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14. ABSTRACT

Genetic instability is a hallmark of tumor development. Mechanisms for maintenance of genomic stability are heterogeneous and identification of the genes responsible a critical goal of cancer biologists. The very large number of genetic alterations in breast tumors and genetic heterogeneity, even within a single breast tumor, strongly suggests that some mutator mechanism must be involved in breast tumorigenesis. Our hypothesis is that a mutator mechanism contributes to the development of breast cancer. However, since breast tumors do not display an obvious phenotype that signals the presence of a mutator defect (such as microsatellite instability), another scheme to identify defects in repair genes and their targets is necessary. Thus, our first objective is to use a novel yeast model system to identify genes that are previously unrecognized targets of mutator mechanisms and to determine whether these genes are altered in breast tumors. Our second objective is to identify genes that function as novel mutators in the yeast system then evaluate whether any are altered in breast tumors. The identification of mutator genes and their targets that contribute to the etiology of breast cancer will enhance our understanding of the genetic mechanisms involved in initiation and progress of disease. These genes will impact drug and biomarker discovery and ultimately, revolutionize patient care.

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Introduction

Genomic instability in human carcinogenesis

Most tumors display a high degree of intercellular genetic and phenotypic heterogeneity, including gene amplifications, deletions, insertions, rearrangements, and point mutations. The observation that cancer cells have many genetic alterations suggests that they have undergone some form of genomic instability that drives the process of carcinogenesis [1, 2]. Mechanisms of genomic instability are themselves heterogeneous. Because genomic stability is under genetic control, identification of genes that maintain stability remains a goal of cancer biologists.

In normal cells, genomic integrity is maintained by a combination of high fidelity DNA replication processes and multiple mechanisms that recognize and repair DNA damage. The mechanisms for DNA repair are based on the size of the DNA affected during the repair process. Small base changes in DNA are repaired predominantly by base excision repair pathways, bulky adducts are targets for nucleotide excision repair, and the mismatch repair enzymes recognize single mismatched nucleotides and small insertion/deletion loops. Inactivation of DNA repair pathways leads to increased mutation rate; chromosomal instability can initiate and accelerate the neoplastic process. Of these, inactivation of mismatch repair in tumor cells is particularly straightforward to detect, because it results in microsatellite instability (MSI), the hallmark of which is an elevated mutation rate in microsatellite tracts of genomic DNA [3].

DNA mismatch repair

Damage to DNA is minimized by systems that recognize and repair the damage. These systems recognize DNA distortions ranging from single base changes such as deamination of cytosine to uracil to structural distortions such as intrastrand pyrimidine dimmers. In *E. coli*, DNA repair systems are grouped into several repair pathways: the *uvr* excision repair pathway, the *dam* replication mismatch repair pathway, and the *recB* and *recF* recombination and recombination-repair pathways.

Mismatch repair was postulated more than 30 years ago [4, 5]. In *E. coli*, GATC sequences are targets of *dam* methylase which recognizes non-methylated GATC sequences and targets the adenine for methylation. Prior to methylation, the nonmethylated strand is the target for repair of mismatched bases [6]. This repair system consists of several proteins encoded by the *mut* genes [7]. Mismatched bases on the unmethylated strand are recognized by MutS [8]. MutS proteins bind to mismatches as homodimers and are joined by MutL homodimers which play a role in signal transduction. MutS can use two DNA-binding sites one of which specifically recognizes the mismatch; the other is not specific for sequence or structure. ATP hydrolysis translocates the complex along the DNA until a GATC sequence is encountered. Recognition of the GATC site causes MutH to bind the complex. MutH is an endonuclease which cleaves the unmethylated strand. Base excision can occur in either direction. Excision in the 5'-3' direction is mediated by RecJ or endonuclease VII with 3'-5' excision accomplished by the helicase UvrD and exonuclease. Repair is completed when DNA Pol III then synthesizes the new DNA strand which is ligated to the adjacent DNA by DNA ligase.

The mismatch repair system is highly conserved across species including yeast and mammals [6, 9]. In human cells, mismatch repair requires two different heterodimeric complexes of MutS-related proteins, MutSα (MSH2-MSH6) and MutSβ (MSH2-MSH3) [10-13]. These two complexes have different mispair recognition properties. MutSα preferentially recognizes single base mismatches whereas insertion/deletion loops containing 2 to 8 bases are recognized by MutSβ [13-15]. The mammalian MSH4 and MSH5 proteins, like their yeast counterparts, appear to be meiosis specific [16-18]. As in yeast, the central mammalian MutL homolog, MLH1, forms pair wise interactions with the remaining three mammalian MutL homologs [19-21]. The heterodimer of MLH1 and PMS2, MutLα, is the major player in mammalian mismatch

repair. Mammalian PMS1 exhibits homology to yeast MLH2 and MLH3, and the mammalian MLH1/PMS1 heterodimers are referred to as MuLβ [20]. The human homolog of yeast MLH3 has been identified [21]. Expression of a dominant negative MLH3 protein is associated with MSI suggesting that in humans, MLH3 plays a role in mismatch repair.

In humans, the mechanism used to discriminate between DNA strands is less well understood. There is no obvious equivalent to the methylation pattern of GATC residues employed by *E. coli*. The observation that human MSH2 interacts with proliferating cell nuclear antigen (PCNA) has lead to the hypothesis that human mismatch repair may use the termini of Okazaki fragments as the strand discrimination signal [22-24]. Interestingly, homologs of the *E. coli* MutH endonuclease, which binds hemi-methylated GATC sites, have only been found in gram negative bacteria. However, the MED1 protein forms a complex with MLH1, binds to methyl-CpG-containing DNA, has homology to bacterial DNA repair glycosylases/lyases, and displays endonuclease activity [25]. Transfection of a MED1 mutant lacking the methyl binding domain was associated with microsatellite instability. These findings suggested that MED1 is a human DNA repair protein that may be involved in mismatch repair and, as such, may be a candidate eukaryotic homolog of the bacterial mismatch repair endonuclease, MutH. In addition, these results suggested that cytosine methylation may play a role in human DNA repair

A number of other proteins have been implicated in human mismatch repair. Human exonuclease I interacts with MSH2 suggesting that is may be involved in the mismatch repair process [26, 27]. Biochemical analysis indicates that DNA polymerase delta is employed in mismatch repair [28]. The possibility that other DNA polymerases may be involved in mismatch repair has been raised with the identification of several novel DNA polymerases [29, 30].

