Published in final edited form as:

Mutat Res. 2018 November ; 835: 16–20. doi:10.1016/j.mrgentox.2018.08.007.

DNA damaging agents trigger the expression of the *HML* silent mating type locus in *Saccharomyces cerevisiae*

Michael Derevensky and

College of Nanoscale Sciences and Engineering SUNY Polytechnic Institute

Michael Fasullo*

College of Nanoscale Sciences and Engineering SUNY Polytechnic Institute

Abstract

Many DNA damaging agents also react with RNA and protein, and could thus cause epigenetic as well as genotoxic changes. To investigate which DNA damaging agents alter epigenetic states, we studied the chemical-induced changes in expression of the yeast silent mating type locus *HML α* , which can be triggered by inhibiting yeast Sir2. We observed that the alkylating agent methyl methane sulfonate (MMS) can result in *HML α* expression, using a colony sector assay that results from expression of a *HML*-positioned *cre* gene. Using single-cell imaging we also observed that alkylating agents, including MMS and methyl-3-nitro-1-nitrosoguanidine (MNNG), as well as short-wave UV, also decreased *HML* silencing. We suggest that chemical-induced alterations in heterochromatin structure could confer transient phenotypic changes that affect the cellular responses to DNA damaging agents.

Keywords

Epigenetics; budding yeast; DNA damaging agent; Sir-mediated gene silencing

1. Introduction

Many potent carcinogens epigenetically alter gene expression by DNA methylation or by affecting the posttranslational modifications of histones and transcription factors. These carcinogens include metals, such as arsenic, cadmium, and nickel, and organic compounds such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), [1]. Recently, increased focus has turned to the epigenetic effects of carcinogens with established genotoxic effects, such as 1,3 butadiene [2], bisphenol A (BPA) [3], and benzene [4,5]. However, the epigenetic alterations caused by many genotoxic agents, including alkylating agents, have yet to be fully established [6].

*Correspondence: 257 Fuller Road Albany, New York 12205 (518) 956-7385 mfasullo@sunypoly.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest
None

Inhibition of deacetylases, such as the evolutionarily conserved Sirtuins, can trigger epigenetic changes, which in turn, can promote carcinogenesis. SIRT1, a homologue of the *Saccharomyces cerevisiae* (budding yeast) *SIR2*, encodes a deacetylase with a broad range of substrates, including NF- κ B, c-Myc Ap-1, and p53, resulting in inhibition. Thus, SIRT1 can function to suppress inflammation as a tumor suppressor, while its role as a tumor promoter has been postulated but is controversial [7]. To gain a better understanding of chemicals that may inhibit Sirtuins, we chose to study expression of the yeast silent mating type locus, which is inhibited by yeast Sir2, along with Sir3 and Sir4 [8].

Sir2 is an NAD-dependent histone deacetylase that is required for silencing of genes at the yeast mating type loci and telomeres [8]. Inhibition of Sir2 results in accelerated cell aging, while introduction of an additional Sir2 gene into the genome is associated with increased lifespan [9]. Commonly-used techniques for evaluating epigenetic response to environmental chemicals may fail to detect transitory alterations in gene expression, which may occur at telomeres and silenced loci. We used yeast strains that detect transient gene expression changes by an easily measured and visible phenotype, described by Dodson and Rine [10]. Briefly, these strains contain the *cre* gene within the silent mating type cassette (*HML α*); epigenetic silencing requires *SIR2*. The gene encoding green fluorescent protein (GFP) is positioned at an unlinked locus, and its promoter is blocked by an insert containing the gene for red fluorescent protein (RFP) and flanked by loxP sites. Transient loss-of-silencing events result in expression of *cre*, which catalyzes recombination at the loxP sites, removing the RFP and resulting in constitutive expression of GFP. We used these strains to show that alkylating agents can trigger loss of silencing using fluorescent imaging microscopy and flow cytometry.

2. Methods

2.1 Media, chemicals, and strains

Standard media was used to culture yeast strains. SC-TRP contained 6.71g/L yeast nitrogen base (YNB), 0.74g/L amino acid supplement lacking tryptophan (Sunrise Science), and 2% glucose (Fisher Scientific); plates contained 2% agar (Fisher Scientific). Cells were grown in media lacking tryptophan, since nicotinamide, a tryptophan metabolite [11], is a Sir2 inhibitor. Chemical genotoxic agents included methyl the alkylating agents methanesulfonate (MMS, Sigma Aldrich) and methyl-3-nitro-1-nitrosoguanidine (MNNG, Sigma Aldrich), and the UV-mimetic agent 4-nitroquinoline 1-oxide (4NQO, Sigma Aldrich). Nicotinamide was purchased from Sigma Aldrich. Yeast α -factor was purchased from Sigma Aldrich. Zeocin (Invivogen, 1 mg/ml) was a gift of T. Begley.

