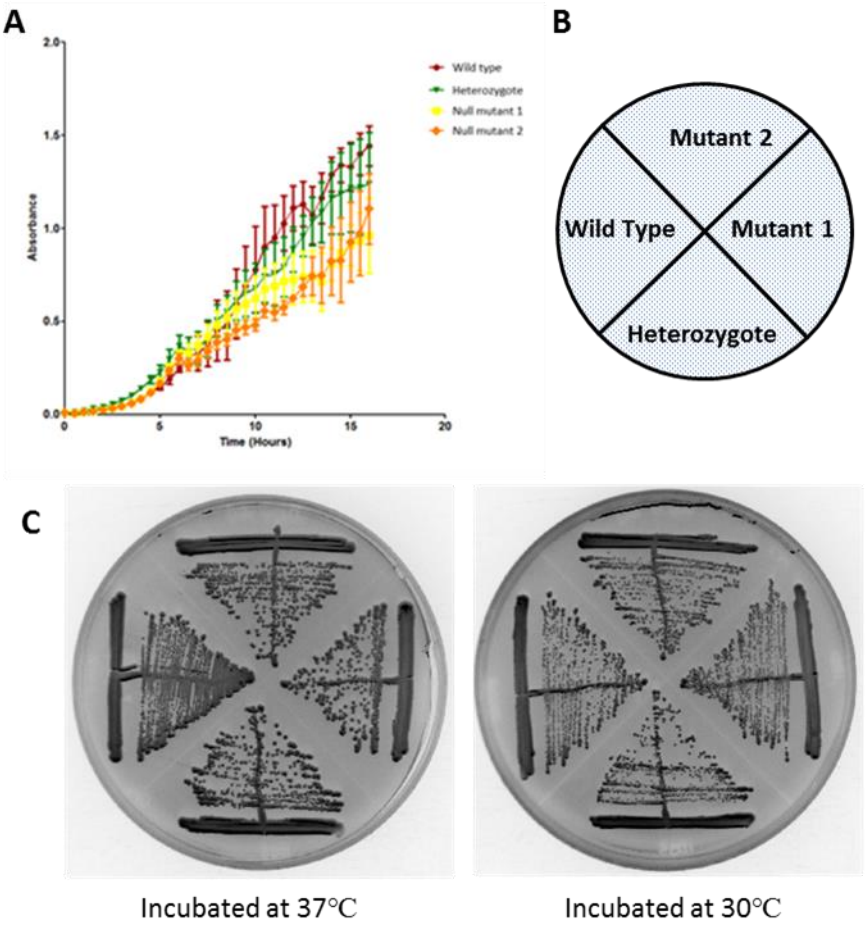


Figure 17: Growth rates of *ago1* null mutants are not affected by temperature. (A) A comparison of growth rates of wild type SN148, *AGO1/ago1* heterozygous deletion and *ago1/ago1* null mutants. As can be seen from the graph, there is a marginal lag in growth observed between the wild type and heterozygous and homozygous deletion mutants. (B and C) Effect of temperature on the growth rates of null mutants. Unlike *DCR1* conditional mutants, the growth rates of *ago1/ago1* null mutants do not appear to be affected by temperature as can be evidenced by the fact that colony sizes of mutants are similar to that of wild type at both temperatures. (B) Schematic of the identity of each strain on the plate.

Further, *DCR1* conditional mutants display an increased cell size upon Dcr1 depletion. No such increase was associated with *ago1* deletion as evidenced from microscopic and flow cytometry observations (Figure 18B). Therefore, from these observations it can be concluded that *ago1* mutants do not show cell cycle or size defects.

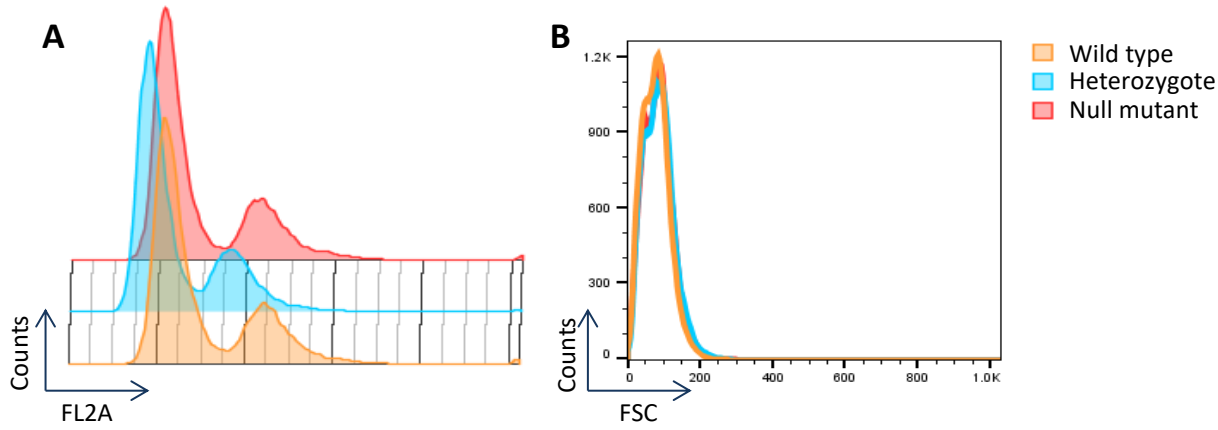


Figure 18: Accumulation of cells in S phase and increase in cell size is not observed upon deletion of *AGO1*. (A) Flow cytometry profiles of the wild type, heterozygous deletion mutant and null mutants. The profiles of null mutants resemble that of the wild type indicating a normal cell cycle in them. (B) Histogram showing distribution of sizes in the above mentioned strains. Size distribution of *ago1/ago1* null mutants is similar to that of wild type signifying no cell size defects. Two transformants of the *ago1/ago1* null mutants were used for the experiment. Colours indicate different strains.

2.9.3 No altered sensitivity to hydroxyurea or dimethyl sulfoxide is observed in case of *AGO1* deletion

Since we observed cells accumulated in S phase in *DCR1* conditional mutants we tested for problems in the two major processes of S phase, namely, DNA replication and DNA repair. This was done by treating the cells with replication inhibitor HU and oxidizing agent DMSO. The mutant cells were seen to be resistant to HU and sensitive to DMSO. To examine whether *AGO1* deletion also causes the same phenotypes, serial dilutions of log phase cultures of the wild type, heterozygous deletion mutants and null mutants were made and spotted onto complete medium plates containing the appropriate drug. The plates were incubated at 30°C for 36 h and then photographed (Figure 19). There was no difference in sensitivity to these drugs between wild type and mutants as can be observed from the plates. Thus, *AGO1* deletion does not alter the sensitivity to these drugs contrary to what was obtained for *DCR1* conditional mutants.