Mismatch repair proteins and carcinogenesis

Genetically, the most compelling explanation for the strong mutator phenotype in neoplasms is the notion that genes involved in mismatch repair of DNA become inactivated by one or another of genetic and/or epigenetic mechanisms. The ensuing mutator phenotype increases the probability (by random or possibly non-random mechanisms) of inactivation of other DNA repair genes [31, 32]. In some tumors, defects in mismatch repair enzymes lead to errors in the replication of simple nucleotide repeats. Some tumors caused by loss of mismatch repair function are characterized by a microsatellite instability phenotype (MSI) and by mutations in target genes (TGFB RII, IGF2R, BAX) with repeated sequences in their coding regions [33-35]. Loss of function of these target genes contributes to neoplastic progression. That inherited mutations can lead to inactivation of the DNA mismatch repair pathway and tumorigenesis was elegantly demonstrated for hereditary nonpolyposis colon cancer (HNPCC) [36-38]. At least four different mismatch repair genes are mutated in HNPCC families. Among HNPCC families with detected inherited mutations in mismatch repair genes, 60% have a mutation in MSH2 [39-41], 30% in MLH1 [42], and less than 10% in PMS1 or PMS2 [43]. Mice with homozygous deletions in either MSH2 or MLH1 also have increased cancer susceptibility.

In many nonfamilial colon tumors with MSI, somatic mutations have been identified in MSH2, in MLH1, and occasionally in PMS2 and MLH6 [44-46]. However, about half of sporadic colorectal tumors with MSI do not have detectable mutations in one of the known mismatch repair genes [47]. Approximately 20% of sporadic endometrial tumors have the MSI phenotype, but few have mutations in known mismatch repair genes [48]. In Japan, many familial gastric tumors have the MSI phenotype, although these families lack germline mutations in known mismatch repair genes [49]. These observations suggest that other types of mutations or alternative mechanisms of inactivation of known mismatch repair genes, or as-yet-unidentified mismatch repair genes may contribute to the MSI phenotype in these tumors.

Inactivation of mismatch repair genes in some tumors is the result of epigenetic silencing. Down regulation of MLH1 expression due to promoter hypermethylation was demonstrated in sporadic colorectal and ovarian tumors and in mismatch repair-defective cell lines [50-52]. A striking new report has demonstrated that some exogenous mutagens can inactive mismatch repair proteins directly [53]. This is the first clear demonstration of a mutagen promoting genomic instability through interaction with MMR proteins and implores that a more emphasis be given to understanding the mechanisms of environmentally induced genomic instability. The observation that for many MSI tumors the underlying genetic defect in mismatch repair has not been identified suggests that additional mismatch repair genes and targets may exist.

Genomic instability in breast tumorigenesis.
Every breast cancer is the result of multiple genetic alterations. These alterations differ among tumors, and among cells in the same tumor. Karyotypic analysis of breast tumors reveals extraordinary heterogeneity, ranging from perfectly diploid tumors to highly aneuploid tumors with complex multiply translocated and unbalanced chromosomes. At the molecular level, genetic alterations commonly detected in breast tumors include point mutation of P53; loss of function mutations of CDH1 (in lobular breast cancer); amplification of oncogenes such as MYC and ERBB2; and loss of heterozygosity (LOH) associated with genetic deletion at numerous chromosomal locales, including 17p (P53), 17q (BRCA1), and 13q (BRCA2) [54].

Whether, and how, defects in mismatch repair contribute to the pathogenesis of breast cancer remains controversial and important. At the most basic level, the very large number of genetic alterations in breast tumors, and their genetic heterogeneity, strongly suggest that some mutator mechanism must be involved in breast tumorigenesis. In breast cancer, microsatellite instability has been detected in 0-30% of cases [55-60]. However, reports of the MSI phenotype in breast tumors are inconsistent. In HNPCC families, some of the few breast cancers that occur are characterized by MSI [61]. On the other hand, MSI was revealed to be rare by recent studies of very large numbers of sporadic breast tumors, evaluated for abnormal amplification at many simple nucleotide repeat markers [62, 63]. Earlier studies yielded widely varying estimates of MSI frequencies in sporadic breast cancers [64, 65]. It is possible that MSI in breast tumors was not detected because normal cells outnumber cancer cells in some primary breast tumor specimens, and hence that microdissection of tumor tissues might reveal more frequent MSI [66]. Thus, for both technical and biological reasons, we believe it is most likely that MSI, at least as defined for colon tumors, is not a useful marker for genomic instability of breast tumors.

Nevertheless, there is excellent, though indirect, biological evidence for the relevance of a mutator mechanism in human breast cancer. The BRCA1 protein is part of a macromolecular complex termed BASC (BRCA1 associated super complex), which includes MSH2, MLH1, MSH6 and several unknown proteins [67]. In this complex, BRCA1 directly interacts with MSH2 [68]. Given the role of BRCA1 in inherited and sporadic breast cancer and the observation that BRCA1 is part of a macromolecular protein complex that includes many of the known mismatch repair genes, it is reasonable to suggest that disruption of this complex may contribute to genomic instability specifically in breast tumorigenesis.

Hypothesis/Objective
All cells are subject to continual DNA damage. For this reason, elaborate pathways have been developed to monitor DNA damage and to coordinate cell cycle progression with DNA repair. To date, over 70 genes involved in DNA damage surveillance and repair have been identified [69]. These genes include those involved in mismatch repair, homologous recombination, non-homologous end joining, and signaling cascades that respond to DNA damage. Of these, only a few (BRCA1, BRCA2, ATM, CHK2, and P53) have been shown to be associated with breast tumor development [70]. However, the very large number of genetic alterations in breast

tumors, and genetic heterogeneity even within a single breast tumor, strongly suggest that other, as yet, unidentified repair genes must play a role in breast tumorigenesis.

Our proposal assumed that loss of function mutations in mutator genes contribute to the genetic heterogeneity observed in breast tumors. However, since breast tumors do not display a convenient phenotype (such as microsatellite instability) to signal the presence of repair defects, another scheme to identify mutator genes, and their targets, was necessary. Thus, we designed a straight forward, yeast-based screen to identify these two classes of genes. Our rationale for this project was based on the evolutionary conservation between DNA repair systems in yeast and humans. This conservation enabled us to detect and measure, in yeast strains, increases in mutation rates in human tumor suppressor genes due to defects in known mismatch repair genes [71].

