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PRINCIPAL INVESTIGATOR: J. Martin Brown, Ph.D.
James A. Brown, Ph.D.

CONTRACTING ORGANIZATION: Leland Stanford Junior University
Stanford, CA 94305-4125

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14. ABSTRACT We are attempting to identify novel genes in the yeast <i>S. cerevisiae</i> that confer gross chromosomal instability (GCI) a hallmark of most breast cancers when deleted. Using a collection of yeast strains carrying the deletion of a unique open reading frame, we have transfected a yeast artificial chromosome (YAC) as a reporter for GCI frequency to determine the quantitative impact of the loss of each gene function. We have constructed the redesigned reporter with all of the components for selection and maintenance. The URA3 gene results in sensitivity to 5-FOA and the methionine regulated BAX gene induces apoptosis in yeast in individual and pooled deletion strains, and the NatMX (nourseothricin resistance) marker for YAC retention... We have introduced the YAC by mating into the entire collection of deletion strains and are currently running the pooled growth assay to identify genes affecting GCI. The mammalian orthologs of the resulting candidate genes methods. There is a crucial need to find new candidate genes for breast cancer susceptibility in women and identifying these human genes can further improve monitoring and treatment guidelines for women with these mutations.					
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INTRODUCTION

We are attempting to identify novel genes in the yeast *S. cerevisiae* that confer gross chromosomal instability (GCI) a hallmark of most breast cancers when deleted. Using a collection of yeast strains carrying the deletion of a unique open reading frame, we will transfect a yeast artificial chromosome (YAC) as a reporter for GCI frequency and determine the quantitative impact of the loss of each gene function. We have constructed the redesigned reporter with all of the components for selection and maintenance. The *URA3* gene results in sensitivity to 5-FOA and the methionine regulated BAX gene induces death in yeast in individual and pooled deletion strains. We have modified the original YAC to incorporate the NatMX (nourseothricin resistance) marker which has been shown to overcome the defects in the original design. We have transfected the final YAC construct into five different yeast deletion strains with well documented genomic instabilities that span the current spectrum of measurable frequencies. We are transfecting the entire collection of deletion strains to assess the pool of yeast deletion strains to identify genes affecting GCI to determine the mammalian orthologs of these genes as well as those of interacting partners using *in silico* methods. There is a crucial need to find new candidate genes for breast cancer susceptibility in women and identifying these genes can further improve monitoring and treatment guidelines for women with these mutations.

BODY

BACKGROUND

The aim of the research covered under this grant is to employ a novel approach to identify genes that are required to maintain genomic stability in yeast that may potentially identify breast cancer susceptibility genes in humans that are currently unknown and not deducible from current methodologies. It is a fundamental requirement for any cell to invoke its protein armamentarium to maintain genomic stability. Failure results in a myriad of disease states. In fact, breast cancers exhibit a high degree of genomic instability(1). The increase in the occurrence of gross chromosomal rearrangements is a common theme of most malignant tumors(2). A large proportion of cancer susceptibility syndromes are the result of mutations in genes in DNA repair or in cell-cycle checkpoint pathways in response to DNA damage. Examples include ataxia telangiectasia (AT), Fanconi's anemia (FA), Bloom's syndrome (BS), hereditary non-polyposis colon cancer (HNPCC), Nijmegen breakage syndrome (NBS), and xeroderma pigmentosum (XP). Mutations in such genes, termed "caretaker" genes, are likely to lead to an increased mutation rate of all genes including those directly responsible for cancer, the so-called "gatekeeper" genes(3). Mutations in the caretakers lead to what has been termed a "mutator phenotype" which allows accumulation of the multiple mutations needed to produce cancer(4).