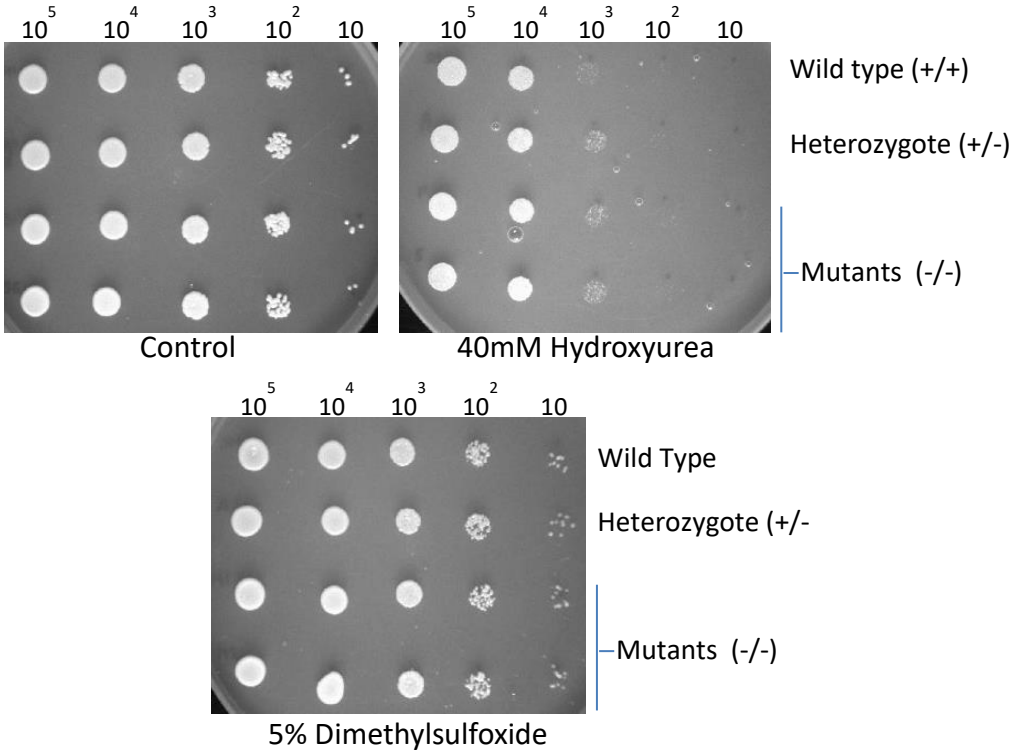


Figure 19: Sensitivity to HU and DMSO is not altered upon *AGO1* deletion. In order to test whether AGO1 deletion confers the same phenotype as Dcr1 depletion, the sensitivity of *ago1/ago1* null mutants to HU and DMSO was tested. However, in contrast to *DCR1* conditional mutants, no change in sensitivity to these drugs was observed in the case of *ago1/ago1* null mutants. Number of cells spotted is mentioned at the top of each column, genotype of the spotted strain is given on the right. Sensitivities of two mutants was assayed.

2.10 *AGO1* deletion like Dcr1 depletion does not affect position-effect variegation at *C. albicans* centromeres

Position-effect variegation at *S. pombe* centromeres was shown to be abrogated by the deletion of both *DCR1* and *AGO1* genes. In section 2.6, it was shown that Dcr1 depletion does not have an effect (as measured through spotting) on position-effect variegation at the *C. albicans* centromeres. We next sought to test the effect of *AGO1* deletion on this phenomenon. To achieve this, both copies of the *AGO1* gene were deleted in a strain having *URA3* integrated in centromere 7 of *C. albicans.* The resultant strain, CaAB011 along with relevant controls was grown in non-selective medium and serial dilutions of log phase cultures were spotted onto complete medium, complete medium lacking uridine and complete medium containing FOA plates. The plates were incubated at 30°C for 36 h and then photographed (Figure 20). No difference in growth was obtained on the FOA plates between

heterozygous control and mutants. Hence, *AGO1* deletion also does not impact position-effect variegation observed at *C. albicans* centromeres.

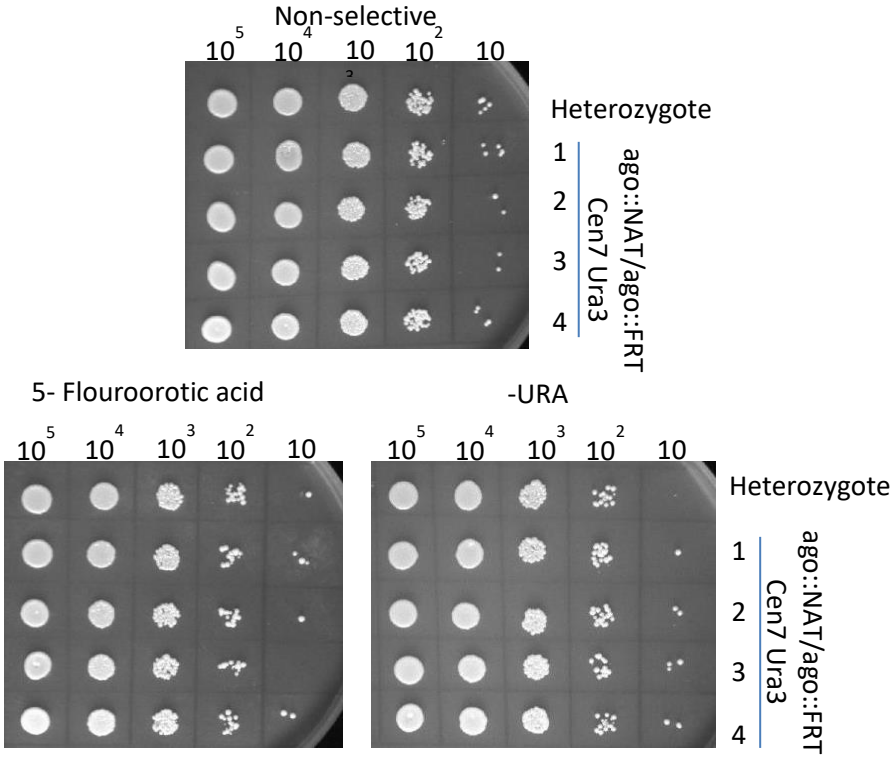


Figure 20: Position-effect variegation at *C. albicans* centromeres is not affected by deletion of *AGO1*. The effect of *AGO1* deletion on position-effect variegation at *C. albicans* centromeres was tested. It was found that like Dcr1 depletion, *AGO1* deletion too does not affect this phenomenon. Number of cells spotted is mentioned at the top of each column, genotype of the spotted strain is given on the right. Four transformants were used for the assay. Genotype of all four was confirmed using PCR amplification and Southern blotting.