Our research plan consisted of two objectives. First, we proposed to use a novel yeast-based screen to identify genes that are previously unrecognized targets of mutator mechanisms. Second, we proposed to use the same yeast-based screen to identify genes that function novel mutators. Our aims included generating a high quality, complex breast cDNA library, using this library in a screen designed to identify genes that are targets of mutators, screening for novel mutator genes using known tumor suppressor genes as targets, and finally, analyzing both target and mutator genes in high-risk breast cancer families and in sporadic tumors.

Body

This final report contains details for all research objectives and progress made. In addition, we include a comprehensive report of preliminary data (all data remains preliminary at this time) and research findings.

Statement of Work for DAMD17-02-1-0615 as actually funded:

Technical objective 1: *Construction of a high quality breast cDNA library*

Months 1-6: Culture normal breast mammary epithelial cells from dissected breast reduction material from premenopausal women. Confirm quality of cultures using immunohistochemical methods. Isolate total RNA, generate poly A+ RNA and convert to cDNA using standard techniques. Clone material into lambda TripLEX. Transduce lambda TripLEX pahgemid library into *E. coli* BNN123 and isolate plasmid DNA.

Studies and Results, June 01, 2002-June 30, 2005

We proposed to construct a high quality normal breast cDNA library using reduction material from 3-4 premenopausal women. Normal mammary epithelial cells were cultured, the quality of the cultures has been confirmed, and poly A+ RNA isolated.

Our rationale for pooling cDNA from different premenopausal women was to normalize the library for genetic contributions that are unique to a particular individual. However, in reviewing our rationale for constructing the breast library solely with cDNA from premenopausal women, we realized that the library should be 'complex' meaning that it should contain cDNA from normal epithelial cells from premenopausal women (as proposed), as well as cDNA from postmenopausal women and breast tumor cell lines. Construction of such a complex breast cDNA library would allow us to perform a more complete screen as mutator target genes may be uniquely expressed following reduction of estrogen levels and/or following initiation of tumorigenesis.

We cultured and isolated poly A+ RNA from a number of breast tumor cell lines including those positive and negative for the estrogen receptor. In addition, we isolated poly A+ RNA from breast tumor cell lines that have no wild-type BRCA1 expression, cell lines that have reduced BRCA1 expression, and cell lines that have wild-type BRCA1 expression. Use of this complex breast cDNA library in our screen would allow for identification of genes that are targets of mutator mechanisms in epithelial cells from premenopausal and postmenopausal women, and from breast tumor cells to be identified.

Technical objective 2: ***In vivo construction of a breast cDNA library in the yeast vector pCI-HA***

Months 7-8: PCR amplify the normal breast cDNA library with PCR primers to facilitate gap repair. Linearize pCI-HA. Use a high efficiency yeast transformation protocol to transform yeast strains deleted for *msh2* and *mlh1* with linearized plasmid and PCR amplified cDNAs.

Studies and Results, June 01, 2002-June 30, 2005

We originally proposed to construct the breast cDNA library during months 7-8. However, this objective was delayed approximately 4 months to allow for the culture of additional breast cDNAs, to make the complex breast cDNA library (see Technical objective 1, Studies and Results, June 01, 2002-June 30, 2005). We have completed construction of the complex breast cDNA library. For reasons outlined below, we have not yet used the library in the proposed screen. Thus, there are no preliminary results to present for this objective.

Technical objective 3: ***Screen for targets of mutator mechanisms in the breast transcriptome.***

Months 9-18: Perform dual plating of the normal breast cDNA library in a yeast strain deleted for *msh2* on -Leu/-Ura and -Leu/FOA plates to constrain mutator phenotype. Replica plate clones onto –Leu plates to allow for mutation of target sequences. Select for clones that have been disrupted due to defective mismatch repair by replica plating onto either –Leu/FOA or –Leu/-Ura. Repeat the screen in the *mlh1* deletion strain.

Studies and Results, June 01, 2002-June 30, 2005

We have obtained yeast strains defective for *msh2* and *mlh1*. We have confirmed that these strains display a mutator phenotype by transforming them with control plasmids (those containing known targets of these mutator genes) and selecting for mutation events by plating transformants on media containing –Leu/FOA. Mutation rates have been determined and agree with published results.

We delayed pursuing this aim following reasons: First, we became aware of two publications that described screens for novel mutator genes [72, 73]. These reports directly impact our proposal. The manuscripts describe the identification of novel mutator genes using approaches that are similar, although not identical to our approach (See technical objective 6 of this proposal). Because others were already publishing their results, we felt that we needed to begin work on objectives 6 and 7 immediately. Second, to our knowledge, no one had performed the type of screen we proposed in objectives 3-5. Finally, because preliminary work on objectives 6 and 7 proved to be highly successful, and, we believe, would have a greater potential to impact breast cancer, we continued to pursue these objectives. We felt that postponing objectives 3-5

and pursuing objectives 6 and 7 worth the risk. Hence, technical objectives 3-5 were postponed indefinitely.

Technical objective 4: ***Identification of target genes and mutations and confirmation of hypermutability in candidate genes***

Studies and Results, July 1 2003-June 30, 2005

Months 19-20: Rescue cDNA clones that are disrupted in *msh2* or *mlh1* strains using standard yeast plasmid rescue protocols. Sequence cDNA inserts to identify the gene and mutation. Use candidate cDNA clones to retransform naïve *msh2* and *mlh1*strains and determine mutation rate using data from several fluctuation analyses.

See above for delay on this aim.

Technical objective 5: ***Analysis of candidate genes in sporadic breast tumors***

Months 21-24: Prepare microdissected material from sporadic and inherited breast tumor samples. Isolate DNA from microdissected tumor and normal tissues (normal breast tissue or blood). Sequence candidate genes in tumor and normal DNAs to identify mutations. Repeat procedure for sporadic ovarian tumor samples.

Studies and Results, July 1 2003-June 30, 2005

See above for delay on this aim.