Yeast strains used in this study included JRY9628 and JRY9627, which were a generous gift of J. Rine. In JRY9628, loss of Sir-mediated silencing at the *HML α* locus results in expression of Cre recombinase, which, in turn, catalyzes excision of an ectopically placed gene encoding red fluorescent protein (RFP); this results in constitutive expression of the gene encoding green fluorescent protein (GFP). Strain JRY9627 is identical to JRY9628 but lacks *cre* at the *HML α* . The genotypes of JRY9628 and JRY9627 are *mat ::natMX lys2 his3-11,15 leu2-3,112 can1-100 HML α - α 2 ::cre ura3 ::PGPD-loxP-yEmRFP-TCYC1-kanMX-loxP-yEGFP-T_{ADH1}* and *mat ::natMX lys2 his3-11,15 leu2-3,112 can1-100*

ura3 ::P_{GPD}-loxP-yEmRFP-TCYC1-kanMX-loxP-yEGFP-TADH1, respectively. The genotype of YB226, which was used a GFP negative control, is *MATa-inc trp1 ura3 his3 ade2 rad4::KanMX rad51::URA3*.

2.2 Exposure to chemical agents

Cells were acutely exposed to chemical agents in liquid medium or chronically exposed to chemical agents in either plates or liquid. For both protocols, single colonies of JRY9627 or JRY9628 were inoculated in 2 mL of SC-TRP liquid media and incubated overnight at 30°C. For acute exposure, cells were exposed to the chemical agent for three hours at 30°C, then washed in sterile water, and re-suspended in fresh SC-TRP and incubated for an additional 24 hours at 30°C. For measuring effects of acute exposure in unbudded cells, diluted log phase cells (10^6) were exposed to 10^{-4} M α -factor for 90 minutes and unbudded cells were visualized in the light microscope. For chronic exposures, $\sim 2 \times 10^5$ cells were inoculated in 2 mL of SC-TRP liquid media and exposed to chemicals in a 30°C incubator for 72 hours.

For chronic exposures on plates, approximately 100 cells were inoculated on each -TRP agar plate supplemented with the appropriate chemical and incubated for 72 hours at 30°C. Colonies were observed with a Nikon SMZ800 low magnification fluorescent microscope to determine GFP expression. Colonies with one quarter, one half, or three quarters green fluorescence were considered 'sectored,' indicating a loss-of-silencing event on the first and/or second division. Statistical significance was determined by the two-tailed t-test.

2.3 Exposure to radiation

Cells were plated on SC-TRP agar, and then exposed to UV radiation at $2\text{J}/\text{M}^2$ for 0, 20, 40, and 60 seconds at 254 nm (short wavelength). Following irradiation, the plates were shielded from light until the end of the 72 hour incubation period at 30°C. After incubation, colonies were selected at random, inoculated in PBS, and analyzed using the Amnis ImageStream^x.

2.4 Measuring GFP Fluorescence by Flow Cytometry

For assessing cell fluorescence using the AMNIS ImageStream^x, cells were resuspended in phosphate-buffered saline (PBS); samples contained approximately 10^4 cells. Cell shape and GFP fluorescence was measured with both a visible light and a 495 nm laser, using the image stream software to calibrate cell shape and fluorescence. Fluorescence was excited with a 50.00 mW laser at 488 nm and collected using 40x magnification with cell classification set between 5 and 125 μm . Data was analyzed using IDEAS software with parameters developed from a negative control strain which lacks any GFP signal (YB226), and nicotinamide-exposed JRY9628 positive control. The net change in GFP signal was obtained by subtracting the positive GFP unexposed cells from that of the exposed sample. Statistical significance was determined by the two-tailed t-test.