CHAPTER 3:
DISCUSSION

CHAPTER 3: DISCUSSION

The aim of this study was to explore possible functions of the cryptic RNAi machinery in *C. albicans.* In order to achieve this aim the approach adopted was to study the consequences of the loss of function of its constituent proteins, Dicer and Argonaute. For this purpose, it was decided to create deletion mutants of the same.

C. albicans is an obligate diploid; consequently, each gene is present in two copies. The first copy of the gene coding for *Dicer* was deleted by replacement with a selectable marker using homologous recombination. However, despite many attempts a null mutant of the gene using the same strategy could not be obtained, raising the possibility that the gene may be essential for viability in this organism. This was not anticipated as Dicer and Argonaute are known to be non-essential in all fungi examined till date (Pickford, Catalanotto et al. 2002, Volpe, Kidner et al. 2002, Catalanotto, Pallotta et al. 2004, Kadotani, Nakayashiki et al. 2004, Drinnenberg, Weinberg et al. 2009, Janbon, Maeng et al. 2010, Dang, Yang et al. 2011, Weiberg, Wang et al. 2013, Nicolás, Vila et al. 2015, Villalobos-Escobedo, Herrera-Estrella et al. 2016). To test this hypothesis, the second copy of the gene was put under the control of the *MET3* promoter (Care, Trevethick et al. 1999). Upon shutting down expression from the promoter it was observed that the *DCR1* conditional mutants were able to grow but their growth was retarded compared to the wild type strain. Further, this phenotype was observed to be temperature dependent with a greater retardation seen at 37℃ as compared to 30℃, the reason for which could not be determined.

A slow growth phenotype can arise either due to cell death or because of an increase in the time taken to complete the cell cycle itself. To assess if Dcr1 depletion resulted in cell death the viability of Dcr1 depleted cells was tested by spotting serial dilutions of these cultures on a permissive medium plate and allowing the cells to grow. Dead cells present in the culture would not divide and form colonies on the plate. If there was a population of dead cells in the mutant cultures over and above what was found in wild type then this increased cell death could be attributed to Dcr1 depletion. It was observed that, the *DCR1* conditional mutants grew comparably to the wild type at both the temperatures tested indicating that there was no significant loss in viability. Thus, the retarded growth was not due to large scale cell death. There could still be a minor loss in cell viability associated with Dcr1 depletion but that cannot be determined from this assay. Since Dcr1 depletion did not lead to a significant loss

in viability, *DC1* is unlikely to be essential for survival and it should be possible to create a null mutant for the same.

Having ruled out death of cells as contributing to slow growth of mutant, we then tested for cell cycle defects. Each stage of the mitotic cell cycle has a corresponding ploidy associated with it. Thus changes in cell stage, that is, progression through cell cycle can be monitored by tracking changes in ploidy of the cells. If the cell cycle length is increased then in an asynchronous culture one would expect a greater proportion of cells to be in the stage where the delay is occurring. Thus, the ploidy corresponding to this stage would be over-represented compared to the wild type. To determine if there is a delay in the cycle and if so at what stage, flow cytometry analysis was carried out. What was observed was that compared to the wild type, which showed a profile in which most cells had 2C genome content and a small proportion had 4C genome content, the mutant displayed a profile in which the peak corresponding to 2C genome content was reduced and ploidies in between 2C and 4C were obtained. Moreover, the 4C peak was slightly broader and the tail of the distribution extended beyond 4C. This phenotype was seen both at 30°C and 37°C. Since a greater proportion of ploidies intermediate between 2C and 4C were observed, we postulated that there is a probable delay in the S phase of the cell cycle. From this observation we hypothesized that either the processes of DNA replication or DNA repair - the two dominant processes of S phase - are themselves taking longer or Dcr1 depletion may have resulted in activation of the intra S-phase checkpoint which in turn has delayed the cell cycle.

DCR1 conditional mutants also display an increased cell size as compared to the wild type. Recently, Chaillot *et al.* performed a comprehensive genome-wide survey in which they identified genes which are haploinsufficient for cell size (Chaillot, Cook et al. 2017). They sub-divided the genes into two classes, one in which haploinsufficiency conferred a smaller size than wild type and the other in which the size was larger as compared to the wild type. Gene ontology enrichment analysis revealed that large sized mutants were predominantly defective in genes related to the mitotic cell cycle (Chaillot, Cook et al. 2017). Thus we speculate that the large size of the *DCR1* conditional mutants may be indicative of a putative function in the cell cycle. Furthermore, under conditions of Dcr1 depletion, apart from large sized individual cells some psuedohyphal cells were also observed which would explain the tail of the distribution beyond 4C that was observed in the flow cytometry profile.

Regulation of the cell cycle by RNase III domain containing enzymes has been reported earlier in literature. *Pac*I in *S. pombe* is an RNase III containing enzyme and is known to regulate meiosis in the fission yeast (Iino, Sugimoto et al. 1991). Further, deletion of *S. cerevisiae* Rnt1, an RNase III enzyme involved in ribosomal RNA biogenesis results in a delay in the G1 and G2/M phases of the cell cycle (Catala, Lamontagne et al. 2004). Deletion of the *Dicer* gene in *S. pombe* affects chromosome segregation resulting in slow growth (Provost, Silverstein et al. 2002, Hall, Noma et al. 2003) while CARPEL FACTORY, a Dicer-homologue in *Arabidopsis thaliana* has been shown to regulate cell division in floral meristems (Jacobsen, Running et al. 1999). Therefore, on the basis of the results obtained by us and these reports linking RNase III enzymes to cell cycle regulation, it is conceivable that *C. albicans DCR1* may have a role in the cell cycle.