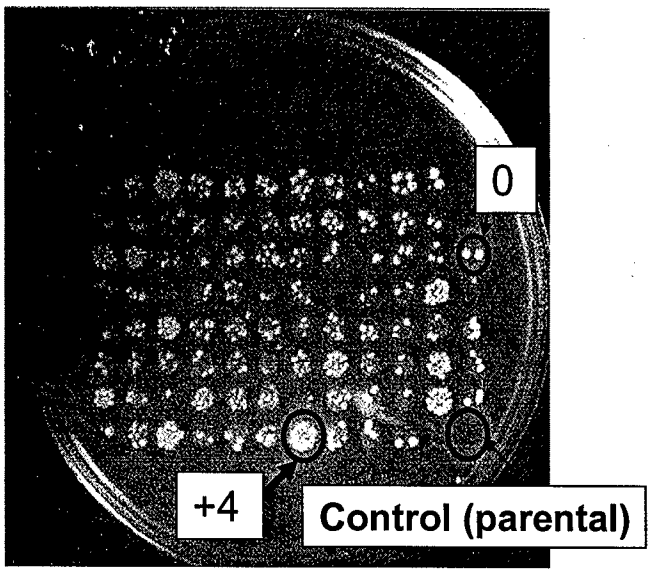
Technical objective 6: ***Screen for novel mutator genes***

Months 25-32: From the haploid set of *Saccharomyces* Genome Project (SGP) deletion strains, remove strains deleted for known mutator genes. Pool remaining clones in subsets. Use high efficiency yeast transformation protocols to introduce plasmid pHJ3. Repeat protocol for plasmids pHJ4 and pHJ9. Identify strains with a mutator phenotype by plating onto –Ura/-Leu to constrain mutator phenotype. Replica plate clones onto –Leu to allow for mutations to occur in target sequences. Select for strains with a mutator phenotype by replica plating onto –Leu/FOA plates. Identify deleted gene in clones which display a mutator phenotype by PCR amplification and sequencing. Determine mutation rates as described.

Studies and Results, July 1 2003-June 30, 2005

We obtained the complete *Saccharomyces cerevisiae* haploid deletion array collection (BY4741: MAT**a** *his3 eu2 met15 ura3*). We did not removed strains deleted for known mutator genes because we realized that these serve as internal positive controls for our screen. We generated 7 pools. Pools A-E contained approximately 700 clones. Pool F contained about 100 slow growing clones. These clones were pooled independently so as to not bias the collection.

We optimized our yeast transformation protocol. This modified, high efficiency protocol was used to transform the pools with plasmid pHJ3. Transformed clones were identified by growth on the appropriate selection media. For each pool, 3000 independent transformants were picked and gridded into 96 well plates. Clones were then replica plated onto media to allow for mutation events to occur. After three days, clones were replica plated onto FOA media to identify FOA^R strains. Strains capable of enhanced growth on FOA due to an acquired mutation in the plasmid target sequence were identified and scored (Figure 1).



Transformants were scored on a scale of 0 to +4 with 0 representing 0-5 colonies per patch, +1 representing 6-12 colonies, +2 represents 13-19 colonies, +3 representing 19-25 colonies, and +4 representing more than 25 colonies or confluent growth. As a control, the parental strain (which does not contain a deletion in any open reading frame) was transformed and carried through the screen (Figure 1). Transformants of the parental strain that grew on FOA media reflect the background mutation rate of the strain. Scoring results for pool A transformants are shown (Table 1).

Figure 1. FOA^R growth of 96 yeast clones transformed with plasmid pHJ3.

Table 1.

Growth score of strain	0	+1	+2	+3	+4
Number of clones	1412	613	145	92	159
% of total clones screened	59%	25%	6%	4%	6%

In the first round of the screen, 396 strains scored +2 or greater, with the majority of strains scored as 0. Interestingly, these results are consist with those reported in a similar screen [72]. All 159 clones with a growth score of +4 were re-analyzed on FOA media to determine if the clones continued to display a mutator phenotype. Of these, approximately half (3.6% of all clones analyzed) appeared to be true mutators. Sequence analysis of these clones revealed a number of interesting candidate genes including those involved in signal transduction, sister chromatid segregation, cytokinesis, sumoylation, neddylation, protein synthesis, and transport. Candidate mutators from pool A are listed in the following table:

Table 2. Candidate mutator genes isolated from pool A.

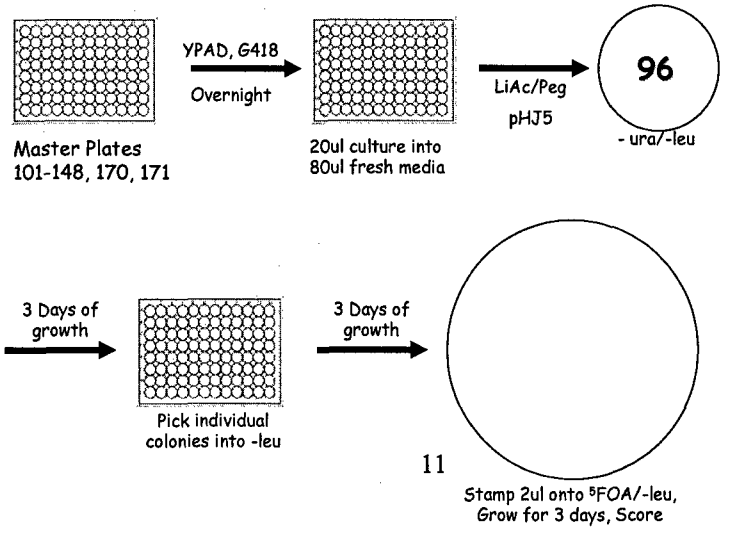
Yeast Gene	**Function**	**Human homolog**
CYK3	SH3-domain protein with possible role in cytokinesis	SH3KBP1
DCS2	protein of unkown function	DCPS
DDP1	member of the MutT family of nucleotide hydrolases	NUDT10
EFT1	elongation factor2	EEF2
ULA1	involved in protein neddylaiton; plays a role in protein degredation	APPBP1
GAC1	regulates protein phosphatase 1	PPP1RC3
IAH1	iso-amyl acetate-hydrolizing esterase	None
LEM1	multidrug resistance transporter	ABCG2
LEU9	alpha-isoproplmalate synthese II	None

NFI1	SUMO-ligase that interacts with UBC9	PIAS4
PPH21	catalytic subunit of protein phosphatase 2A; regulation of mitosis	PPP2CB
RBS1	RNA-binding suppressor of PAS kinase	KIAA1002
SEY1	protein involved in membrane organization and biogenesis	MYH9
SIA1	suppressor of eIF5A	None
SRC1	protein involved in sister chromatid segregation	LEMD2
STE7	signal transducing MAP kinase kinase activity	MAP2K1
TRS33	a subunit of Trapp, a transport complex involved in ER to Golgi trafficking	TRAPPC6B
YRM1	zinc finger transcription factor; activates genes in multidrug resistance	None
YDL124W	hypothetical open reading frame	AKR1A1
YDL172C	hypothetical open reading frame	None
YOR105W	hypothetical open reading frame	None
YOR111W	hypothetical open reading frame	ASMTL
YOR112W	hypothetical open reading frame	SCYL1
YOR118W	hypothetical open reading frame	None
YOR121C	hypothetical open reading frame	None
YOR129C	component of spindle pole body	None
YOR161C	hypothetical open reading frame	C6orf29
YOR164C	hypothetical open reading frame	C7orf20
YOR166C	hypothetical open reading frame	C1orf26

We repeated the screen for pool B. However, upon sequencing candidate mutators from this collection of deletion strains, we found many of the candidate genes identified in pool A were also identified in pool B. Thus, we concluded that in pooling and library, amplification bias had occurred so that all pools consisted of a small collection of yeast deletion strains. In the process of amplifying and pooling the library, only clones that grew well were amplified thus resulting in pools that were biased and only consisted of a few deletion strains.