One of the most important DNA lesions that the cell has to repair is the double-strand break (DSB), a lesion that can arise following exposure to genotoxic agents such as ionizing radiation (IR) but also as a consequence of normal cellular processes including DNA replication, meiosis and lymphocyte development(5). Eukaryotic cells have two major pathways for repairing DSB's: Non-homologous end-joining (NHEJ) which rejoins the two broken ends, often with significant loss of sequence, and homologous recombination (HR) which uses the homologous chromosome, or chromatid, as a template to faithfully repair the broken strand. In human cancer it is loss of HR, rather than NHEJ, that is more important in increasing cancer susceptibility (6) (as evidenced by mutations in the ATM, BLM, BRCA-1, BRCA-2, hMRE11, and NBS1 genes, all of which are involved in HR). Although yeast and mammalian cells use both HR and NHEJ to repair DSB's induced by DNA damaging agents, their relative importance is different: Mammalian cells primarily use NHEJ to repair

DSB's following IR, whereas yeast primarily use HR. Consequently, mutation of genes affecting HR has a greater impact on radiation sensitivity in yeast than in mammalian cells. A further consequence of the reliance of HR to repair DSB's in yeast is the greater importance of cell-cycle checkpoints in affecting sensitivity to DNA damaging agents in yeast than in mammalian cells. Consequently, yeast (but not mammalian cells) with defects in cell-cycle checkpoints are sensitive to DNA damaging agents, making them excellent models to study defects in these checkpoints.

There is a large degree of homology between genes in yeast and in humans, particularly in the basic cellular processes of DNA repair and cell cycle checkpoints with most of the genes involved being homologous between the two species(7, 8). Consequently, systematically screening yeast for those genes required to minimize gross chromosomal rearrangements will identify those genes which when mutated in humans fail to maintain genomic fidelity, and hence predispose to cancer.

Finally, we are working with a powerful new resource in yeast: Namely pools of strains with homozygous deletion of all non-essential genes (9). This has allowed us to identify all genes whose deletion causes sensitivity to UV and other DNA damaging agents(10). The power of this system is the ability to interrogate the genome for similar phenotypic profiles to identify functional pathways. The impact of this system and the connection between yeast and cancer is strengthened by our finding that two of the new genes that we identified as producing sensitivity to UV radiation have human homologs that are involved in cancer(8). Using this system to directly determine the genes causing genomic instability in yeast will dramatically advance our understanding of the genetic factors leading to cancer progression.

HYPOTHESIS

The hypothesis to be tested is that novel, human cancer susceptibility genes can be identified by determining novel genes in the budding yeast, *S. cerevisiae*, that confer increases in gross chromosomal instability. As this type of genomic instability is a hallmark of most human cancers and mutations in the corresponding mammalian cells orthologs of yeast genes previously shown to confer genomic instability, also cause genomic instability, we anticipate that our method will identify new genes in mammalian cells, inactivation of which causes genomic instability. We further propose that proteins encoded by these genes that have interactions with known genes involved in DNA repair mechanisms, DNA damage checkpoint functions, telomeric maintenance or fidelity of chromosomal integrity will be candidate breast cancer susceptibility genes and will predispose human cell lines to chromosomal aberrations

OBJECTIVES

Our Specific Aims are:

Specific Aim 1: To construct a yeast artificial chromosome which allows for the direct measurement of the occurrence of gross chromosomal rearrangements (YAC-GCR) and transfect it into yeast strains deleted for an individual gene?

Specific Aim 2: To identify novel genes affecting the frequency of gross chromosomal rearrangements in *S. cerevisiae*. Pools of the YAC transected yeast will be grown under non-selective conditions and the rates of spontaneous loss of two individually selectable markers. By hybridization of the DNA from these pools to high density oligonucleotide arrays, we will determine all the genes whose deletion confers increased loss of both markers.

Specific Aim 3: To reconfirm which of the deletion strains in the pooled YAC study also show native chromosomal instability. We will use an established assay for GCR's that we know identifies yeast genes, the human orthologs of which are involved in cancer.

Specific Aim 4: To identify proteins interacting with the proteins encoded by the genes whose deletion produces genomic instability. We will identify interacting proteins by *in silico* methods which exploits the enormous wealth of yeast data of protein-protein interactions identified from systematic yeast two-hybrid studies. Any such proteins which interact with known genes involved in genomic stability or whose homolog is transcriptionally biased in human breast cancer cell lines would go on to further testing.