3. Results

3.1 MMS chronic and acute exposure decreases HML silencing as indicated by colony sectors

We measured chemical-induced gene expression changes using a GFP reporter that is constitutively expressed when SIR-mediated silencing of *HML* is inhibited. In JRY9628, *cre* is positioned at the *HML* locus (Figure 1). The expression of this GFP reporter is blocked by an intervening sequence containing RFP and flanked by loxP sites; thus, inhibition of silencing results in expression of Cre recombinase and excision of the intervening sequences. Cells were directly inoculated on SC-TRP plates containing MMS and colony sectors were counted. Among cells that were not exposed to any chemical agent, approximately 5–10% of the resulting yeast colonies exhibit fluorescence ($n=3$). As a positive control, we first verified that nicotinamide exposure resulted in 100% of the yeast colonies expressing GFP. As a negative control we exposed JRY1927 cells, which lacked *cre* at *HML*, to 0.005% and 0.01% MMS and no sectors were observed ($< .04\%$, 473 colonies). However, in JYR1928 cells that contained *cre* at *HML*, after exposure to 0.01% MMS for three days, the percent of sectorized colonies increased to 10%, approximately a three-fold increase. Colonies derived from unbudded (α -factor arrested) cells acutely exposed to 0.01% MMS also exhibited about a three-fold increase ($1.9\%/0.6\%$), compared to the control ($N=3$, $P=0.003$). These studies indicated that chronic and acute exposures to MMS increased *HML* expression, as indicated by colony sectoring.

Considering that MMS is considered a radiomimetic agent but does not directly cause double-strand breaks, we also measured increases in sectoring after chronic exposure to zeocin ($30\mu\text{g/ml}$), an agent which does induce double-strand breaks. We observed sectoring in $(0.6 \pm 0.2)\%$ colonies ($N=3$, 522 colonies) without zeocin exposure and in $(0.7 \pm 0.2)\%$ colonies ($N=3$, 490) with zeocin exposure. We conclude that $30\mu\text{g/ml}$ chronic zeocin exposure does not directly induce loss of silencing at the *HML* locus.

3.2 Chronic exposure to alkylating agents decreased HML α silencing

To quantify the number of GFP-expressing cells after chronic exposure to alkylating agents and 4-NQO, we measured GFP fluorescence using the Amnis Image Stream (Figure 2). The concentration of the agent was chosen so that lethality did not exceed 50%. The highest-fold increases were observed following exposure to MNNG, which exhibits a strong dose-dependent response ($P < 0.05$, ANOVA analysis). Exposure to MMS resulted in significantly increased GFP expression relatively independent of exposure dose ($p=0.00135$); the net increase in MMS-exposed cells expressing GFP was greater than for those exposed to MNNG. Exposure to 4NQO also resulted in an increase in GFP expression; however, the dose-dependence of the response was less than the response in MNNG. While the increased GFP expression was not significant when each dose was compared to the blank ($P=0.38$, ANOVA analysis), the average increase in GFP expression for all three doses, compared to the blank, was significant ($p=0.00960$).

3.3 Acute exposures to alkylating agents decreased HML silencing

We exposed independent cultures to alkylating agents for three hrs and measured GFP fluorescence (Figure 2). Cells that were not exposed indicated that 0.05% (± 0.01) expressed GFP. Cells that were exposed to MNNG and MMS exhibited higher numbers of fluorescent cells, compared to those that were not exposed, while the increase in the number of fluorescent cells after exposure to 4NQO was not significant. Nonetheless, the net increases in the number of fluorescent cells were relatively small (1–2%). No GFP fluorescent signal was detected in the JRY9627 strain after cells were acutely exposed to 4NQO, MMS, or MNNG ($n > 10,000$ cells/exposure). These studies indicate that shorter term exposures can also decrease *HML* silencing, which is likely dependent on the dose and the chemical agent.

3.4 Exposure to short-wave UV light decreased HML silencing:

To determine whether UV exposure also leads to loss of silencing, cells were plated on SC-TRP and exposed to 40 J/M², 80 J/M², and 120 J/M² of UV. After three days, colonies were arbitrarily selected and cells expressing green fluorescence were counted in the Amnis ImageStream ($N = 3$, Figure 2E). Measured increases in the number of GFP-expressing cells averaged 4.3% for all UV exposures and there was no significant difference between exposures ($P > 0.05$, $N = 3$). These data indicate that UV exposure can decrease *HML* silencing. However, if only sectoring colonies were counted 3.4% of the UV-irradiated cells (73/2118, $N = 3$) exhibited sectoring while 2.2% (23/1050, $N = 3$) of the non-irradiated cells exhibited sectoring. We suggest that the increase in GFP cells after UV exposure occurred after the third division in the clonal growth of the colony.