Upon realizing that the pools consisted of only a few of the over 4000 yeast deletion strains, we realized that we had to develop a high throughput transformation protocol that would allow us to independently transform and screen each of the yeast deletion strains. A flow chart of our methodology is diagramed below in Figure 2.

High Throughput Transformation of Yeast Deletion Strains



Each master plate consisted of 95 independent yeast deletions. Fifty master plates were carried through the protocol. Briefly, following culture overnight in YPAD with G418, cells were diluted into fresh media, allowed to grow for 5 hours. Following incubation, cells were treated with lithium acetate and polyethylene glycol and plated onto selection media. After colonies appeared (3 days) they were manually picked and gridded into –leu media. Cells were stamped onto plates containing FOA after a three day incubation. Cell growth on FOA was scored and recorded as number of FOA^R colonies per patch. Table 3 summarizes our results.

Number of FOA^r colonies	**0-1**	**1-2**	**3-6**	**>6**
Number of strains	**3612**	**912**	**65**	**36**
% of total strains screened	**79**	**19.7**	**1.4**	**0.8**

We were able to successfully screen 99% of the yeast deletion collection (4672 different yeast deletions). Of these, less than 3% (101 strains) exhibited an elevated rate of mutation. The tags sequences for these strains were sequenced to confirm their identity. All candidate mutators strains were confirmed.

In order to verify that deletion of given yeast gene resulted in an elevated mutation rate, fluctuation analysis was performed with the 101 strains that exhibited an elevated rate of mutation. In order to determine mutation rate, single colonies were re-suspended in rich media. An aliquot was plated directly onto an FOA plate while another aliquot was subjected to serial dilution. An aliquot of the final dilution was plated onto a –leu plate. Thus, we were able to calculate mutation rate for a given yeast deletion strain. An example of this procedure is shown in Figure 3.

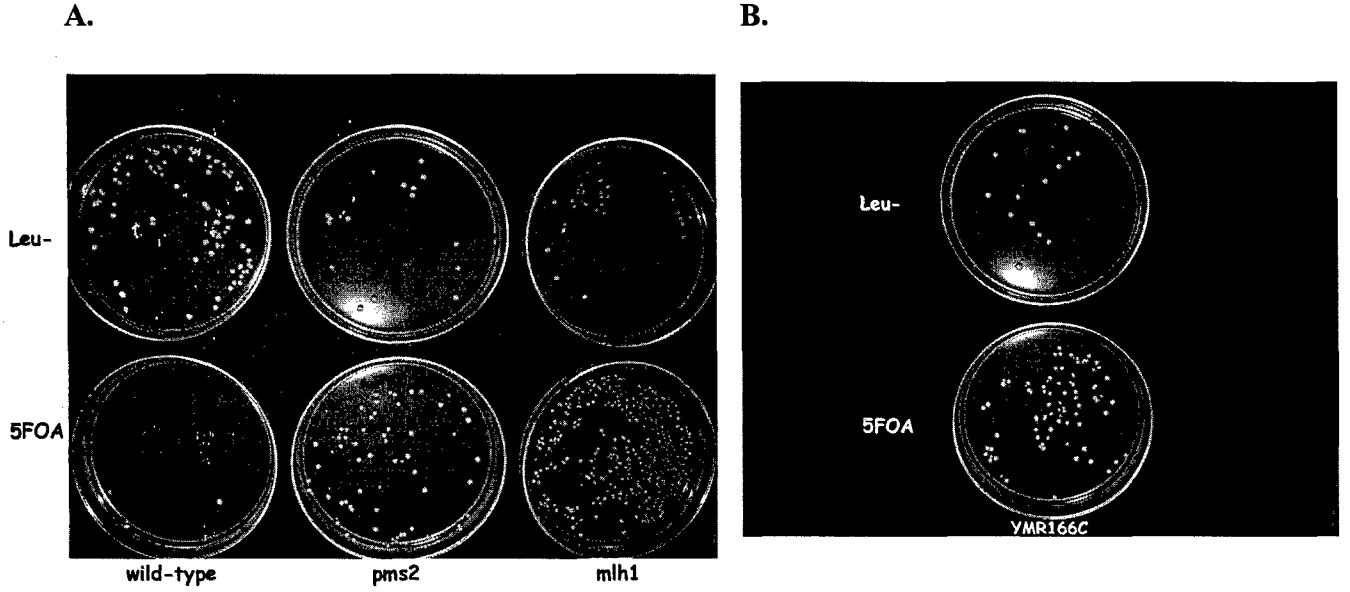


Figure 3. Fluctuation analysis for three yeast deletion strains using plasmid pHJ3.

Total cell numbers for a given yeast colony are determined by plating a serial dilution of cells onto –leu plates. The number of clones, having undergone mutation of the reported plasmid, is revealed by growth on FOA. Panel A contains the wild-type strain and two strains deleted for known mutator genes, pms2 and mlh1. Note the elevated number of FOA^R colonies arising in

strains deleted for known mutator genes. Panel B contains a yeast strain deleted for a gene of unknown function. This gene is a candidate mutator given the elevated mutation rate (compare growth of known mutators pms2 and mlh1 on FOA). Table 4 the results of fluctuation analysis for all candidate mutators. The mutation rate for each yeast deletion strain is given as well as fold induction (of mutation) over wild-type. It also includes the best human homologs for the yeast genes (if known) as well as genomic location.