Specific Aim 5: To determine whether mammalian cells with inhibition of the potential breast cancer susceptibility gene identified in Specific Aims 4 produces genomic instability in the normal human cells in culture. Utilizing the rapidly evolving RNAi technology to selectively reduce the expression of a single gene, we will determine by SKY analysis those candidate genes which demonstrate an increase in chromosomal aberrations.

RESULTS

The specific aims for the first year of the project was to construct a yeast artificial chromosome which allows for the direct measurement of the occurrence of gross chromosomal rearrangements (GCR) and transfect it into yeast strains deleted for an individual gene. We constructed the YAC-GCR as originally proposed consisting of the *URA3* gene that causes sensitivity to 5-FOA and the *HSV-TK* gene which causes sensitivity to ganciclovir as shown in figure1.

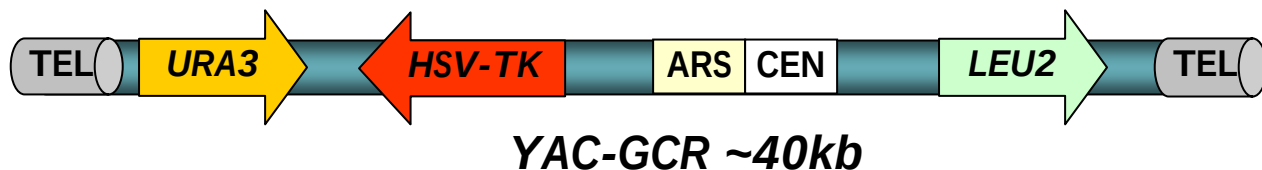


Figure 1: Original design for the yeast artificial chromosome for measuring the rates of gross chromosomal rearrangement in yeast.

In order to determine the dynamic range for this construct, we transfected it into 78 different deletion strains which have a wide spectrum of previously reported GCR frequencies. We tested each strain individually to determine the GCR frequencies derived from the YAC reporter to calibrate these results with a genomically integrated reporter (11). The YAC mediated sensitivity to *URA3* expression in the presence of 5-FOA was highly consistent with previously published results (12). The mutation rates identified with this construct for the resistance to 5-FOA were an order of magnitude higher when compared to the chromosome V inserted *URA3* with an increase linear range making it a very good genome wide screening tool.

The original design called for the use of the *HSV-TK* reporter gene from pTK1 (13) as a negative selection marker. In constructing the YAC we discovered that in contrast to previous reports (14), no inhibition of cells growth was seen with gancyclovir even at very high concentrations which necessitated the use of a different counter-selectable marker. We attempted to use a double *URA3* marker but for reason unclear at present, we were unable to clone the construct in *E. coli*. We obtained the *Serratia* NucA gene Michael Benedik at Texas A&M and cloned it into the YAC under the GAL promoter. Although this worked very well in selecting for loss of the marker, subsequent

amplifications of the barcodes for enumeration failed due to carry over of the nuclease in the media. Recent work by Denko and coworkers who expressed the human ^{hs}BAX gene in yeast demonstrated the induction of apoptotic cell death (15). They used this construct in the yeast deletion pool and identified all of the genes which were resistant to ^{hs}BAX expression. Only the genes required for the regulated expression from the GAL promoter were resistant to ^{hs}BAX. Due to the problems inherent in galactose induction with the concomitant changes in cellular metabolism and growth rates we sought to modify the expression vector in order to better utilize this gene in the YAC assay.

We cloned the *MET15* promoter (16) from genomic DNA as well as the *ADH1* terminator which has been used successfully in many expression vectors (17). In order to positively identify the expression of the ^{hs}BAX protein we also incorporated a FLAG epitope tag for western analysis. The expression plasmid containing the prom*MET15*, FLAG epitope and the term*ADH1* with a useful multiple cloning and restriction sites was constructed for any future expression cassettes which may be required for this or future studies.

