Mutational analysis of N-ethyl-N-nitrosourea (ENU) in the fission yeast *Schizosaccharomyces pombe*.

Rafael Hoyos-Manchado[1], Juan Jiménez[1*] and Víctor A. Tallada[1*]

[1]Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide/Consejo Superior de Investigaciones Científicas, Carretera de Utrera Km1, 41013 Seville, Spain.

*Corresponding authors

E-mail: valvtal@upo.es

Abstract

Forward genetics has boosted our knowledge on genic function in a multitude of biological models and it has significantly contributed to the understanding of genetic bases of development, ageing and human diseases. With the advent of the next generation sequencing and use of powerful bioinformatic tools, this *traditional* genetic strategy has acquired a new impulse. At present, whole genome sequencing assisted by *in silico* analysis allows the rapid and efficient identification of gene variants that are responsible for a particular phenotype. In this experimental pipeline, it is crucial to start by provoking a large number of random changes in the genome of the model organisms to be screened. A range of chemical mutagens are used to this end because most of them display particular reactivity properties and act differently over DNA. Here we use N-ethyl-N-nitrosourea (ENU) as a mutagen for the first time to our knowledge in the fission yeast *Schizosaccharomyces pombe*. By comparison to the extensively used Ethyl methanesulfonate (EMS) in a phenotype-based study, we conclude that ENU is a very potent and easy-to-use mutagen. Judging from DNA sequence analysis of the identified mutants, ENU induces base changes rather than *indels* and the mutational spectrum in the fission yeast seems similar to that found in mice but different to that described in other single-celled organisms such as budding yeast and *E. coli*. Using ENU in *S. pombe*, we have gathered a collection of 49 auxotrophic mutants including two deleterious alleles of ATIC human ortholog. Defective alleles of this gene are causative of AICA-Ribosiduria, a severe genetic disease. We have also identified 5 aminoglycoside-resistance inactivating mutations in APH genes. All these mutations reported here may be of interest in the metabolism and antibiotic resistance research fields.

Introduction

The search of a particular phenotype of interest after natural or induced random mutation in biological models, and the consequent identification of the underlying genetic variant, is known as *forward genetics*. This strategy provides an extremely powerful tool to characterise individual gene functions as well as complex genetic pathways interplay, when two or more mutations coexist into the same cell. Human genetic diseases are usually provoked by hypomorphic mutations (partial loss of function) that allow embryo development and birth but become deleterious at later stages. Therefore, by inducing DNA changes into model organisms, genetic bases of human diseases can be very closely reproduced to be studied; even in single-celled organisms such as yeast [1].

A number of physical and chemical mutagens have been used to boost the number of random changes over the natural spontaneous mutation rate. In addition to single base substitutions, most of these agents also provoke small insertions and deletions (generically known as *indels*) [2]. Indels usually lead to frame shifts that generate either truncated or aberrant polypeptides. These provide very valuable information on non-essential gene functions because very often abolish the corresponding protein function entirely or disrupt complexes where they work in, mimicking the full gene deletion phenotype. However, the ideal genetic variants to study essential genes consist of mutations that enable an “on-off” function switch under given conditions. So called conditional alleles are very often generated by individual amino acid substitutions which “chirurgeonly” destroy postraductional modification sites, unwind binding motives, impair the active centre, etc. without dramatically affecting the overall polypeptide´s structure and/or complexes they are part of. For

theses alleles, the switch to destroy function, activity or structure when desired can be turned off simply modulating the culture conditions (temperature, media, presence of chemicals, etc.) [3-5]. These mutations enable elegant genetic studies -otherwise impossible- of essential genes applied to structure-function relationships, biochemical regulation, protein-protein and protein to other partners interactions, etc. Therefore, depending on the aim of the study, it would be desirable to use specific mutagens to enrich the screens for single point substitutions.

Ethyl methanesulfonate (EMS), N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG) and Methyl methanesulfonate (MMS) are within the most common alkylating agents used in yeast and other model organisms as mutation inducing agents [4, 6-8]. Another alkylating agent, N-ethyl-N-nitrosourea (ENU), has also been extensively used for a long time for random mutagenesis screens, especially in mice [9]. However, as far as we know, we are the only ones to use it as a mutagen in fission yeast [3]. As other alkylating counterparts, ENU does not need to be metabolised to alkylate DNA proactively *in vivo* [10-12]. Resulting ethylated nucleotides are mispaired, leading to base pair substitutions after two replication rounds. Importantly, the chemical reactivity described for this molecule remarkably differs from EMS and MMS in the Swain-Scott substrate constant [13, 14]. An especially attractive feature of this molecule is that *Indels* are extremely rare in its mutagenic repertoire [15, 16]; We report here the use of N-ethyl-N-nitrosourea (ENU) as an effective mutagen in the model yeast *Schizosaccharomyces pombe* which may significantly enrich the isolation of new single-base mutant alleles.

Materials and methods

Media and growth conditions

Standard fission yeast growth media and molecular biology approaches were used throughout [17]. For qualitative spot tests to assess auxotrophy, every candidate strain was inoculated from a plate in a unique well of a 96-well plate in YES liquid media. After 13 hours, they were diluted 10-fold and plated onto YES and supplemented minimal media lacking adenine, uridine, histidine or leucine in each case. Sporulation agar (SPA) was used for mating and sporulation. Tetrad pulling for segregation analyses was performed in a Singer MSM 400 automated dissection microscope (Singer Instruments).