To test whether the processes of DNA Replication and/or DNA repair are affected under conditions of Dcr1 depletion we decided to use drugs which adversely affect these processes. As mentioned in Chapter 3, if there are problems in a certain process in the cell, then treating with drugs which are known to affect the process negatively acts as an insult resulting in a more severe phenotype which was otherwise mild or undetected. As a result, mutant cells in which the process is defective are more sensitive to these drugs than wild type. We tested the sensitivity of these mutants to the replication inhibitor hydroxyurea (HU) and oxidizing agent dimethyl sulfoxide (DMSO). DMSO is a commonly used organic solvent and an oxidizing agent. Treatment of yeast cells with DMSO has been demonstrated to induce oxidative stress (Sadowska-Bartosz, Pączka et al. 2013). Generation of oxidative radicals by DMSO can lead to DNA damage. The *DCR1* conditional mutants were found to be a fold more sensitive to DMSO as compared to wild type at a concentration of 5%. Thus, it can be concluded that Dcr1 is required to protect the cell against oxidative damage. Its role, however, cannot be yet narrowed down to DNA damage repair as DMSO is not specific for DNA. To arrive at this conclusion, sensitivity of *DCR1* conditional mutants to damaging agents more specific to DNA such as methyl methane sulphonate (MMS) needs to be tested. Nevertheless such a role may be probable as Dicer is known to be involved in the DNA damage response in *A. thaliana* (Burger and Gullerova 2015) and *N. crassa* (Lee, Chang et al. 2009). Furthermore, DNA damage has been shown to accumulate in Dicer-deficient cells in mice and humans (Tang and Ren 2012, Swahari, Nakamura et al. 2016).

It has been shown that in α factor synchronised *S. cerevisiae* cells, onset of S phase is accompanied by an increase in the cellular deoxyribonucleotide triphosphate (dNTP) pool

(Koç, Wheeler et al. 2004). These dNTPs are then utilized for chain elongation in replication. The enzyme responsible for the synthesis of dNTPs, Ribonucleotide reductase (RNR) is the target of HU. By inhibiting the activity of this enzyme, HU effectively inhibits the increase in the cellular dNTP pool. This is sensed by the cell which then results in the activation of the intra S-phase checkpoint leading to cell cycle arrest (Koç, Wheeler et al. 2004). Cells having mutations in proteins involved in the process of replication such as DNA polymerase are more sensitive to HU than wild type (Gerik, Li et al. 1998). When cells were treated with HU at a concentration of 40mM, *DCR1* conditional mutants were observed to be resistant to the drug compared to the wild type. To our knowledge, such a phenotype has not been reported for Dicers till now. We carried out a gene ontology analysis on the genes catalogued in Saccharomyces Genome Database whose deletion confers resistance to HU. The aim of this exercise was that if there is a particular class of genes which is over-represented amongst those analysed then probably the functions associated with that class of genes are also associated with *C. albicans DCR1*. However, upon doing the analysis it was found that there was no single class which was over-represented but instead there were five classes which had maximal and equal representation (http://yeastgenome.org/cgi-bin/GO/goSlimMapper.pl). These contained genes involved in chromatin re-organisation, response to chemicals, regulation of cellular organelle organization, transcription from RNA polymerase II promoter and some genes of unknown function. Thus, from this analysis we cannot conclude much about the function that *C. albicans* Dcr1 may be performing. We, however, hypothesize that maybe Dcr1 depletion is - (i) preventing the entry of HU inside the cell, or (ii) interfering with its action on RNR, or (iii) leading to an increased amount of RNR in the cell or (iv) combinations of the preceding factors. Further experimentation will be required to shed more light on the causes of HU resistance in Dcr1 depleted cells.

The phenotypes described above implicate Dcr1 in functions which may or may not involve the RNase III activity of the protein. Indeed studies on yeast Rnt1 and bacterial RNase III have revealed functions of the protein which do not require the protein's catalytic cleavage activity (Catala, Lamontagne et al. 2004). Domain analysis studies wherein particular domains are mutated and the consequence on the phenotype studied need to be carried out to determine whether a similar scenario exists for *C. albicans DCR1*.

The other component of the cryptic RNAi machinery of *C. albicans* is the Argonaute protein. We were able to generate homozygous deletion mutants for *AGO1* indicating that this gene is non-essential for viability in this organism. This was concordant with what is observed in the

rest of the fungal kingdom (Pickford, Catalanotto et al. 2002, Volpe, Kidner et al. 2002, Drinnenberg, Weinberg et al. 2009, Janbon, Maeng et al. 2010, Dang, Yang et al. 2011, Nicolás, Vila et al. 2015, Villalobos-Escobedo, Herrera-Estrella et al. 2016). In most organisms with functional RNAi machinery, Dicer and Argonaute are known to work together to bring about the functions associated with the machinery. It is thus expected that a mutation in either protein should affect the downstream function. To see if this was the case in *C. albicans* we tested whether the phenotypes observed for Dcr1 depletion were also observed for *AGO1* deletion. We however found that *AGO1* deletion did not confer the same phenotypes as observed for Dcr1 depletion. Thus, Argonaute is either redundant or not required for the phenotypes that have been tested for Dicer in *C. albicans.* In case it is redundant, then one would expect a protein of similar domain architecture (and thus capable of performing similar functions) to be present in the *C. albicans* genome; however, BLAST analysis using *AGO1* as query have found no such candidate. Therefore, based on these evidences, we believe that Dicer and Argonaute may have functionally diverged at least with respect to these phenotypes in this yeast pathogen of humans.

When exploring possible roles of this machinery in *C. albicans*, in specific, we wished to test whether the RNAi machinery was involved in regulating position-effect variegation at the *C. albicans* centromeres as is documented in *S. pombe* (Volpe, Kidner et al. 2002). As has been described in the first chapter, *S. pombe* has three centromeres; each comprising a central core flanked by two kinds of inverted repeats, the innermost (*imr*) and the outermost (*otr*) repeats. *Ura4*$^{+}$ transgenes inserted in any region of the centromere exhibit position-effect variegation wherein a subpopulation of cells has the transgene silenced (Allshire, Javerzat et al. 1994, Volpe, Kidner et al. 2002). When the RNAi proteins of *S. pombe*- Dicer, Argonaute and RNA dependent RNA polymerase are deleted, silencing is de-repressed for genes located in the *otr* region but not in the central core or *imr* regions (Volpe, Kidner et al. 2002). *C. albicans* has eight centromeres, with one having a structure like *S. pombe* with a central core and flanking inverted repeats and other seven comprising just a central core region (Sanyal, Baum et al. 2004). All eight centromeres are clustered in space (Sanyal and Carbon 2002). A *URA3* transgene inserted in the central core of *C. albicans* centromeres also exhibits position-effect variegation (Thakur and Sanyal 2013). To see whether RNAi was responsible for regulating this phenomenon, we depleted Dcr1 and deleted *AGO1* separately in a background having *URA3* integrated at centromere 7. It was observed that in neither of the two cases, was there any effect on position-effect variegation as measured through spotting assays. Thus,

analogous to *S. pombe*, the position-effect variegation at *C. albicans* central core is not alleviated when Dcr1 and *AGO1* are depleted or deleted, respectively.