The original design for the YAC called for the *LEU2* gene which is essential for growth on media lacking leucine and absent in all of the deletion library strains, to be included on the opposite arm of the YAC. While the YAC acts like an artificial chromosome in that it carries an ARS so that it can replicate, and a centromere to insure equal distribution during mitotic division, there is a greater propensity for complete loss of the YAC when compared to *bona fide* chromosomes carrying large numbers of essential genes. We have measured the complete loss of the YAC with the *LEU2* marker and found it to be unacceptably high. The reasons are unknown but may be due to the long half life of the protein which would persist in daughter cells as well as large internal pools of leucine which permits recycling until critical depletion causes a cell division arrest.

To overcome this limitation we have incorporated the *NatMX* selection marker(18) which encodes nourseothricin N-acetyltransferase which inactivates nourseothricin (ClonNat obtained from Werner BioAgents) which is cytotoxic to the cells by interfering with protein synthesis. When the *NatMX* marker resides on one arm of the YACs, selection is maintained such that less than 0.01% of the viable cells grown in the presence of ClonNat do not carry the *URA3* marker as well. The short half life of this protein and the continuous demand for its activity insures the faithful retention of the YAC in all of the strains to the levels required in this assay.

The current version of the YAC is shown in figure2. We have transfected the wild type diploid strain with the YAC and all of the elements have been tested and are functional in the circular (plasmid-like) as well as linear form (chromosome-like). In order to determine the necessary drug concentrations and the selection parameters for the pooled growth assay, we have transfected five benchmark strains with the YAC and are currently characterizing chromosomal instability.

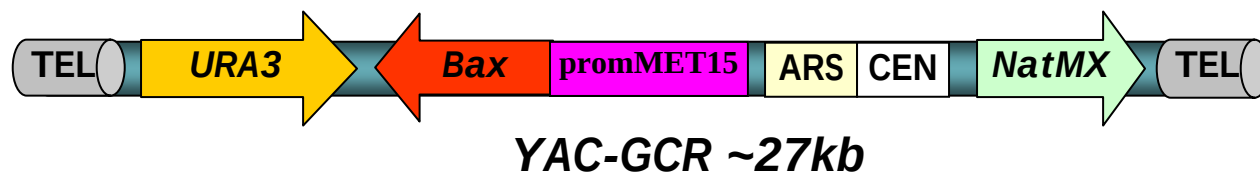


Figure 2. Basic layout of the new yeast artificial chromosomal for measuring the frequency of Gross Chromosomal Rearrangements.

We have transfected in addition to the wild type BY4743 isogenic parental strain, the *yku70Δ/Δ*, *sgs1Δ/Δ*, *mec3Δ/Δ* and *xrs2Δ/Δ* homozygous diploid null strains. They were chosen due to their well studied genomic instability frequencies based on the gold standard ChrV based Kolodner Assay (11). The dynamic range of the ChrV based assay is between 10^{-7} down to the 10^{-10} range of the wild type strain. Greater frequencies have been shown when multiple deletions are combined, as with the *pif1-m1* mutation that increases de-novo telomere additions, but does little to increase our

understanding of the basic mechanisms involved (19). We have independently identified these strains to be true deletions without any obvious genomic defects and the individual gross chromosomal instability frequencies overlap those shown by the Kolodner Lab (11, 20). These five strains BY4743 (3.5×10^{-10}), *yku70* Δ/Δ (1.1×10^{-9}), *sgs1* Δ/Δ (7.7×10^{-9}), *mec3* Δ/Δ (1.9×10^{-8}), and *xrs2* Δ/Δ (1.9×10^{-7}) run the scale from the most stable to the most unstable in the ChrV assay. The magnitude of the rearrangement frequency in the YAC strains is over 2 orders more frequent than the chromosomal reporter. The relative order is preserved with the exception of the *yku70* Δ/Δ strains which shows a much higher instability in the YAC assay comparable to the *mec3* Δ/Δ strain levels whereas in the Kolodner assay it fails to show much instability over that of the wild type strain. We are in the process of characterizing the spectrum of rearrangement events to determine the possible mechanism of this difference. It is however intriguing that haploinsufficiency of Ku86 in human somatic cells resulted in an increase in chromosomal fusions, translocations, and genomic instability suggesting that in some cases the YAC based assay may be an improvement over the Kolodner assay (21).