Mutagenesis

A reference strain, bearing G418 and Hygromycin B resistance markers integrated into chromosome I and II respectively, was mutagenized using N-ethyl-N-nitrosourea (ENU; Sigma N3385) or ethyl methanesulfonate (EMS; Sigma M0880) as in Winston, 2008 with minor variations [18]. Cells were grown overnight to mid log phase in 500 ml YES medium at 30°C. Cells were concentrated to $2 \cdot 10^8$ cells/ml and 2 ml aliquots were washed twice with 2 ml of water (centrifuge 5-10 seconds maximum speed), resuspended in 7 ml 0.1 M sodium phosphate buffer (pH 7.0) and split into four aliquots. One of them was treated with 50 ul EMS (0.3 M final) for 1 hour at 30ºC, and another one was treated with 60 µl ENU (0.03 M final) for 20 minutes at 30ºC. The other two were not treated with mutagens to be used as controls. For ENU treatment and its control, 0.2 ml of each were taken and washed three times with Minimal Medium

lacking amino acids and NH_4 in order to stop mutagenesis. EMS and control aliquots were diluted in 8 ml $Na_2S_2O_3$ 5% and washed twice in minimal medium lacking amino acids and NH_4. Cell numbers were scored in Neubauer´s chamber and plated in corresponding dilutions for experiments depicted in Fig 1 (I, II and III).

Sequencing

Genomic DNA was extracted from selected mutants (Hph^s, Kan^s and Ade/His auxotrophs). Antibiotic resistance cassettes and *ade10* open reading frame were amplified by PCR. Resulting products were sequenced using forward and reverse primers, to ensure total open reading frame coverage in both strands to identify the bases substitutions. Parental strain marker sequencing was used as a control to discard putative previous polymorphism regarding to database sequence.

Results

Cellular viability upon ENU vs EMS treatment

In a previous trial for a random mutagenesis study [3] we had roughly adjusted ENU concentration to allow a comparable survival rate to a standard EMS mutagenesis protocol for yeast [18]. Therefore, in the first place, we aimed to finely assess cell viability after ENU treatment in our experimental conditions as compared to well characterised EMS. We cultured our prototroph reference strain (975 background) in yeast extract medium (YES) until log phase ($2x10^6$ cells/ml). Cells were harvested and split into four different aliquots of $1x10^8$ cells each (see materials and methods for details and Fig 1) and treated as follows: Aliquot 2 was treated, as in the reference protocol, with 0,3 M EMS for one hour at 30º [18]. Aliquot 4 was treated with 0,03M

ENU for 20 minutes at 30º. To serve as untreated controls, Aliquots 1 and 3 were processed and washed as aliquots 2 and 4 respectively; except for the lack of the alkylating agent. Afterwards, aliquots 1 and 2 were added into 8 ml of 5% $Na_2S_2O_3$ to inactivate EMS reactivity and washed immediately with fresh media. We had also found in previous trials that KOH solution, which is used in other systems to inactivate ENU´s reactivity, resulted rather toxic for yeast cells. Thus, aliquots 3 and 4 were washed three times with only minimal medium lacking nitrogen source to avoid cell division before plating. After the last wash, cells were resuspended in fresh media and the number of cells per millilitre was scored in all tubes, considering the average of three independent counts in Neubauer´s chamber as the reference cell number for each tube (Fig 1, Table 1).

Table 1. Survival rate after EMS and ENU treatment.

	Expected cells/plate	**Observed cells/plate (3 repeats)**	**Observed average**	**%Survival control vs experiment**	**χ^2 p-value**
Control EMS	500	350/399/390	379.67	61.72%	89.6 <0.00001
EMS 0.3M	500	206/266/231	234.33		
Control ENU	500	520/524/553	532.33	59.17%	7.57 0.05579
ENU 0.03M	500	310/314/321	315		

Cell number per ml was estimated for each control and mutagen-containing tubes after the procedure described in materials and methods. Results from three different platings

147 are shown. Respective untreated number of colonies obtained were compared to the
148 expected goal by a chi square test. The result suggests that the treatment used to stop
149 EMS reactivity significantly reduces viability (associated p value<0.00001).

150 **Fig 1. Schematics of the experimental design.** (A) Fission yeast cells (975 taxon)
151 bearing KanMX6 and HphMX6 resistance markers were grown in rich media (YES)
152 until early log phase and then split into four tubes. The first and third represent
153 untreated controls while second and fourth were treated with EMS and ENU
154 respectively. (B) Same number of cells, aiming to 500, from each tube in "A" were
155 plated in three technical repeats. Difference in control vs experiment plates was
156 calculated for an estimation of cell survival after exposure to either alkylating agent. (C)
157 Cells exposed to EMS and ENU respectively were grown in solid rich media (YES).
158 Resulting colonies (3840 EMS-treated and 4353 ENU-treated) were replica-plated to
159 synthetic minimal media (MM) without any supplement to identify auxotrophic
160 mutants. (D) Another batch of 45000 ENU-treated colonies were grown in YES media
161 plates as in "C" but replica-plated to YES media containing G418 (indicated as Kan)
162 and Hygromycin B (Hph) antibiotics respectively. Sensitive colonies to either antibiotic
163 were selected to amplify and sequence the resistance marker gene.

164 To assess survival rate, we plated three technical repetitions for each treatment
165 and controls, aiming to 500 colonies per plate (Fig 1B). As shown in Table 1, both
166 alkylating treatments allow a very similar viability in comparison to their respective
167 untreated control (around 60%); although the exposure time is one third and the molar
168 concentration of ENU is one order of magnitude lower than EMS in this experiment. It
169 is to note as well that the number of viable colonies in aliquot 3 plates (untreated ENU
170 control) are significantly and consistently much closer to the expected goal (500

171 colonies) than in aliquot 1 (untreated EMS control) (Table 1). This suggests that
172 $Na_2S_2O_3$ used to inactivate EMS may be also affecting viability itself in spite of such a
173 short exposure time.