4. Discussion

Many DNA damaging agents may not only be genotoxic but also cause epigenetic alterations. Although DNA methylation is a well-noted biomarker for epigenetic silencing, many potent DNA damaging agents also cause protein damage [12,13,14,15], which could inhibit transcription factors and histone modification enzymes, as well as directly modify chromatin. These DNA damaging agents include MMS, 4-NQO, and short-wave UV [15]. Because protein turnover is generally fast, many of the alterations in gene expression could be transitory and difficult to detect. In this study, we took advantage of a previously constructed yeast strain that measures expression of the silent mating type locus (*HML*) to demonstrate that a subset of DNA damaging agents can trigger alterations in epigenetic gene silencing. We showed that single-cell imaging could expeditiously quantify fluorescence in greater than ten thousand cells, and thus allow more robust quantification of both the time course and concentration required to elicit an epigenetic response. The primary conclusion is that alkylating agents can elicit alterations in gene silencing and this effect can be detected after chronic and acute exposures.

Single-cell imaging techniques using the Amnis image flow cytometer allow us to detect alterations in epigenetic silencing that otherwise might go undetected measuring colony sectors. The sensitivity of the colony sectoring assay is limited to the first three divisions; i.e., one eighth sectors. Although we did not detect colony sectors after UV exposure, we did detect an increase in GFP expressing cells from UV-exposed colonies. An interesting

interpretation is that some agent-induced changes may require several cell divisions and not occur until after the first three divisions after exposure, rendering it difficult to quantify smaller sectors. Alternatively, chronic exposure to particular DNA damaging agents may be important to observe sectoring phenotypes.

The primary limitation of measuring Cre-mediated activation of GFP fluorescence is the inability to differentiate between transient and more prolonged loss-of-silencing events. Since the gene encoding GFP is positioned at an ectopic locus, loss of silencing at *HML* and transient Cre expression, leads to constitutive expression of GFP. Nonetheless, Dodson and Rine [10] report that when the GFP gene is positioned at *HML*, no spontaneous GFP fluorescent colonies are observed, suggesting that prolonged loss-of-silencing events are less common than transient gene expression changes. Since expression of both *MATa* and *MATα* has been shown to increase radiation resistance and enhance recombinational repair [16], it will be important to determine whether even transient expression of the silent mating types locus could confer DNA repair phenotypes. Further studies would be necessary to determine the duration of *HML* expression, which could be manifested by the number of cells that express a *Mata* phenotype.

These results provide new insights into the alkylating agents MMS and MNNG. Direct effect of the exposure to MMS and MNNG is protein alkylation. While many protein targets are possible, Sir2, Sir3, and Sir4 are possible targets, whose inactivation would lead to *HML* expression. While direct chemical agents, such as nicotinamide, can directly inhibit Sir2, DNA damaging agents, such as MMS, likely inhibit Sir function by triggering protein turnover [14]. Other direct targets on histones are less probable due to the required specificity. An alternative explanation is that MMS triggers a *MEC1*-dependent checkpoint response that is initiated by DNA damage. However, we did not observe increased sectoring when cells were exposed to zeocin, nor did Burgis and Samson [15] observe a decrease in protein turnover in a *mec1* mutant. Further experiments would be necessary to determine the mechanism by which alkylating agents trigger transient heterochromatic changes at the *HML* locus.

As ongoing research continues to develop new insights into the functions of Sir2 and mammalian homologues, a thorough analysis of environmental influences on sirtuin activity could provide valuable information on risk factors for cancer and age-related disease and analysis of therapeutic targets. Future studies could expand on the number of agents that cause epigenetic changes and correlations between epigenetic changes and other genetic instability phenotypes.

Acknowledgements:

We thank N. Cady for assistance with microscopy for analyzing sectoring colonies, J. Rine for JRY9628 and JRY9627, S. Nevins for zeocin, and A. Burch for useful discussions. This research was funded by grant 2 R15ES023685 from the National Institutes of Health.

References

- [1]. Baccarelli A V. Bollati, Epigenetics and Environmental Chemicals, Current opinion in pediatrics 21 (2009) 243–251. [PubMed: 19663042]