Transfection of the YAC into the library of individual deletion strains revealed additional concerns regarding the inherent mutagenic events induced by transfection. In the first pass of strain constructions, we transfected two 96-well plates of strains with purified linear YAC with a 74% efficiency. Unfortunately, over half of these carried a YAC which had lost the *URA3* marker indicating a re-arrangement event occurring during transfection. To eliminate this unacceptable level of failed transfections, we designed an alternative method of strain construction.

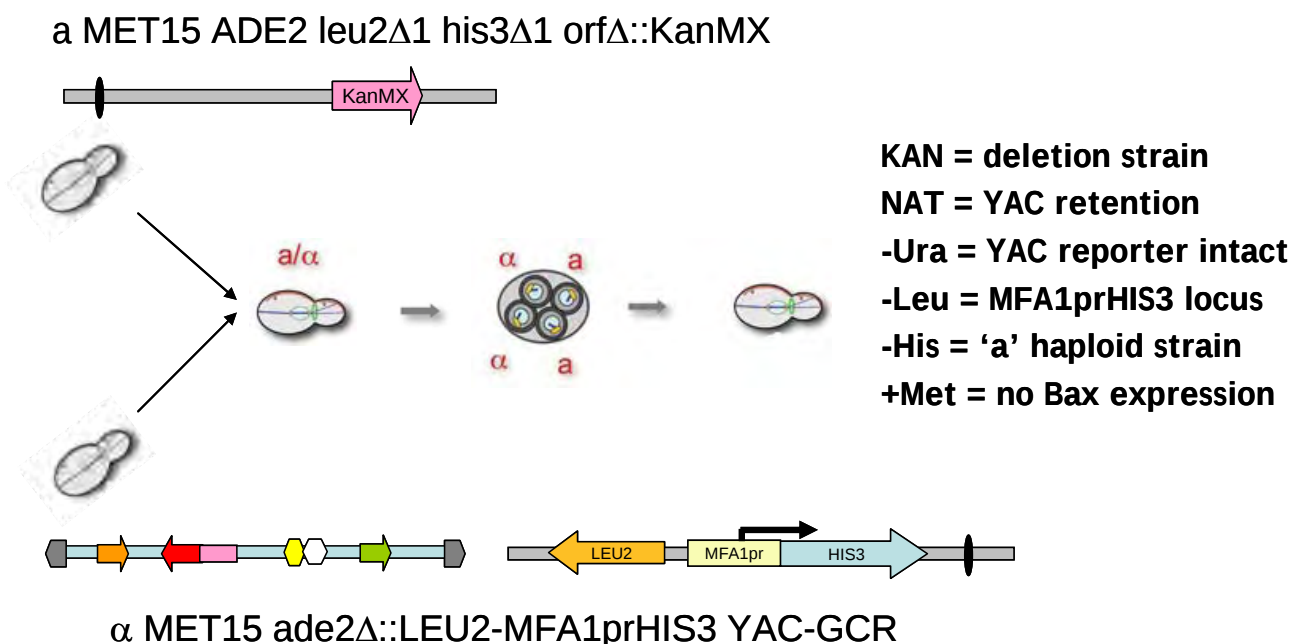


Figure 3. Construction of the YAC deletion library by mating with a donor strain, sporulation and selection of the desired progeny.

The concept, shown in figure 3, was to construct a mating donor strain that would mate with the individual 'alpha' haploid deletion strain library, undergo meiosis and allow for the selection of the desired haploid progeny carrying the deleted ORF and intact YAC in the manner used successfully during the construction of the synthetic lethality screens (22). We constructed an 'a' mating type donor strain which carried a verified YAC, an intact *MET15* allele, and a *LEU2* linked *HIS3* gene under the

tightly regulated *MFA1* promoter. We inserted the *LEU2-MFA1pr-HIS3* construct into the *ADE2* locus.