174 Loss-of-function mutation frequencies

175 In order to compare the mutagenic potential of ENU and EMS in fission yeast, we
176 screened for auxotrophy-causing mutations as a gene loss-of-function readout over the
177 same number of genomic targets. After EMS and ENU mutagenesis of prototrophic
178 cells explained above (see materials and methods and Fig 1C), we plated them onto
179 YES media. Resulting colonies were counted up and replica-plated onto synthetic
180 minimal media. We then searched for any auxotrophic mutant growing in rich media
181 but unable to proliferate in media with no supplements (EMM). We found 21 such
182 mutants out of 3840 colonies (0,54%) in the case of EMS, and 28 mutants out of 4353
183 colonies for ENU treatment (0,64%) (Table 2). We further checked if some of these
184 mutants really interrupted specific metabolic pathways and whether any of these could
185 have mutations in more than one metabolic pathway. Mutants were plated onto minimal
186 media lacking just the final product for one of the most common auxotrophic markers
187 used in this yeast: leucine, adenine, uracil and histidine respectively. We found
188 particular auxotrophs for all these metabolites, except for uracil in the case of ENU
189 (Table 2).

190 **Table 2. Auxotrophic mutants frequency**

	EMS	**ENU**
Leu-	1	1
Ura-	2	0

Ade-	4	8*
His-	2	3*
Other	12	16
Total auxotrophs	21	28
Colonies analysed	3840	4353
Metabolic loss-of-function frequency (%)	0.55%	0.64%

* Two of them are both ade- and his-.

Interestingly, two adenine-dependent mutants turned out to be histidine-dependent as well. It is known that both purine and L-histidine synthesis routes share a number of intermediates which make them interdependent [19]. Furthermore, fission yeast *ade9* and *ade10* deletion mutants (adenine dependent) -and their orthologues knock-out in the Baker´s yeast- become histidine dependent as well [19-22]. It is then possible that a single mutation in one of these genes accounts for both auxotrophies. Thus, these could represent ideal candidates for going down to the specific mutation in the DNA to discover new deleterious alleles for both or any of such genes, contributing also to assess the ENU´s mutational spectrum in fission yeast. Therefore, we first crossed both mutants to a wild type strain to check out whether the two metabolic deficiencies segregate together. In both cases, in 16 pulled tetrads, 100% of adenine requiring spores need histidine as well. We then crossed both mutants to each other and did not find any prototroph within the offspring (10 tetrads). These data indicate that there is only one single locus affected and this one is the same in both mutants.

To distinguish among the two putative mutated loci (*ade9* and *ade10*), we checked out genetic linkage to *csi1*, which is just 8 Kb apart from *ade9*

208 (www.pombase.org). Neither mutation showed linkage to *csi1* deletion marker, leaving
209 *ade10* as the only candidate. We therefore sequenced *ade10* coding locus in both
210 mutants. We found a different single base pair substitution in each strain (Table 3),
211 confirming that both mutants are allelic to *ade10* (named *ade10.68* and *ade10.424*
212 respectively) and that the double auxotrophic deficiency is due to a single defective
213 gene rather than two independent mutations in different pathways.

214 **Table 3. Mutational spectrum of ENU in fission yeast.**

Marker	Base pair change	Coding nucleotide change (pFA6a numbering)	Amino acid change
Kan	A-T→G-C	T375C	Val192Ala
Kan	A-T→T-A	T626A	Leu209STOP
Kan	A-T→G-C	T634C	Cys212Arg
Hph	A-T→C-G	A182C	Asp61Ala
Hph	G-C→A-T	G293A	Gly98Asp
Ade10	A-T→G-C	T1270C	Ser424Pro
Ade10	A-T→G-C	T203C	Val68Ala

215 Base pair substitutions found after ENU treatment are summarized along with the
216 change that abolish function of corresponding protein product.

217

Mutational spectrum of ENU in fission yeast

The reference strain used in this study was originally chosen to bear both G418 and Hygromycin B resistance markers integrated into chromosome I and II respectively. To survey for the type of mutations induced by ENU in the fission yeast genome, we selected loss-of-function mutations in those dominant markers after the treatment. To this end, we obtained 45000 colonies in regular YES rich media after ENU treatment described before (tube 4) (Fig 1D). These were replica-plated back to regular YES and YES containing either G418 or Hygromycin B antibiotics (50 mg/ml). Sensitive colonies (three $G418^s$ and two Hph^s) were picked for marker sequencing.

Sequence comparison to wild type control markers identified mutations leading to antibiotic resistance loss (listed in Table 3). Both enzymes conferring G418 and Hygromycin B resistance encode for aminoglycoside phosphotransferases (APH(3')-Ia and APH(4)-Ia types respectively) [23]. Despite a weak sequence identity (12.23%), the spatial protein structure results strikingly similar with two big lobes corresponding roughly to the Amino and Carboxy halves of the protein [24, 25]. Four out of the five mutations target an A-T base pair. All three mutations found in kanMX6 (APH(3')-Ia) lay within a 20 amino acid stretch in the C-terminal domain. T626A base change (KanMX6 numbering) generates a nonsense codon (L209STOP) predicting a 60 amino acid C-terminal truncation. The remaining V192A and C212R amino acid changes fall in close proximity to predicted residues of the aminoglycoside and ATP pockets respectively. In contrast, D61A and G98D changes inactivating HphMX6 (APH(4)-Ia) are located at the N-terminus half, although Glycine 98 is also at the inner face of the nucleotide pocket (https://www.rcsb.org/pdb/protein/P00557?addPDB=3TYK; [24]).

241 These mutations may be of interest in clinic to reveal protein structural changes that
242 inactivate resistance of pathogens to this antibiotic family.