Pericentric regions in *C. albicans* are not defined by repeats as is the case in *S. pombe* with the exception of chromosome 5. Instead, most centromeres are flanked by ORF-free regions of varying size (Sanyal, Baum et al. 2004). Recently, the chromatin state of the regions flanking centromeres in *C. albicans* was probed and it was discovered that chromatin in the pericentric region was highly acetylated on histone H3 lysine 9 and 16 which are marks of euchromatin and hypomethylated on histone H3 lysine 4 which is a mark of heterochromatin (Freire-Benéitez, Price et al. 2016). Further it was found that genes in the vicinity of centromeres had an inherently low level of expression compared to the rest of the genome and this repressive environment could also lower the expression of a transgene inserted in that region. This chromatin state, which bears features of both euchromatin and heterochromatin, was observed to require the histone methyltransferase Set1 for it to be formed (Freire-Benéitez, Price et al. 2016). It is not yet known as to what regulates this unusual chromatin flanking *C. albicans* centromeres and it would be interesting to study the role of RNAi, if any, in this process.

The overall goal of this study was to characterize the role of the cryptic RNAi machinery in the human pathogenic yeast *C. albicans*. There have been two more studies that have applied themselves with the RNAi machinery of *C. albicans* which have not been referred in detail in this thesis so far. The first study was carried out by Bernstein *et al.* wherein they also tried to characterize the RNAi proteins by adopting the loss of function approach (Bernstein, Vyas et al. 2012). They too were able to obtain homozygous deletion mutants for *AGO1* but could not do so for *DCR1*. They were only able to knock out both the endogenous copies of *DCR1* when Dcr1 was expressed *in trans* from an inducible promoter. Like us they observed a slow growth phenotype upon shutting down gene expression. Initially, based on their inability to delete the second copy of the gene coupled with retardation in growth of the conditional mutant they concluded that the gene is essential for viability. Later on however, they have attributed the slow growth of the mutant to defects in ribosome biogenesis as they found that Dcr1 was performing functions similar to *S. cerevisiae* Rnt1 (Bernstein, Vyas et al. 2012). We took the approach of deleting the first copy of *DCR1*and putting the remaining one under the control of the *MET3* promoter. The results obtained by us agree with theirs as we have also found that the slow growth is not due to a loss in viability but instead due to a longer cell cycle. Further, a reduction in global protein translation due to defects in ribosome biogenesis

would certainly result in a longer time taken to complete the cell cycle; however, it cannot explain the suspected delay in the S-phase of the cell cycle that we have observed. Thus, we believe that Dicer may be affecting the cell cycle by means apart from its role in ribosome biogenesis.

The second report, also by the same group described the introduction of the CRISPR-Cas9 system in *C. albicans* (Vyas, Barrasa et al. 2015). In this they utilized the CRISPR-Cas9 system to create loss of function mutations in *DCR1*, confirming that this gene is not essential for viability. Further, they observed that these null mutants of *DCR1* are viable at high temperatures and inviable at lower ones. As an example, they showed that the *DCR1* null mutants grew normally at 37℃ but had a slower growth at 16℃ (Vyas, Barrasa et al. 2015). This result of theirs contrasts with our observations that the growth of the conditional mutants is further retarded at 37℃ compared to 30℃. Thus, there exists some controversy regarding the temperature sensitivity of Dcr1 which will require further experimentation to be resolved.

There are some results obtained by their group which along with ours point to a common inference. The Rnt1 like function observed for *DCR1* mutants was not associated with mutants of *AGO1* (Bernstein, Vyas et al. 2012). Additionally, they discovered that depletion of Dcr1 but not the deletion of *AGO1* reduced the amount of siRNAs generated as compared to the wild type. These two lines of evidence lend further support to our hypothesis that Dicer and Argonaute may have functionally diverged in *C. albicans*.

In conclusion, in this work we have attempted to characterize the functions of the RNAi protein in *C. albicans*. We have identified some phenotypes associated with the depletion of Dcr1 and based on them speculate that it may be playing a role in the cell cycle. Deletion of *AGO1* does not confer the same phenotypes as depletion of Dcr1; this fact along with other evidences suggests that they may have functionally diverged at least with respect to these phenotypes. Further, we have found that unlike in *S. pombe*, the position-effect variegation observed at the *C. albicans* centromeres is not under the control of the RNAi machinery.

CHAPTER 4:
MATERIALS AND METHODS

CHAPTER 4: MATERIALS AND METHODS

4.1 Strain construction

4.1.1 Construction of a heterozygous deletion mutant of *DCR1*

The first copy of *DCR1* was deleted in the *C. albicans* strain SN148 using the *SAT1* flipper cassette (pSFS2a) (Reuß, Vik et al. 2004). Upstream (US) and Downstream (DS) sequences of the *DCR1* ORF (CaCh4 ORF 19.3796, assembly 19/21 identifier) were amplified from the *C. albicans* genome using primer pairs AB27/AB28 and AB29/AB30, respectively. They were cloned in pSFS2a as *Kpn*I/*Xho*I and *Sac*II/*Sac*I fragments of 438 bp and 538 bp, to obtain the plasmid pAB1. The clones were confirmed using restriction analysis and PCR amplification using specific primers as mentioned in Table M1. The plasmid was digested with the enzymes *Kpn*I and *Sac*I and the resulting fragment was transformed into *C. albicans* strain SN148. Transformants were selected using nourseothricin resistance. For this purpose, nourseothricin (Werner Bioagents, CAS 96736) was added to YPDU agar plates to a final concentration of 100µg/ml. The resulting strain was named CaAB001 (*DCR1/dcr1::FRT-NAT-FRT*). Deletion was confirmed using primer pair AB27/AB30. To obtain nourseothricin sensitive derivatives, transformants were grown in YPMU to obtain the strain CaAB002 (*DCR1/dcr1::FRT*).

4.1.2 Construction of a conditional mutant of *DCR1*

In CaAB002 (*DCR1/dcr1::FRT*), the remaining wild type allele was placed under the regulation of the conditional *MET3* promoter (Care, Trevethick et al. 1999). A 584 bp *DCR1* ORF fragment at the 5' end was amplified from the genome using the primer pair AB31/AB32. A tag was included in the forward primer to N-terminally tag the protein with the V5 epitope. The resultant PCR fragment carried the restriction site for *Age*I (which would later be used to linearize the plasmid) and was digested with the enzymes *Pst*I/*Bgl*II followed by cloning into the plasmid pCaDis to create the plasmid pAB2. The auxotrophic selection marker in pAB2, *URA3* was replaced by *HIS1* to yield the plasmid pAB3. This was then linearized with the enzyme *Age*I and transformed into CaAB002 (*DCR1/dcr1::FRT*) to obtain the strain CaAB003 (*MET3pDCR1/dcr1*). Transformants were selected for histidine prototrophy. Transformants were confirmed by PCR amplification and Southern hybridization using primers and probes as given in Table M1, M4. Presence of the V5 tag was confirmed by western blotting.