Table 4. Candidate yeast mutator genes and their human homologs

Yeast Gene	Human homolog	Location	Rate	Induction
YLR454W	KIAA0100	17q11.2	1.5E-06	1.2
YJL188C(RPL39)	RPL39	Xq24	1.6E-06	1.4
ATG20	SNX4	3q21.2	1.7E-06	1.4
RPSOB	LAMR1	3p22.2	1.8E-06	1.5
HXT1	SLC2A8	9q33.3	1.8E-06	1.5
YNL254C			2.0E-06	1.6
NTH2	TREH	11q23.3	2.0E-06	1.7
YLR456W			2.3E-06	1.9
YNL143C			2.5E-06	2.1
YHR095W			2.5E-06	2.1
YJL084C	DSPP	4q22.1	2.5E-06	2.1
YPL014W			2.6E-06	2.2
YNR040W			2.6E-06	2.2
YBR047W			2.7E-06	2.2
YKR065C			2.9E-06	2.4
URK1	UCKL1	20q13.33	2.9E-06	2.4
CHS3	HAS3	16q22.1	3.0E-06	2.5
URA8	CTPS	1p34.2	3.3E-06	2.7
MSH3	MSH3	5q14.1	3.5E-06	2.9
RPS9B	RPS9	19q13.42	3.6E-06	3.0
FUR4			6.3E-06	5.2
DUS1	DUS1L	17q25.3	8.4E-06	7.0
YMR166C	SLC25A26	3p14.1	2.1E-05	17.0
	SLC25A28	10q24.2	2.1E-05	17.0
	SLC25A21	14q11.2	2.1E-05	17.0
	SLC25A13	7q21	2.1E-05	17.0
	MSCP	8p21.2	2.1E-05	17.0
PMS1	PMS2	7p22.1	5.1E-05	42.6
MSH2	MSH2	2p21	1.1E-04	93.0
MLH1	MLH1	3p22.3	1.3E-04	107.1
WT			1.2E-06	-

To confirm that mutations arising in our reporter plasmid were of the sort predicted (insertion or deletion of a CA repeat) and not due to mutation of the URA reporter, we rescued and sequenced our reporter construct from selected strains. For all strains analyzed, the nature of the mutation that silenced the URA reporter gene was either and insertion or deletion of a CA repeat. Thus, the screen as performed, identified candidate mutator genes in yeast that when deleted resulted in an elevated rate of microsatellite instability.

Technical objective 7: ***Analysis of candidate mutator genes in sporadic breast tumors.***

Months 33-36: Use bioinformatic analyses to identify human homologs of novel yeast mutator genes. Prepare microdissected material from sporadic and inherited breast tumor samples. Isolate DNA from microdissected tumor and normal tissues (normal breast tissue or blood). Sequence candidate genes in tumor and normal DNAs to identify mutations. Repeat procedure for sporadic ovarian tumor samples.

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For our initial studies in tumors we chose to evaluate the human homologs of CHS3 and DUS1. Our rationale for choosing to focus on the human homologs of these genes (HAS3 and DUS1L respectively) included the elevated rate of mutation (2.5 sand 7 fold) for the corresponding yeast deletions.

Within tumors there appears to be an intricate balance between hyaluronan (HA) synthesis and degradation where the invading edges display increased HA metabolism. Three eukaryotic HAS isoforms have been identified, termed HAS1, HAS2 and HAS3. These proteins are believed to participate in tumor growth and cancer progression. HAS3 maps to 16q22. LOH of this region is a frequent event in breast cancer [74-76]. In addition, in colon carcinoma cells, HAS3 is upregulated [77] and overexpression of HAS3 in prostrate cancer cells leads to larger tumors in mice [78]. LOH analysis in sporadic breast tumors of the region on 16q containing HAS3 is shown in Table 5. These results indicate that loss of HAS3 is not common in sporadic tumors.

Table 5. LOH of 16q in 41 sporadic breast tumors

Tumor	Grade	ER Status	LOH
1	III	+	
2	III	+	
3	III	+	
4	III	+	
5	III	+	
6	III	+	
7	III	+	
8	III	+	
9	III	+	
10	III	+	
11	III		
12	III		
13	III		
14	III		
15	III		
16	III		
17	III		
18	III	+	
19	III	-	
20	III	-	
21	III	+	+
22	III	+	
23	III	+	
24	I		

25	III		
26	III		
27	III		+
28	III		
29	III		
30	III		
31	III		
32	III		
33	III		
34	III		+
35	III	+	
36	III	+	
37	III	-	
38	III	-	
39	III	-	+
40	III	+	
41	III	+	

All 41 breast tumors were also sequence for the HAS3 gene. No sequence variants other than common SNPs were revealed in this analysis. Thus, it does not appear that mutation of HAS3 is a common event in sporadic breast tumorigenesis.

We have a large collection of high-risk breast cancer families for which no mutation in known breast cancer predisposing genes (BRCA1, BRCA2, CHK2, p53) have been identified. We reasoned that while mutation of HAS3 is not a common event in sporadic breast tumors, inherited mutations in HAS3 may explain some of these families. Genome-wide linkage analysis was performed on many of these families. Families with positive LOD scores at candidate mutator genes are listed in Table 6.

Table 6. Preliminary linkage analysis of CIDR markers in high-risk breast cancer families

Hum. Hom.	Location	CIDR markers	Fams with positive LODs
KIAA0100	17q11.2	D17S975; D17S1880	41, 267, 284, 554, 568,689, 711, 910, 778, 821, 832, 43, 62, 472, 54, 61, 94,248
RPL39	Xq24	CXS1725; DXS1001	41, 60, 568, 579, 16,689, 778, 804, 831, 821
SNX4	3q21.2	D3S4523; D3S1764	41, 60, 267, 711, 681, 737, 821,55, 94, 248, 265, 472,
LAMR1	3p22.2	D3S1768; D3S2409	43, 47, 94, 265, 472, 512, 590, 267, 284, 554, 568, 579, 689, 737, 16, 910, 821, 831, 832
SLC2A8	9q33.3	D9S1825; D9S2157	450, 681, 689, 16, 47, 55, 248, 265, 472, 590
TREH	11q23.3	D11S1998; D11S4464	590, 43, 47, 55, 61, 94, 265, 472, 512, 821, 831, 41, 60, 267, 284, 450, 554, 681, 737, 910
DSPP	4q22.1		
UCKL1	20q13.33	D20S451	60, 284, 568, 689, 711, 804, 62,