243 Likewise, the two mutations found in *ade10* open reading frame target two different A-
244 T base pairs (transition A-T→G-C) (Table 3). *sp*Ade10 and orthologs encode for a
245 bifunctional enzyme (AICAR formyl transferase/IMP cyclohydrolase) well conserved
246 in prokaryotes and eukaryotes. It uses AICAR (5-Aminoimidazole-4-carboxamide 1-β-
247 D-ribofuranoside, Acadesine, N1-(β-D-Ribofuranosyl) -5-aminoimidazole-4-
248 carboxamide) as the substrate in the last steps of the de novo purine biosynthetic
249 pathway [26, 27]. There are evidences pointing out that an excess of AICAR
250 downregulates histidine synthesis, though the actual biochemical mechanism remains
251 unknown [20]. The deleterious alleles identified in the present study correspond to
252 changes in two very well conserved residues that lead to missense amino acid
253 substitutions with a different expressivity: Val68Ala results a bit leaky while Ser424Pro
254 becomes totally unfunctional. Alignment of 170 orthologs recovered from Ensembl
255 database
256 (https://fungi.ensembl.org/Schizosaccharomyces_pombe/Gene/Compara_Tree/pan_com
257 para?g=SPCPB16A4.03c;r=III:947597-949685;t=SPCPB16A4.03c.1) reveals that
258 Valine is the most common residue in position 68 (in 56% of sequences; S1A Fig) and
259 it is present in most eukaryotes within a very well conserved motif (S1B Fig). In
260 position 424, Alanine is the most frequent (57%) residue within all available sequences.
261 However, this residue is present in prokaryotes, mosses and a few plants whilst Serine
262 present in S. pombe is also conserved in nearly all higher eukaryotes including human
263 (31% of database sequences) (S1 Fig). Compound heterozygous deficiency of the
264 human ortholog (ATIC) causes AICA-ribosiduria, a devastating disease characterised
265 by severe neurological defects and congenital blindness. An abnormal accumulation of

AICAR and defective purinosome formation is observed in these patients´ fibroblasts [28, 29]. Therefore, the new alleles isolated in this screen could indeed shed light in the study of purine-histidine metabolic pathways crosstalk as well as ATIC-associated ribosiduria.

Discussion

In order to perform genetic screenings in fission yeast, Ethyl methanesulfonate (EMS), Methyl methanesulfonate (MMS), Nitrosoguanidine (NG), UV radiation and insertional mutagenesis had extensively been used for a long time to cause mutations [4, 30-33]. In high doses, these agents guarantee the generation of a large number of mutants at the cost of a low survival rate. Nevertheless, the spectrum of mutations resulting from UV radiation becomes quite limited and biased [34] and insertional mutagenesis is very useful to disrupt functional elements instead of causing single-base substitutions [33]. On the other hand, DNA alkylating chemicals by no means represent a homogeneous class but differ greatly in their specific bases and atoms attacked and consequently in the potential downstream mutation caused. Alkylation sites in cellular DNA include adenine N^1, N^3 and N^7, guanine N^3, 0^6, and N^7, thymine N^3, 0^2 and 0^4 and cytosine 0^2 and N^3. The preference to these sites is largely determined by the nucleophilic selectivity of the agent [35, 36]. It is also well documented that genetic effects of chemical mutagens may vary in different species, strains, sex, age and individuals, even in different cell stages and cell types within the same pluricellular organism [14, 37, 38]. Thus, to enlarge the chemicals shelf that we could use in the fission yeast to induce genetic variations, we decided to implement another alkylating agent, N-ethyl-N-nitrosourea (ENU), since the chemical reactivity of this molecule is qualitatively different from the others mentioned above. Although this agent has been profusely used

290 as a mutagen in mice [39, 40] and to a lesser extent also in other models, including fly
291 [41], worm [42], fish [43], plants [44], bacteria [45] and budding yeast [46]; to our
292 knowledge, this molecule has only been used very few times previously in *S. pombe,*
293 and not as mutagen but as a genotoxin to show sensitivity of a repairing enzyme´s null
294 allele (Atl1) [47, 48]

295 A very well-established empirical expression of alkylating agents' reactivity is
296 the Swain-Scott substrate constant (*s*). It is a measure of the sensitivity of the alkyl
297 agent to the strength (n) of various nucleophilic reagents with a series of substrates in
298 water solution [13]. Alkylation reactions are then classified in S_N1-type (low *s*) and S_N2-
299 type (high *s*). Most of alkylating compounds used as mutagens range from *s*=0,26 to
300 *s*=0,86. Both EMS and ENU act by transferring the ethyl group into nucleotides.
301 However, there is a remarkable difference in their reactivity: whilst EMS act by a mixed
302 S_N1/S_N2-type reaction and shows a relatively high Swain-Scott constant (*s*=0.67), ENU
303 only acts by a S_N1-type reaction with a much lower *s* constant (*s*=0.26) [49]. This
304 translates into EMS attacking mostly high nucleophilicity ring N sites (mostly the N^7
305 position of guanine) and O-atoms up to certain extent. Therefore, EMS mutagenesis is
306 biased towards G/C to A/T transitions which often leads to the generation of stop
307 codons [50]. It has also been reported that EMS can generate up to 13% of deletions and
308 other chromosome rearrangements [51], some double strand breaks and attacking also
309 proteins to a considerable extent [52]. On the other hand, ENU acts preferentially
310 towards low nucleophilicity O-atoms in DNA (mainly O^2 and O^4 in thymine and O^6-
311 deoxyguanosine) [14, 53]. In order to compare the action of two chemicals it is
312 important to consider the trade-off between genotoxic power and population survival.
313 For this comparison, we worked out the conditions allowing almost identical survival
314 rate treating with EMS and ENU respectively. This implied that the molar concentration

315 of ENU had to be adjusted at one order of magnitude lower than EMS and time of
316 exposure reduced to a third. This suggests that ENU results a rather more genotoxic
317 agent than EMS in fission yeast. Both the higher killing power and mutagenic potential
318 over EMS have been previously observed in other systems [14, 54].