4.1.3 Construction of a heterozygous deletion mutant of *AGO1*

Similar to *DCR1*, the first copy of *AGO1* was deleted in the *C. albicans* strain SN148 using the *SAT1* flipper cassette (pSFS2a) (Reuss, Vik et al. 2004). Upstream (US) and Downstream (DS) sequences of *AGO1* ORF (CaCh4 ORF 19.2903, assembly 19/21 identifier) were amplified from the genome using primer pairs NV92/NV93 and NV94/NV95, respectively. They were cloned in pSFS2a as *Kpn*I/*Xho*I and *Sac*II/*Sac*I fragments of 878 bp and 705 bp, to obtain the plasmid pAB4. The clones were confirmed using restriction analysis and PCR amplification using specific primers as mentioned in Table M1. The plasmid was digested with the enzymes *Kpn*I and *Sac*I and the resulting fragment was transformed into *C. albicans* strain SN148. Transformants were selected using nourseothricin resistance (final concentration 100µg/ml). The resulting strain was named CaAB004 (*AGO1/ago1::FRT-NAT-FRT*). To obtain nourseothricin sensitive derivatives, transformants were grown in YPMU as earlier to obtain the strain CaAB005 (*AGO1/ago1::FRT*).

4.1.4 Construction of a homozygous deletion mutant of *AGO1*

In the plasmid pAB4, the region downstream to the *AGO1* ORF, cloned within the enzyme sites *Sac*II/*Sac*I, was excised out by restriction digestion and was replaced with a region within the *AGO1* ORF at its 3' end to yield the plasmid pAB5. This internal homology region of size 238bp was amplified from the genome using primer pair AB3/AB4. The resulting PCR fragment was digested with the enzymes *Sac*II/*Sac*I and cloned into plasmid pAB4. The *Kpn*I/*Sac*I fragment from pAB5 was transformed into CaAB005 (*AGO1/ago1::FRT*) to yield the *AGO1* homozygous deletion mutant CaAB006 (*ago1::FRT-NAT-FRT/ago1::FRT*). Transformants were confirmed by PCR amplification and Southern hybridization using primers and probes as given in Table M1, M4.

4.1.5 Insertion of *URA3* ORF in centromere 7 of the *DCR1* conditional mutant

To integrate the *URA3* ORF in one copy of the centromere 7 in the strain CaAB003 (*MET3pDCR1/dcr1*) the plasmid pCEN7URA3 was used. The plasmid was digested with the enzymes *Hind*III and *Sac*I and the resultant fragment was transformed into CaAB003 (*MET3pDCR1/dcr1*) to yield the strain CaAB007 (*MET3prDCR1/dcr1, CEN7/CEN7::URA3).* Primer pair NV22/KG201 was used to confirm the integration.

4.1.6 Deletion of *AGO1* in a strain having *URA3* inserted in centromere 7

The *URA3* ORF was integrated in one copy of the centromere 7 in *C. albicans* strain SN148 using the plasmid pCEN7URA3 as described above to yield the strain CaAB008 (*CEN7/CEN7::URA3*). In this strain, the first copy of *AGO1* was deleted by transforming with the *Kpn*I/*Sac*I fragment from pAB4 to give rise to the strain CaAB009 (*AGO1/ago1::FRT-NAT-FRT, CEN7/CEN7::URA3*). The gene coding for nourseothricin acetyl transferase (NAT) was then recycled by growing the transformants in YPMU to obtain the strain CaAB010 (*AGO1/ago1::FRT, CEN7/CEN7::URA3*). The second copy of the *AGO1* allele was deleted by transforming CaAB010 (*AGO1/ago1::FRT, CEN7/CEN7::URA3*) with the *Kpn*I/*Sac*I fragment from pAB5 yielding the strain CaAB011 (*ago1::FRT-NAT-FRT/ago1::FRT, CEN7/CEN7::URA3*). The strains were confirmed by PCR amplification and Southern hybridization using primers and probes as given in Table M1, M4.

4.2 Southern hybridization

All deletion strains were confirmed using standard Southern hybridization technique as described in Southern (1975). Genomic DNA was isolated from yeast cells by the glass beads (Sigma G8772) method (Hanna and Xiao 2006) and digested with the enzyme *Eco*RV. Digested DNA was separated on an agarose gel and transferred by means of capillary action onto the Zeta probe membrane (BioRad, 162-0153). The blots were hybridized with specific probes (Table M4), washed, dried and exposed on a scintillation plate. Image viewing and analysis were done using the Phosphorimager (Fujifilm).

4.3 Media, transformation and growth conditions

Except the conditional mutant strain CaAB003 (*MET3pDCR1/dcr1*), all other strains were grown in YPDU (1% yeast extract, 2% peptone, 2% dextrose, 0.01% uracil). CaAB003 (*MET3pDCR1/dcr1*), where *DCR1* is under the regulation of the *MET3* promoter, was grown in Complete Media (CM) as permissive media and CM + 1 mM methionine and 1 mM cysteine as non-permissive media. Recycling of the nourseothricin resistance gene, Nourseothricin Acetyl Transferase (NAT) was done by growing the strains in YPMU (1% yeast extract, 2% peptone, 2% maltose, 0.01% uracil). DNA transformation was performed by standard Lithium acetate transformation method as described in (Daniel Gietz and Woods 2002). All strains were grown at 30°C unless specified otherwise.

4.4 Western Blotting

To confirm expression of the tagged protein from the *MET3* promoter and validate promoter shutdown in non-permissive medium western blotting was carried out. The strain CaAB003 (*MET3pDCR1/dcr1*) was grown for 12-14 h in permissive medium after which it was re-inoculated into permissive and non-permissive media at an initial OD_{600} of 0.2. The cultures were incubated at 30°C and 200 rpm and 8 x 10^7cells were harvested for western blotting every 4 h up till a span of 12 h. Cell lysates were prepared using a modification of the tri-chloroacetic acid (Himedia, GRM7570) method (Link and LaBaer 2011). The proteins were separated by electrophoresis in a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane (Amersham Protran Premium, Cat No.10600003) using electrophoretic transfer. Blocking was done in 5% skimmed milk followed by incubation with anti-V5 antibody (Invitrogen, Cat No. R96025, diluted 1:5000 in 2.5% skimmed milk) overnight at 4°C. Anti-PSTAIRE antibody (Abcam, Cat No. ab10345, diluted 1:5000 in 2.5% skimmed milk) was used to probe for *PSTAIRE,* which served as a loading control. The blots were washed, incubated with secondary antibody washed again and developed using Clarity Western ECL Substrate, Bio-Rad and imaged using VersaDoc imaging system, Bio-Rad.