			512,
HAS3	16q22.1	D16S1385; D16S2624	592, 43, 47, 54, 57, 94, 512, 41, 60, 284, 554, 579, 711, 783, 16, 910
CTPS	1p34.2	D1S255; D1S3721	43, 94, 590, 592, 284, 554, 568, 579, 681, 689, 711, 737, 783, 16, 804, 821, 831, 898
MSH3	5q14.1		
RPS9	19q13.42	D19S589; D19S254	821, 832, 804, 267, 284, 450, 681, 783, 16, 47, 54, 62, 590
DUS1L	17q25.3	D17S784; D17S928	284, 450, 579, 681, 711, 737, 16, 778, 804, 832, 898, 47, 54, 55, 265, 590, 592
SLC25A26	3p14.1	D3S4542; D3S2406	592, 590, 43, 47, 54, 55, 62, 94, 248, 265, 821, 832, 41, 450, 554, 568 16, 778
SLC25A28	10q24.2	D10S2470: D10S1239	267, 284, 450, 554, 579, 681, 16, 910, 804, 831, 832
SLC25A21	14q11.2	D14S599; D14S306	284, 554, 568, 689, 711, 783, 910, 778, 804, 821
SLC25A13	7q21	D7S2212: D7S821	832, 910, 16, 737, 711, 689, 579, 568, 450, 284, 267, 60, 47, 62, 94, 512,
MSCP	8p21.2	D8S560; D8S1771	590, 43, 54, 55, 248, 472, 512, 41, 60, 284, 579, 711, 737, 778, 804
PMS2	7p22.1		
MSH2	2p21		
MLH1	3p22.3		

Fine linkage mapping of the region on chromosome 16 containing HAS3 was performed. Four families showed strong linkage to HAS3. The complete HAS3 coding sequence was determined for the proband for each of these families. No mutations in HAS3 were identified.

In addition to sequencing HAS3 in high-risk families for which we have genome-wide linkage data, we also sequenced HAS3 in the proband from 94 high-risk breast cancer families. All exons for HAS3 were wild-type sequence for all cancer family probands. Our sequencing analysis of HAS3 revealed a rare SNP in exon 4 variant 1. Results of this analysis are shown in Table 7.

Table 7. Sequence analysis of HAS3 in high-risk breast cancer family probands.

BC Family	ex2	ex3	ex4	ex4v1	ex4v2	ex4v3
1	WT	WT	WT	WT	WT	WT
2	WT	WT	WT	WT	WT	WT
3	WT	WT	WT	WT	WT	WT
4	WT	WT	WT	WT	WT	WT
5	WT	WT	WT	WT	WT	WT
6	WT	WT	WT	WT	WT	WT
7	WT	WT	WT	WT	WT	WT
8	WT	WT	WT	WT	WT	WT
9	WT	WT	WT	WT	WT	WT
10	WT	WT	WT	WT	WT	WT
11	WT	WT	WT	WT	WT	WT
12	WT	WT	WT	WT	WT	WT
13	WT	WT	WT	WT	WT	WT
14	WT	WT	WT	WT	WT	WT
15	WT	WT	WT	WT	WT	WT
16	WT	WT	WT	WT	WT	WT
17	WT	WT	WT	WT	WT	WT
18	WT	WT	WT	WT	WT	WT

19	WT	WT	WT	WT	WT	WT
20	WT	WT	WT	WT	WT	WT
21	WT	WT	WT	WT	WT	WT
22	WT	WT	WT	WT	WT	WT
23	WT	WT	WT	WT	WT	WT
24	WT	WT	WT	WT	WT	WT
25	WT	WT	WT	WT	WT	WT
26	WT	WT	WT	WT	WT	WT
27	WT	WT	WT	WT	WT	WT
28	WT	WT	WT	WT	WT	WT
29	WT	WT	WT	WT	WT	WT
30	WT	WT	WT	WT	WT	WT
31	WT	WT	WT	WT	WT	WT
32	WT	WT	WT	WT	WT	WT
33	WT	WT	WT	WT	WT	WT
34	WT	WT	WT	7707 A/G	WT	WT
35	WT	WT	WT	WT	WT	WT
36	WT	WT	WT	WT	WT	WT
37	WT	WT	WT	WT	WT	WT
38	WT	WT	WT	WT	WT	WT
39	WT	WT	WT	WT	WT	WT
40	WT	WT	WT	7707 A/G	WT	WT
41	WT	WT	WT	WT	WT	WT
42	WT	WT	WT	WT	WT	WT
43	WT	WT	WT	WT	WT	WT
44	WT	WT	WT	WT	WT	WT
45	WT	WT	WT	WT	WT	WT
46	WT	WT	WT	WT	WT	WT
47	WT	WT	WT	WT	WT	WT
48	WT	WT	WT	WT	WT	WT
49	WT	WT	WT	WT	WT	WT
50	WT	WT	WT	WT	WT	WT
51	WT	WT	WT	WT	WT	WT
52	WT	WT	WT	WT	WT	WT
53	WT	WT	WT	WT	WT	WT
54	WT	WT	WT	WT	WT	WT
55	WT	WT	WT	WT	WT	WT
56	WT	WT	WT	WT	WT	WT
57	WT	WT	WT	WT	WT	WT
58	WT	WT	WT	WT	WT	WT
59	WT	WT	WT	WT	WT	WT
60	WT	WT	WT	WT	WT	WT
61	WT	WT	WT	WT	WT	WT
62	WT	WT	WT	WT	WT	WT
63	WT	WT	WT	WT	WT	WT
64	WT	WT	WT	WT	WT	WT
65	WT	WT	WT	WT	WT	WT
66	WT	WT	WT	WT	WT	WT

67	WT	WT	WT	WT	WT	WT
68	WT	WT	WT	WT	WT	WT
69	WT	WT	WT	WT	WT	WT
70	WT	WT	WT	WT	WT	WT
71	WT	WT	WT	WT	WT	WT
72	WT	WT	WT	WT	WT	WT
73	WT	WT	WT	WT	WT	WT
74	WT	WT	WT	WT	WT	WT
75	WT	WT	WT	WT	WT	WT
76	WT	WT	WT	WT	WT	WT
77	WT	WT	WT	WT	WT	WT
78	WT	WT	WT	WT	WT	WT
79	WT	WT	WT	WT	WT	WT
80	WT	WT	WT	WT	WT	WT
81	WT	WT	WT	WT	WT	WT
82	WT	WT	WT	WT	WT	WT
83	WT	WT	WT	WT	WT	WT
84	WT	WT	WT	WT	WT	WT
85	WT	WT	WT	WT	WT	WT
86	WT	WT	WT	WT	WT	WT
87	WT	WT	WT	WT	WT	WT
88	WT	WT	WT	WT	WT	WT
89	WT	WT	WT	WT	WT	WT
90	WT	WT	WT	WT	WT	WT
91	WT	WT	WT	WT	WT	WT
92	WT	WT	WT	WT	WT	WT
93	WT	WT	W	WT	WT	WT
94	WT	WT	W	WT	WT	WT
95	WT	WT	W	WT	WT	WT

In summary, our analysis of HAS3 in sporadic breast tumors and in high-risk breast cancer families did not reveal any variants of the gene sequence that would implicate it in breast tumorigenesis.