319 In the present gene-based study we have also surveyed for ENU´s mutational
320 spectrum. A number systematic and gene/phenotype-based studies reports on the range
321 of ENU induced substitutions have been previously carried out with different outcomes.
322 In *E. coli*, *S. cerevisiae* and *C. elegans* over 70% of changes corresponded to G-C to A-
323 T transitions while almost the remaining 30% were A-T to T-A transversions [46, 55,
324 56]. However other studies in mouse (compiled in [15, 57, 58]) and a systematic study
325 in toxoplasma, revealed opposite proportions: more than 75% of changes affected A-T
326 pairs [59]. Furthermore, in *Drosophila* germ cells selectivity became one or another
327 depending on the pre-or-post meiotic stage [41, 60, 61]. This could be explained under a
328 generally accepted concept suggesting that 0^6-alkylguanine and 0^4-alkylthymine have
329 promutagenic potential towards transition mutations (GC→AT and AT→GC,
330 respectively) [62, 63] and 0^2-alkylthymine to AT→TA transversions [64]. In this
331 scenario, the protective and repairing enzymes background, cell cycle stage and
332 mutagen doses might be important bias factors [48]. In this study all mutations
333 identified correspond to single base pair substitutions (Two in *ade10*, two in APH(4)-Ia
334 and three in APH(3')-Ia). Six out of seven mutations inactivating each
335 phosphotransferase conferring antibiotic resistance or the *ade10* gene, are transitions or
336 transversions from a native A-T pair (Table 3). In spite of the limited number of
337 mutations analysed, all observed changes in other models are represented in this study
338 but the preference in fission yeast seems to be in line with models such as mouse and
339 toxoplasma rather than *E. coli* or *S. cerevisiae*. As mentioned above, this could be

related with the specificity and efficiency of alkyltransferase-like protective proteins in *S. pombe* such as Atl1 against 0^6-alkylguanine adducts [47, 48, 65].

Nevertheless, in contrast to other alkylating agents, indels imputed to ENU are extremely rare or absent in literature in any model, [16, 46]. This implies that loss-of-function mutations generated by ENU are caused by single base substitutions rather than by frame shifts that usually result more deleterious. Furthermore, in phenotype and gene-driven screens it is also evident that, within the substitutions category, missense mutations are much more likely to occur than protein-truncating nonsense mutations [15]. This sets a remarkable difference between ENU and other alkylating counterparts to be used to complement each other to enlarge the changes spectrum in mutational studies. It has also been shown by whole genome sequencing studies that ENU induced mutations are randomly distributed over a whole genome [59]. In our loss-of-function mutations screens, on the one hand, over about 93 potential target genes (listed in fission yeast phenotype ontology database as auxotrophy-causing, PomBase FYPO:0000128) we found 28 auxotrophic mutants in 4353 colonies. On the other hand, over two target dominant markers, we identified 5 mutants in 45000 colonies. Therefore, these experimental conditions would give a rough estimation between 14520 and 18000 colonies to be screened to find a knock-out hit in a given average gene. Slight variations are to be considered by factors such as G-C content or gene´s size.

Taking all this together, we propose that this molecule can be very efficiently used in mutagenesis studies in *Schizosaccharomyces pombe* (and very likely in other prominent yeast and fungi models such as *Candida albicans*, *Ustilago maydis,* etc.), either as an alternative or as a complement to its sulfonate and nitroso counterparts. In addition to finding new valuable conditional mutations in forward genetics screens, this

could contribute in directed evolution experiments, suppression studies etc. where subtle changes are desired. As a matter of fact, we have gathered in this study a collection of 49 auxotrophic mutants and identified 3 and 2 base substitutions which inactivate respectively APH(3')-Ia and APH(4)-Ia phosphotransferases genes. In the case of auxotrophs, it becomes somehow surprising that among a limited (and non-saturating) number of mutations generated here, two of them fall down within the same locus (*ade10*). However in a meta-study of mutagenic spectrum of ENU in mouse, Barbaric et al found that genes with higher G-C content become more likely to be mutated by ENU and showed that A-T pairs flanked by C or G bases were more prone to mutation [15]. Notably, *ade10* open reading frame contains the highest G-C percentage (46%) of the whole adenine pathway and significantly higher than fission yeast protein-coding gene average G-C content (37.08%) [66]. This might suggest that gene-wise, ENU-induced changes frequency could be biased by G-C content also in fission yeast. Although not in the scope of this work, all mutants reported here might be of interest in the study of metabolic diseases and antibiotic resistance research fields.

Acknowledgments

We thank Víctor Carranco and Anabel Lopez for excellent technical support, Alejandro Rubio for sequence alignment assistance and Andrés Garzón for critical reading of the manuscript.

References

1. Tenreiro S, Franssens V, Winderickx J, Outeiro TF. Yeast models of Parkinson's disease-associated molecular pathologies. Current Opinion in Genetics and

386 Development. 2017;44:74-83. doi: 10.1016/j.gde.2017.01.013. PubMed PMID:
387 28232272.

388 2. Henry IM, Nagalakshmi U, Lieberman MC, Ngo KJ, Krasileva KV, Vasquez-
389 Gross H, et al. Efficient Genome-Wide Detection and Cataloging of EMS-Induced
390 Mutations Using Exome Capture and Next-Generation Sequencing. The Plant Cell.
391 2014;26(4):1382-97. doi: 10.1105/tpc.113.121590. PubMed PMID: 24728647; PubMed
392 Central PMCID: PMC4036560.

393 3. Hoyos-Manchado R, Reyes-Martin F, Rallis C, Gamero-Estevez E, Rodriguez-
394 Gomez P, Quintero-Blanco J, et al. RNA metabolism is the primary target of formamide
395 in vivo. Sci Rep. 2017;7(1):15895. doi: 10.1038/s41598-017-16291-8. PubMed PMID:
396 29162938; PubMed Central PMCID: PMC5698326.

397 4. Bonatti S, Simili M, Abbondandolo A. Isolation of temperature-sensitive
398 mutants of Schizosaccharomyces pombe. J Bacteriol. 1972;109(2):484-91. PubMed
399 PMID: 4110142; PubMed Central PMCID: PMC285166.

400 5. Willis JH, Munro E, Lyczak R, Bowerman B. Conditional dominant mutations
401 in the Caenorhabditis elegans gene act-2 identify cytoplasmic and muscle roles for a
402 redundant actin isoform. Molecular biology of the cell. 2006;17(3):1051-64. doi:
403 10.1091/mbc.e05-09-0886. PubMed PMID: 16407404; PubMed Central PMCID:
404 PMC1382297.