For analysis of protein levels from cells collected every sub-culturing while growing cells in non-permissive medium longer (see below) the same protocol was followed.

4.5 Growth analysis

4.5.1 Construction of growth curve of *DCR1* conditional mutants

The growth of the *DCR1* conditional mutant strain CaAB003 (*MET3pDCR1/dcr1*) was assayed when the protein was depleted as confirmed through western blotting. To deplete the protein the following experimental design was followed. Conditional mutant strains alongside heterozygous deletion mutants and wild type controls were inoculated into permissive media and allowed to grow for 12-14 h following which they were re-inoculated into permissive and non-permissive media at an initial OD_{600} of 0.2. These cultures were allowed to grow for 12 h after which they were sub-cultured. Cells grown in permissive and non-permissive media were re-inoculated into permissive and non-permissive media, respectively at a starting OD_{600} of 0.2. These were again sub-cultured after being allowed to grow for 12 h. This was done up

to 48 h. At every sub-culturing 12 x 10^7 cells were collected for western blotting and flow cytometry analysis.

At 48 h post transfer into non-permissive medium, the wild type and heterozygous deletion mutant were re-inoculated into non-permissive medium and the conditional mutant into permissive and non-permissive media at a starting OD_{600} of 0.1. These were grown at 30°C and 300 rpm and OD_{600} was measured every half hour using Varioskan Flash (Thermo Fisher Scientific). OD_{600} was plotted against time to obtain the growth curve.

4.5.2 Construction of growth curve of *AGO1* deletion mutants

Wild type, heterozygous and homozygous deletion mutants for *AGO1* were taken and grown in complete medium for 12-14 h and then re-inoculated into YPDU at starting OD_{600} of 0.1. These were grown at 30°C and 300 rpm and OD_{600} was measured every half hour using Varioskan Flash (Thermo Fisher Scientific). OD_{600} was plotted against time to obtain the growth curve.

4.5.3 Evaluation of effect of temperature on growth of *DCR1* conditional mutants and *AGO1* deletion mutants

To study the effect of temperature on the growth of *DCR1* conditional mutants, the mutant strains alongside the heterozygous deletion strain were streaked on permissive (CM) and non-permissive (CM + 2.5 mM methionine and cysteine) media plates and one set was incubated at 30°C while the other at 37°C. To ensure depletion of the protein, the strains were re-streaked from permissive to permissive and non-permissive to non-permissive media plates and incubated at 30°C and 37°C as before for 24-36 h.

The effect of temperature on *DCR1* conditional mutants was also studied in liquid media wherein an assay similar to that described in subheading 5.1. was carried out except that the cultures were incubated at 37°C rather than 30°C.

To see if temperature affects the growth of *AGO1* deletion mutants, wildtype, heterozygous and homozygous deletion strains were streaked on CM plates and one set incubated at 30°C while the other at 37°C for 24-36 h.

4.6 Viability

The viability of the *DCR1* conditional mutants was assayed 48 h post transfer into non-permissive medium. Cultures of wild type, heterozygous deletion mutants and conditional mutants grown in non-permissive medium were taken and diluted to 2 x 10^7 cells per ml. From this, serial dilutions were made diluting 1 in 10 at each step till a dilution of 2 x 10^3 cells per ml was obtained. From each dilution 5 µl was spotted onto a permissive medium plate to obtain spots of 10^5, 10^4, 10^3, 10^2 and 10 cells. The plates were incubated at 30°C for 24-36 h. Viability was evaluated in the same manner for cultures grown at 37°C except that the plates were incubated at 37°C in that case.

4.7 Flow Cytometry

For analysis of genome content of *DCR1* conditional mutants by flow cytometry, cells collected at 48 h post transfer into non-permissive medium were taken. Approximately 3 x 10^7 cells each from wild type grown non-permissive medium and from conditional mutant grown in permissive and non-permissive medium were taken, washed with 1X Phosphate Buffer Saline (PBS) and fixed with 70% ethanol. These were then treated with RNase (Sigma-Aldrich R4875, final concentration 1µg/µl) for 4 h at 37°C. Following this, the cells were stained with Propidium Iodide (Himedia TC252, final concentration 0.005µg/µl) for half an hour. Stained cells were diluted 1 in 4 in 1X PBS, sonicated and analysed using FACS Calibur, Becton Dickinson. This was done for cultures grown at 30°C and 37°C both.

Analysis of genome content of *AGO1* homozygous deletion was carried out in a similar fashion. Wild type, heterozygous and homozygous deletion mutants were inoculated into CM and grown for 12-14 h. From these cultures, secondary cultures were established with a starting OD_{600} of 0.2 and were grown till mid-log phase. Approximately 3 x 10^7 cells were harvested from each culture and processed for flow cytometry analysis as described above.

4.8 Treatment with hydroxyurea and dimethyl sulfoxide

To assess response of *DCR1* conditional mutants and *AGO1* homozygous deletion mutants to the replication inhibitor hydroxyurea (HU) and the oxidizing agent dimethyl sulfoxide (DMSO) spotting assays were carried out. For *DCR1*, cultures of heterozygous deletion mutant and conditional mutants grown in non-permissive medium for 48 h were taken and diluted to a concentration of 2 x 10^7 cells per ml. These were then serially diluted as described for viability and spotted onto HU (final concentration 40 mM) and DMSO (final

concentration 5%) plates made in non-permissive medium. The plates were incubated at 30°C for 36-48 h.

For *AGO1*, wild type, heterozygous and homozygous deletion mutants were inoculated into CM and grown for 12-14 h. From these cultures, secondary cultures were established with a starting OD_{600} of 0.2 and were grown to late log phase. These were then processed as described for *DCR1* above.

4.9 Assaying position-effect variegation at *C. albicans* centromeres

To evaluate whether absence of Dcr1 or Ago1 has any effect on the position effect variegation observed at *C. albicans* centromeres the following experimental design was used. In case of *DCR1*, heterozygous deletion mutants and conditional mutants having *URA3* ORF integrated in one copy of centromere 7 were grown in both permissive and non-permissive media and post 48 h of transfer to non-permissive medium these were diluted as described earlier and spotted onto CM, CM-URA and CM+ FOA plates. For cultures grown in non-permissive medium, methionine and cysteine to a final concentration of 2.5 mM were added to these media. The plates were incubated at 30^{O}C for 36-48 h.