- [2]. Israel JW, Chappell GA, Simon JM, et al., Tissue-and strain-specific effects of a genotoxic carcinogen 1, 3-butadiene on chromatin and transcription, *Mammalian Genome* 29(2018) 153–167. [PubMed: 29429127]
- [3]. Ferreira LL, Couto R, Oliveira PJ, Bisphenol A as epigenetic modulator: setting the stage for carcinogenesis?, *European journal of clinical investigation* 45(2015)32–36. [PubMed: 25524584]
- [4]. Smith MT, (1996). The mechanism of benzene-induced leukemia: a hypothesis and speculations on the causes of leukemia, *Environmental health perspectives* 104 (1996) 1219–1225. [PubMed: 9118896]
- [5]. Peng C, Ng JC, The Role of Epigenetic Changes in Benzene-Induced Acute Myeloid Leukaemia, *Journal of Clinical Epigenetics* 2(2016) 2 DOI: 10.21767/2472-1158.100019.
- [6]. Chappell G, Pogribny IP, Guyton KZ Rusyn, Epigenetic alterations induced by genotoxic occupational and environmental human chemical carcinogens: a systematic literature review, *Mutation Research/Reviews in Mutation Research* 768 (2016) 27–45. [PubMed: 27234561]
- [7]. Saunders LR, Verdin E, Sirtuins: critical regulators at the crossroads between cancer and aging, *Oncogene* 26(2007), 5489–5504. [PubMed: 17694089]
- [8]. Rine J, Herskowitz I, Four genes responsible for a position effect on expression from HML and HMR in *Saccharomyces cerevisiae*, *Genetics* 116 (1987), 9–22. [PubMed: 3297920]
- [9]. Blander G, Guarente L, The Sir2 family of protein deacetylases, *Annual review of biochemistry* 73(2004) 417–435.
10. Dodson AE, Rine J, Heritable capture of heterochromatin dynamics in *Saccharomyces cerevisiae*. *Elife*, 4 (2015) e05007. [PubMed: 25581000]
- [11]. Dietrich LS, Regulation of nicotinamide metabolism. *The American Journal of Clinical Nutrition*, 24(1971), 800–804.
- [12]. Lee MY, Kim MA, Kim HJ, Bae YS, Park JI, Kwak JY, Chung JH, Yun J, Alkylating agent methyl methanesulfonate (MMS) induces a wave of global protein hyperacetylation: implications in cancer cell death. *Biochem Biophys Res Commun* 2360(2007) 483–489.
- [13]. Roberts JJ, Pascoe JM, Plant JE, Sturrock JE, Crathorn AR, Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. I. the effect on I. he effect on I. lei .a and Chinese hamster cell survival of alkylation of cellular macromolecules. *Chem.-Biol. Interact*, 3 (1971) 29–47. [PubMed: 5156325]
- [14]. Jelinsky SA, Samson LD, Global response of *Saccharomyces cerevisiae* to an alkylating agent. *Proc Natl Acad Sci U S A* 96(1999) 1486–91. [PubMed: 9990050]
- [15]. Burgis N, Samson LD, The protein degradation response of *Saccharomyces cerevisiae* to classical DNA-damaging agents. *Chem.Res.Toxicol* 20(2007) 183–1853
- [16]. Fasullo MT, Dave P, Mating type regulates the radiation-associated stimulation of reciprocal events in *Saccharomyces cerevisiae*. *Mol. and Gen. Genet* 243 (1994)63–70. [PubMed: 8190072]

Highlights

- Acute and chronic exposure to alkylating agents trigger expression of *HMLα* in yeast
- Chemical-associated activation of *HMLα* can be observed by yeast colony sectors
- Flow cytometry can be used to identify agents that trigger epigenetic expression

Figure 1:

(Left, A) Strain construction used to monitor epigenetic effects of alkylating agents. The GFP reporter gene used to monitor HML expression, using *HML::cre*. Expression of Cre catalyzes intrachromosomal recombination of the *loxP* sites and deletes the intervening stop signal, allowing for GFP expression. *HML-E* and *HML-I* are effective silencers of the HML locus. The direction of gene transcription is shown by the arrow of *HML α 1* and *HML α 2*. *HML::cre* is inserted into *HML α 2*. The feathers represent *loxP* sites that are recombined upon expression of Cre. The figure is modified from one described in Dodson and Rine [10]. **(Right, B)** Percent colonies exhibiting sectoring phenotypes after three day exposure to methylmethane sulfonate. **(Right, C)** Below is shown an example of sectoring colonies; clockwise, a fully sector colony, a half-sector colony, a quarter sector colony and a multi-sector colony are shown. Statistical significance was determined by the two-tailed t-test.

Figure 2:

Net increase in GFP-fluorescent cells after chronic exposure to chemical agents (top A, B,C), acute exposures (bottom, D), and UV exposure (bottom, E). The chemical agents tested include, MNNG, MMS and 4-NQO. Mean baseline GFP expression was <0.01%, 2.9%, and 0.01% for the chemical agents and ~7% for the UV experiments in a survey of greater than ten thousand cells. Statistical significance was determined by the two-tailed *t*-test and ANOVA multivariant analysis