405 6. Segal E, Munz P, Leupold U. Characterization of chemically induced mutations
406 in the ad-1 locus of Schizosaccharomyces pombe. Mutation Research - Fundamental
407 and Molecular Mechanisms of Mutagenesis. 1973;18(1):15-24. doi: 10.1016/0027-
408 5107(73)90017-1.

409 7. Spencer JF, Spencer DM. Mutagenesis in yeast. Methods in molecular biology.
410 1996;53:17-38. doi: 10.1385/0-89603-319-8:17. PubMed PMID: 8924979.

411 8. Sabatinos SA, Forsburg SL. Chapter 32 - Molecular Genetics of
412 Schizosaccharomyces pombe. Methods in Enzymology. 470: Academic Press; 2010. p.
413 759-95.

414 9. Russell WL, Kelly EM, Hunsicker PR, Bangham JW, Maddux SC, Phipps EL.
415 Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse.
416 Proceedings of the National Academy of Sciences. 1979;76(11):5818-9. doi:
417 10.1073/pnas.76.11.5818. PubMed PMID: 293686; PubMed Central PMCID:
418 PMC411742.

419 10. Haglund J, Van Dongen W, Lemière F, Esmans EL. Analysis of DNA-
420 phosphate adducts in vitro using miniaturized LC-ESI-MS/MS and column switching:
421 Phosphotriesters and alkyl cobalamins. Journal of the American Society for Mass
422 Spectrometry. 2004;15(4):593-606. doi: 10.1016/j.jasms.2003.12.012. PubMed PMID:
423 15047064.

424 11. Wani AA, Gibson-D'Ambrosio RE, D'Ambrosio SM. Quantitation of O6-
425 ethyldeoxyguanosine in ENU alkylated DNA by polyclonal and monoclonal antibodies.
426 Carcinogenesis. 1984;5(9):1145-50. Epub 1984/09/01. PubMed PMID: 6467504.

427 12. Scalera SE, Ward OG. A quantitative study of ethyl methanesulfonate-induced
428 alkylation of Vicia faba DNA. Mutation Research/Fundamental and Molecular
429 Mechanisms of Mutagenesis. 1971;12(1):71-9. doi: 10.1016/0027-5107(71)90074-1.

430 13. Swain CG, Scott CB. Quantitative Correlation of Relative Rates. Comparison of
431 Hydroxide Ion with Other Nucleophilic Reagents toward Alkyl Halides, Esters,

Epoxides and Acyl Halides1. Journal of the American Chemical Society. 1953;75(1):141-7. doi: 10.1021/ja01097a041.

14. Vogel EW, Natarajan AT. The Relation between Reaction Kinetics and Mutagenic Action of Monofunctional Alkylating Agents in Higher Eukaryotic Systems: Interspecies Comparisons. In: Serres FJ, Hollaender A, editors. Chemical Mutagens. 7. New York: Plenum Press; 1982. p. 295-336.

15. Barbaric I, Wells S, Russ A, Dear TN. Spectrum of ENU-induced mutations in phenotype-driven and gene-driven screens in the mouse. Environmental and molecular mutagenesis. 2007;48(2):124-42. doi: 10.1002/em.20286. PubMed PMID: 17295309.

16. Takahasi KR, Sakuraba Y, Gondo Y. Mutational pattern and frequency of induced nucleotide changes in mouse ENU mutagenesis. BMC Molecular Biology. 2007;8:1-10. doi: 10.1186/1471-2199-8-52. PubMed PMID: 17584492; PubMed Central PMCID: PMC1914352.

17. Moreno S, Klar A, Nurse P. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods in enzymology. 1991;194:795-823. PubMed PMID: 2005825.

18. Winston F. EMS and UV mutagenesis in yeast. Current Protocols in Molecular Biology. 2008;Chapter 13:1-5. doi: 10.1002/0471142727.mb1303bs82. PubMed PMID: 18425760.

19. Tibbetts AS, Appling DR. Characterization of Two 5-Aminoimidazole-4-carboxamide Ribonucleotide Transformylase/Inosine Monophosphate Cyclohydrolase Isozymes from Saccharomyces cerevisiae. Journal of Biological Chemistry. 2000;275(27):20920-7. doi: 10.1074/jbc.M909851199. PubMed PMID: 10877846.

455 20. Rébora K, Laloo B, Daignan-Fornier B. Revisiting Purine-Histidine Cross-
456 Pathway Regulation in Saccharomyces cerevisiae. A Central Role for a Small Molecule.
457 2005;170(1):61-70. doi: 10.1534/genetics.104.039396. PubMed PMID: 15744050;
458 PubMed Central PMCID: PMC1449729.

459 21. Jones EW, Fink GR. Regulation of Amino Acid and Nucleotide Biosynthesis in
460 Yeast. In: Strathern JN, Jones EW, Broach JR, editors. The Molecular Biology of the
461 Yeast Saccharomyces: Metabolism and Gene Expression. 11B: Cold Spring Harbor;
462 1982.

463 22. Whitehead E, Nagy M, Heslot H. Interactions entre la biosynlhèse des purines
464 nucléotides et celle de l'histidine chez le Schizosaccharomyces pombe. Comptes rendus
465 hebdomadaires des seances de l'Academie des sciences Serie D: Sciences naturelles.
466 1966;263:819-21.

467 23. Wright GD, Thompson PR. Aminoglycoside phosphotransferases: proteins,
468 structure, and mechanism. Front Biosci. 1999;4:D9-21. doi: 10.2741/Wright. PubMed
469 PMID: 9872733.

470 24. Stogios PJ, Shakya T, Evdokimova E, Savchenko A, Wright GD. Structure and
471 function of APH(4)-Ia, a hygromycin B resistance enzyme. Journal of Biological
472 Chemistry. 2011;286(3):1966-75. doi: 10.1074/jbc.M110.194266. PubMed PMID:
473 21084294; PubMed Central PMCID: PMC3023493.