For *AGO1*, heterozygous and homozygous deletion strains having *URA3* ORF integrated in one copy of centromere 7 were grown in YPDU for 12-14 h. From these cultures, secondary cultures were established with a starting OD_{600} of 0.2 and were grown to late log phase. These were then spotted on CM, CM-URA and CM+FOA as described for *DCR1* above.

Table M1: Primers used in the study

Primer	**Sequence (5'→ 3')**	**Description**
AB27	AGTGGTACCTTAGATACTTGTACCTTCCCG	Forward primer for *DCR1* deletion cassette (US cloning)
AB28	ATGCCTCGAGTAATCCCAAACTGAATAAGG TG	Reverse primer for *DCR1* deletion cassette (US cloning)
AB29	ATGCCCGCGGCCAGCTTACCTTACATCTCAG	Forward primer for *DCR1* deletion cassette (DS cloning)
AB30	ATGCGAGCTCGAAGGTAGAAGAGAAGACG ATG	Reverse primer *DCR1* deletion cassette (DS cloning)
AB31	AGCTAGATCTATGGGTAAGCCTATCCCTAA CCCT	Forward primer for *MET3p* construct

	CTCCTCGGTCTCGATTCTACGGGATCCTCATCGTT TTCTCAAAAAAATCTAGC	cloning
AB32	ACGTCTGCAGCCCGGGTGATGCCGTGTCTATCAATGG	Reverse primer for *MET3p* construct cloning
AB7	ATTGCTGTGGATCACGTGCG	Forward primer for integration of *MET3p* construct at the right locus
AB9	GTCATTTATTGGTGGTAACGGTGG	Reverse primer for integration of *MET3p* construct at the right locus
AB16	CCAACCTGCTCTTTGAATAGC	Forward primer for *DCR1* Southern probe
AB17	ACTGCTTTCCAATTCTTGGGAG	Reverse primer for *DCR1* Southern probe
NV92	AGTGGTACCGTTTAAATCCCAACCCAATATTG	Forward primer for *AGO1* deletion cassette (US cloning)
NV93	CAGCTCGAGGTCGGAGGATTGATACAACTAT	Reverse primer for *AGO1* deletion cassette (US cloning)
NV94	TCCCCGCGGGGTAAGCCATGGTAAACAA	Forward primer for *AGO1* deletion cassette (DS cloning)
NV95	ATCGAGCTCGCCAAAGAAACAAACATCTCAAG	Reverse primer for *AGO1* deletion cassette (DS cloning)
AB3	AAGTCCGCGGTGATTTATGTCATACTTTTAGTAGAGCTAC	Forward primer for *AGO1* deletion cassette (internal DS cloning)
AB4	GACTGAGCTCCTAACTATCACCATTAAATATAAAACATGGTG	Reverse primer for *AGO1* deletion cassette (internal DS cloning)
AB5	ATCGAGATCTAGTGATTTGGTTAAATTTTCAACACCAACC	Forward primer to amplify *AGO1* N-Terminus ORF fragment
AB6	ATGCCCCGGGCGAACATCATCAAATGAACATCTAACC	Reverse primer to amplify *AGO1* N-Terminus ORF fragment
AB21	TGATGATTGTGGGTTTGAATC	Forward primer for

		AGO1 Southern probe
AB22	CAAAGTACCTTGATTCATGATACTTC	Reverse primer for *AGO1* Southern probe
NV22	CCTGACACTGTCGTTTCCCATAGC	Forward primer for confirming *URA3* integration
KG201	ATCCTTCTTCTTGGCCACCC	Reverse primer for confirming *URA3* integration

Table M2: Plasmids used in the study

Plasmid name	**Description**
pAB1	Cassette for *DCR1* deletion using external homology regions
pAB2	Cassette for integration of *MET3* promoter with *URA3* as marker
pAB3	Cassette for integration of *MET3* promoter with *HIS1* as marker
pAB4	Cassette for *AGO1* deletion using external homology regions
pAB5	Cassette for *AGO1* deletion using one external and one internal homology region.
pCEN7URA3	Cassette for integration of *URA3* in centromere 7

Table M3: Strains used in the study

Strain name	**Genotype**	**Source/Reference**
SN148	*arg4*/*arg4 leu2*/*leu2 his1*/*his1 ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434}	Noble and Johnson , 2004
CaAB001	*arg4*/*arg4 leu2*/*leu2 his1*/*his1 ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *DCR1/dcr1::FRT-NAT-FRT*	This study
CaAB002	*arg4*/*arg4 leu2*/*leu2 his1*/*his1 ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *DCR1/dcr1::FRT*	This study
CaAB003	*arg4*/*arg4 leu2*/*leu2 his1*/*his1*	This study

	ura3::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *MET3prDCR1/dcr1::FRT*	
CaAB004	*arg4*/*arg4 leu2*/*leu2 his1*/*his1* *ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *AGO1/ago1::FRT-NAT-FRT*	This study
CaAB005	*arg4*/*arg4 leu2*/*leu2 his1*/*his1* *ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *AGO1/ago1::FRT*	This study
CaAB006	*arg4*/*arg4 leu2*/*leu2 his1*/*his1* *ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *ago1::FRT-NAT-FRT/ago1::FRT*	This study
CaAB007	*arg4*/*arg4 leu2*/*leu2 his1*/*his1* *ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *MET3prDCR1/dcr1, CEN7/CEN7::URA3*	This study
CaAB008	*arg4*/*arg4 leu2*/*leu2 his1*/*his1* *ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *CEN7/CEN7::URA3*	This study
CaAB009	*arg4*/*arg4 leu2*/*leu2 his1*/*his1* *ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *AGO1/ago1::FRT-NAT-FRT, CEN7/CEN7::URA3*	This study
CaAB010	*arg4*/*arg4 leu2*/*leu2 his1*/*his1* *ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *AGO1/ago1::FRT, CEN7/CEN7::URA3*	This study
CaAB011	*arg4*/*arg4 leu2*/*leu2 his1*/*his1* *ura3*::*imm*^{434}/*ura3*::*imm*^{434} *iro1*::*imm*^{434}/*iro1*::*imm*^{434} *ago::FRT-NAT-FRT1/ago1::FRT, CEN7/CEN7::URA3*	This study

Table M4: Probes used for Southern hybridization

Experiment	**Enzyme Used**	**Length of probe**
Confirmation of deletion of first copy of *DCR1* and integration of *MET3p* at *DCR1* ORF	*Eco*RV	447bp
Confirmation of homozygous deletion mutant of *AGO1*	*Eco*RV	474bp

CHAPTER 5:
REFERENCES

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