We replicated the ‘alpha’ mating type deletion library into 96-well plates adding the donor strain to each well. After 6 hours of incubation, the media was changed to select for Nat and Kan resistant diploid only strains. These diploid strains were shifted to starvation media to induce sporulation. Five days after induction of sporulation, the strains were shifted to media containing Nat, Kan and methionine but lacking uracil, leucine and histidine. Only the strains that were of the ‘a’ mating type, carried the intact YAC and the original ORF deletion were capable of growth. This first pass bulk library construct resulted in 3,733 positive strains confirmed random individual strain testing and by PCR amplification of the deletion barcode tags from the entire pool and hybridization to an array. A second pass is underway for the remaining strains. The construction of strains that are mating, sporulation or germination defective as well as those defective in leucine or histidine biosynthesis are not possible with this method and will require individual transfection and isolation followed by verification of the of full length YAC

A pilot experiment with the 96 positive strains was conducted to test the growth and selection parameters of the protocol. However, unlike that seen in plate growth assays, the cells that are unable to convert 5-FOA to 5-FU are still growth inhibited in the mix culture conditions. Our assay is liquid based and the presence of large numbers of stable YAC containing strains results in high levels of 5-FOA conversion to 5-FU. We believe this is the result of 5-FU generated by the non mutated strains being capable of freely diffusing into the media thereby inhibiting growth of the strains unable to convert 5-FOA (23). Trials are underway to minimize this bystander effect through lower concentrations of 5-FOA and frequent media replacements to lower free 5-FU. In addition, we are exploring the efficacy of inserting an interim plating step to minimize diffusion and allow controlled expansion of unstable cells.

We are currently adjusting the assay conditions to maximize the linear range of the YAC assay for individual mutation frequency of the *URA3* gene, frequency of chromosome loss and more importantly the frequency of gross chromosomal instability demonstrated by inactivation of both the *URA3* and ^{hs}*BAX* genes. With this information we can more accurately delineate the contribution each gene deletion has on the frequency of genomic instability.

When we have identified the individual novel genes, progress on specific aim 3 will begin. We have constructed a library of yeast deletion strains which carry the chromosomal GCR reporter developed by the Kolodner lab by a similar “mating-in” method shown above. The donor strain can be used to quickly produce the strains need to proceed with specific aim 3 with the completion of specific aim 2.

KEY RESEARCH ACCOMPLISHMENTS

- Constructed a yeast artificial chromosome (YAC) that is capable of stable retention in the yeast deletion mutants as a backbone for further studies.
- Demonstrated the utility of the *URA3* reporter in the YAC as a means of measuring the mutational frequency of individual deletion strains in a pool.
- Construction of cloning plasmids for the flag-tagged expression of open reading frames in yeast in the absence of methionine.

- Demonstrated the utility of the ^{hs}BAX reporter in the YAC as a means of measuring the mutational frequency of individual deletion strains in a pool.
- Demonstrated the utility of the *NatMX* reporter in maintaining YAC retention in individual deletion strains when grown in a pool as superior to that of a nutritional marker.
- Constructed a donor strain for the efficient transferal of the YAC to the individual deletion strains.
- Transfected benchmark gene deletion strains to optimize the assay conditions for the widest possible linear range.
- Created a library of yeast deletion strains carrying a chromosomal GCR reporter for screening positive strains.
- Created over 75% of the possible individual deletion strains harboring the YAC reporter construct.

REPORTABLE OUTCOMES:

No reportable outcomes at this point

CONCLUSIONS

With the failure of the HSV-TK reporter construct to adequately discriminate between “mutant” high GCR strains and “wild type” survivor strains we have been forced to completely redesign the YAC-GCR. Thorough testing of the individual components, the new ^{hs}BAX reporter construct and the existing *URA3* reporter, has demonstrated the utility of the current design. Replacement of the *LEU2* nutritional marker with the *NatMX* drug resistance marker keeps yeast that have loss the YAC to adequately low background levels. We have completed Specific aim 1 and are optimizing conditions to reproducibly complete specific aim 2. We have created the library of yeast strains harboring a chromosomal GCR reporter to screen the strains identified in specific aim 3.

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