474 25. Stogios PJ, Spanogiannopoulos P, Evdokimova E, Egorova O, Shakya T,
475 Todorovic N, et al. Structure-guided optimization of protein kinase inhibitors reverses
476 aminoglycoside antibiotic resistance. The Biochemical journal. 2013;454(2):191-200.

477 doi: 10.1042/BJ20130317. PubMed PMID: 23758273; PubMed Central PMCID:
478 PMC3743924.

479 26. Rayl EA, Moroson BA, Beardsley GP. The Human purH Gene Product, 5-
480 Aminoimidazole-4-carboxamide Ribonucleotide Formyltransferase/IMP
481 Cyclohydrolase. Cloning, sequencing, expression, purification, kinetic analysis, and
482 domain mapping. Journal of Biological Chemistry. 1996;271(4):2225-33. doi:
483 10.1074/jbc.271.4.2225. PubMed PMID: 8567683.

484 27. Richter R, Heslot H. Genetic and functional analysis of the complex locus ade10
485 in Schizosaccharomyces pombe. Current Genetics. 1982;5(3):233-44. doi:
486 10.1007/BF00391812. PubMed PMID: 24186301.

487 28. Baresova V, Skopova V, Sikora J, Patterson D, Sovova J, Zikanova M, et al.
488 Mutations of ATIC and ADSL affect purinosome assembly in cultured skin fibroblasts
489 from patients with AICA-ribosiduria and ADSL deficiency. Hum Mol Genet.
490 2012;21(7):1534-43. doi: 10.1093/hmg/ddr591. PubMed PMID: 22180458.

491 29. Marie S, Heron B, Bitoun P, Timmerman T, Van den Berghe G, Vincent M-F.
492 AICA-Ribosiduria: A Novel, Neurologically Devastating Inborn Error of Purine
493 Biosynthesis Caused by Mutation of ATIC. The American Journal of Human Genetics.
494 2004;74(6):1276-81. doi: 10.1086/421475. PubMed PMID: 15114530; PubMed Central
495 PMCID: PMC1182092.

496 30. Berry CH, Ibrahim MA, Coddington A. Characterisation of ribosomes from drug
497 resistant strains of Schizosaccharomyces pombe in a poly U directed cell free protein
498 synthesising system. Mol Gen Genet. 1978;167(2):217-25. doi: 10.1007/BF00266915.
499 PubMed PMID: 732808.

500 31. Loprieno N, Guglielminetti R, Bonatti S, Abbondandolo A. Evaluation of the
501 genetic alterations induced by chemical mutagens in Schizosaccharomyces pombe.
502 Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.
503 1969;8(1):65-71. doi: http://dx.doi.org/10.1016/0027-5107(69)90141-9. PubMed PMID:
504 5796945.

505 32. Potashkin J, Li R, Frendewey D. Pre-mRNA splicing mutants of
506 Schizosaccharomyces pombe. The EMBO Journal. 1989;8(2):551-9. PubMed PMID:
507 400840; PubMed Central PMCID: PMC400840.

508 33. Chua G, Taricani L, Stangle W, Young PG. Insertional mutagenesis based on
509 illegitimate recombination in Schizosaccharomyces pombe. Nucleic Acids Res.
510 2000;28(11):E53. PubMed PMID: 10871352; PubMed Central PMCID: PMC102638.

511 34. Pfeifer GP, You YH, Besaratinia A. Mutations induced by ultraviolet light.
512 Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis.
513 2005;571(1-2):19-31. doi: 10.1016/j.mrfmmm.2004.06.057. PubMed PMID: 15748635.

514 35. Beranek DT. Distribution of methyl and ethyl adducts following alkylation with
515 monofunctional alkylating agents. Mutation Research/Fundamental and Molecular
516 Mechanisms of Mutagenesis. 1990;231(1):11-30. doi: https://doi.org/10.1016/0027-
517 5107(90)90173-2. PubMed PMID: 2195323.

518 36. Vogel EW, Natarajan AT. DNA damage and repair in somatic and germ cells in
519 vivo. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis.
520 1995;330(1-2):183-208. doi: 10.1016/0027-5107(95)00040-P. PubMed PMID:
521 7623865.

522 37. Gibson-D'Ambrosio RE, Leong Y, D'Ambrosio SM. DNA Repair following
523 Ultraviolet and N-Ethyl-N-nitrosourea Treatment of Cells Cultured from Human Fetal
524 Brain, Intestine, Kidney, Liver, and Skin. Cancer Research. 1983;43(12 Part 1):5846-
525 50.

526 38. Slikker W, Andersen ME, Bogdanffy MS, Bus JS, Cohen SD, Conolly RB, et al.
527 Dose-dependent transitions in mechanisms of toxicity. Toxicology and Applied
528 Pharmacology. 2004;201(3):203-25. doi: https://doi.org/10.1016/j.taap.2004.06.019.
529 PubMed PMID: 15582645.

530 39. Masumura K, Matsui M, Katoh M, Horiya N, Ueda O, Tanabe H, et al. Spectra
531 of gpt mutations in ethylnitrosourea-treated and untreated transgenic mice.
532 Environmental and molecular mutagenesis. 1999;34(1):1-8. doi:
533 doi:10.1002/(SICI)1098-2280(1999)34:1<1::AID-EM1>3.0.CO;2-P. PubMed PMID:
534 10462717.

535 40. Salinger AP, Justice MJ. Mouse Mutagenesis Using N-Ethyl-N-Nitrosourea
536 (ENU). Cold Spring Harbor Protocols. 2008;2008(4):pdb.prot4985. doi:
537 10.1101/pdb.prot4985. PubMed PMID: 21356809.

538 41. Pastink A, Vreeken C, Nivard MJ, Searles LL, Vogel EW. Sequence analysis of
539 N-ethyl-N-nitrosourea-induced vermilion mutations in Drosophila melanogaster.
540 Genetics. 1989;123(1):123-9. PubMed PMID: 2572507; PubMed Central PMCID:
541 PMC1203775.