In yeast, DUS1 encodes a tRNA dihydrouridine synthase, an enzyme that catalyzes the synthesis of dihydrouridine, a modified base found in the D-loop of most tRNAs. DUS1L maps to chromosome 17q. Frequent allelic losses within chromosomal band 17q25.1 in a variety of human cancers have suggested the presence of one or more tumor suppressor genes in this region. Evaluation of aberrations in breast cancers often reveals loss of 17q25-ter [79]. We evaluated linkage of 17q25, which contains the human homolog of the yeast DUS1 gene, DUS1L, in high-risk breast cancer families. No families from our collection showed strong linkage to this region on chromosome 17 and thus sequence analysis of DUS1L was not perused in this sample set.

Key Research Accomplishments:

- We have cultured and confirmed the quality of poly A+ RNA from cultured normal mammary epithelial cells from pre- and postmenopausal women. In addition, we have cultured and confirmed the quality of poly A+ RNA from a variety of breast tumor cells lines

- We have constructed a high quality complex breast cDNA library using poly A+ RNA isolated from normal mammary epithelial cells from pre- and postmenopausal women and from various breast tumor cell lines.

- We have confirmed the mutator phenotype of yeast strains defective in *msh2* and *mlh1*.

- We have developed a successful high-through put screening protocol that has allowed us to successfully screen 4625 individual yeast deletion strains and evaluate them for elevated rates of mutation.

- We have completed a screen of the haploid set of SGP deletion strains. Approximately 0.5% of all clones analyzed were positive in our screen and thus are candidate mutators. This has been an incredibly successful screen generating a significant number of biologically interesting candidate genes. We will continue to evaluate these genes in sporadic breast tumors and in high-risk breast cancer families to determine if they play a role in development of inherited and/or sporadic disease.

- We have evaluated HAS3 in high-risk breast cancer families and in sporadic tumors. To date, no mutations in HAS3 have been identified in our cases or in sporadic tumors.

- We have evaluated linked to DUS1L in high-risk breast cancer families. No families revealed strong linkage for this region on 17q25.

- We have continued to expand our breast tissue bank. To date, our bank consists of over 400 matched breast tumor and normal tissue samples.

Reportable Outcomes:

We report here the progress this three-year award. All results remain preliminary thus we have no reportable outcomes. Given that the screen for novel mutator genes is completed and we have begun evaluation of candidate genes, we are preparing a manuscript detailing the work described to date to e submitted in the fall of 2005.

The research described here was high-risk. Because analysis of candidate mutators in families and tumors is still on going, we can not determine the final outcome. If we do not reveal novel breast cancer genes, this body of work will still be of great interest to cancer researchers in other fields and thus anticipate publication in a high-impact journal.

Conclusions:

Tumor development is the result of an imbalance between mechanisms controlling gene regulation and genomic stability. Genomic stability is under genetic control. Thus, identification of genes that maintain stability is a goal of cancer biologists. Because a mutator mechanism contributes to the development of breast cancer, we have initiated research designed to identify heretofore, unrecognized targets of mutator mechanisms as well as novel mutators and to determine whether these genes are altered in breast tumors.

The scope of our research includes the evaluation of newly identified genes to determine whether any are altered in breast tumors. Discovery and functional assessment of these genes is essential for understanding the biology of breast cancer and for clinical applications, including

identification of therapeutic targets, early breast cancer detection and improved prediction of breast cancer risk and disease course. If, when we complete evaluation of all candidate mutator genes we have identified in our screen, we discover novel mutators with consequences for breast tumor development, we will open new pathways for investigation into detection and treatment of breast cancer.

There are no publications to report at this time. A manuscript describing the screen and preliminary characterization of candidate mutator genes is currently being prepared.

This work was presented at the 2005 Era of Hope Meeting held in Philadelphia, PA. The abstract is attached.

List of personnel receiving salary from this work:

Piri L. Welcsh, PhD

Krister Freese, BA

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A novel functional screen for mutator genes in breast cancer

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Genetic instability is a hallmark of tumor development. Mechanisms for maintenance of genomic stability are heterogeneous and identification of the genes responsible is a critical goal of cancer biologists. The very large number of genetic alterations in breast tumors and genetic heterogeneity, even within a single breast tumor, strongly suggest that some mutator mechanism may be involved in breast tumorigenesis. Our hypothesis is that a mutator mechanism contributes to the development of breast cancer. However, since breast tumors do not display an obvious phenotype (such as microsatellite instability) that signals the presence of a mutator defect, another scheme to identify defects in repair genes is necessary. DNA repair systems are highly conserved across species including yeast and mammals. Thus, we developed a functional genomics screen to identify novel genes in yeast required for suppression of mutations in reporter plasmids. In our genomewide screen of a collection of over 4500 yeast gene deletion mutants we identified 86 genes that influence genomic stability. Analysis of these deletion mutants revealed that we have (1) identified most of the known mutator mutants including *mlh1, msh2, msh3,* and *psm1* suggesting that the screen was effective. Genes with known function are involved in DNA metabolism, cell cycle signaling and regulation, cellular transport, amino acid synthesis, and transcription. The screen also revealed genes not previously known to be involved in suppressing mutation. Among the 82 previously uncharacterized genes were 34 open reading frames of unknown function. The large number of uncharacterized ORFs suggests that many genes critical to maintaining genomic stability remain to be described. Strikingly, most (81%) of the yeast genes with known function had readily identifiable human homologs, indicating that these genes are involved in important, evolutionarily conserved functions. Our results provide a global view of the nonessential genes involved in protecting against mutagenesis. We are currently analyzing the human homologs of these novel yeast mutator genes in high-risk breast cancer families and in sporadic breast tumors. Identification and characterization of these genes in families and tumors should provide useful clues to understanding cancer predisposition in these families and the consequences of inactivation of these mutator genes to breast tumorigenesis in general.