542 42. De Stasio EA, Dorman S. Optimization of ENU mutagenesis of Caenorhabditis
543 elegans. Mutation Research/Genetic Toxicology and Environmental Mutagenesis.

544 2001;495(1):81-8. doi: https://doi.org/10.1016/S1383-5718(01)00198-X. PubMed
545 PMID: 11448645.

546 43. de Bruijn E, Cuppen E, Feitsma H. Highly Efficient ENU Mutagenesis in
547 Zebrafish. In: Lieschke GJ, Oates AC, Kawakami K, editors. Zebrafish: Methods and
548 Protocols. Totowa, NJ: Humana Press; 2009. p. 3-12.

549 44. Gichner T. Differential genotoxicity of ethyl methanesulphonate, N-ethyl-N-
550 nitrosourea and maleic hydrazide in tobacco seedlings based on data of the Comet assay
551 and two recombination assays. Mutation Research/Genetic Toxicology and
552 Environmental Mutagenesis. 2003;538(1):171-9. doi: https://doi.org/10.1016/S1383-
553 5718(03)00117-7. PubMed PMID: 12834766.

554 45. Hince TA, Neale S. A comparison of the mutagenic action of the methyl and
555 ethyl derivatives of nitrosamides and nitrosamidines on Escherichia coli. Mutation
556 Research/Fundamental and Molecular Mechanisms of Mutagenesis. 1974;24(3):383-7.
557 doi: https://doi.org/10.1016/0027-5107(74)90183-3. PubMed PMID: 4606103.

558 46. Lee GSF, Blonsky KS, Van On DL, Savage EA, Morgan AR, von Borstel RC.
559 Base alterations in yeast induced by alkylating agents with differing Swain-Scott
560 substrate constants. Journal of Molecular Biology. 1992;223(3):617-26. doi:
561 https://doi.org/10.1016/0022-2836(92)90978-S.

562 47. Latypov VF, Tubbs JL, Watson AJ, Marriott AS, McGown G, Thorncroft M, et
563 al. Atl1 regulates choice between global genome and transcription-coupled repair of
564 O(6)-alkylguanines. Molecular cell. 2012;47(1):50-60. Epub 05/31. doi:
565 10.1016/j.molcel.2012.04.028. PubMed PMID: 22658721.

566 48. Pearson SJ, Wharton S, Watson AJ, Begum G, Butt A, Glynn N, et al. A novel
567 DNA damage recognition protein in Schizosaccharomyces pombe. Nucleic acids
568 research. 2006;34(8):2347-54. doi: 10.1093/nar/gkl270. PubMed PMID: 16679453;
569 PubMed Central PMCID: PMC1458281.

570 49. Boffa LC, Bolognesi C, Mariani MR. Specific targets of alkylating agents in
571 nuclear proteins of cultured hepatocytes. Mutation research. 1987;190(2):119-23. doi:
572 10.1016/0165-7992(87)90042-X. PubMed PMID: 3821770.

573 50. Flibotte S, Edgley ML, Chaudhry I, Taylor J, Neil SE, Rogula A, et al. Whole-
574 Genome Profiling of Mutagenesis in Caenorhabditis elegans. Genetics.
575 2010;185(2):431-41. doi: 10.1534/genetics.110.116616. PubMed PMID: 20439774;
576 PubMed Central PMCID: PMC2881127.

577 51. Anderson P. Mutagenesis. Methods in cell biology. 1995;48:31-58. Epub
578 1995/01/01. PubMed PMID: 8531732.

579 52. Sega GA. A review of the genetic effects of ethyl methanesulfonate. Mutation
580 Research/Reviews in Genetic Toxicology. 1984;134(2-3):113-42. doi:
581 https://doi.org/10.1016/0165-1110(84)90007-1. PubMed PMID: 6390190.

582 53. Loveless A. Possible relevance of O-6 alkylation of deoxyguanosine to the
583 mutagenicity and carcinogenicity of nitrosamines and nitrosamides. Nature.
584 1969;223(5202):206-7. Epub 1969/07/12. PubMed PMID: 5791738.

585 54. Natarajan AT, Simons JWIM, Vogel EW, van Zeeland AA. Relationship
586 between cell killing, chromosomal aberrations, sister-chromatid exchanges and point
587 mutations induced by monofunctional alkylating agents in Chinese hamster cells a
588 correlation with different ethylation products in DNA. Mutation Research -

589 Fundamental and Molecular Mechanisms of Mutagenesis. 1984;128(1):31-40. doi:
590 10.1016/0027-5107(84)90044-7. PubMed PMID: 6472304.

591 55. Burns PA, Gordon AJE, Kunsmann K, Glickman BW. Influence of Neighboring
592 Base Sequence on the Distribution and Repair of N-Ethyl-N-nitrosourea-induced
593 Lesions in Escherichia coli. Cancer Research. 1988;48(16):4455-8.

594 56. Sarin S, Bertrand V, Bigelow H, Boyanov A, Doitsidou M, Poole RJ, et al.
595 Analysis of multiple ethyl methanesulfonate-mutagenized Caenorhabditis elegans
596 strains by whole-genome sequencing. Genetics. 2010;185(2):417-30. doi:
597 10.1534/genetics.110.116319. PubMed PMID: 20439776; PubMed Central PMCID:
598 PMC2881126.

599 57. Arnold CN, Barnes MJ, Berger M, Blasius AL, Brandl K, Croker B, et al. ENU-
600 induced phenovariance in mice: inferences from 587 mutations. BMC Res Notes.
601 2012;5:577. doi: 10.1186/1756-0500-5-577. PubMed PMID: 23095377; PubMed
602 Central PMCID: PMC3532239.

603 58. Noveroske JK, Weber JS, Justice MJ. The mutagenic action of N-ethyl-N-
604 nitrosourea in the mouse. Mammalian Genome. 2000;11(7):478-83. doi:
605 10.1007/s003350010093. PubMed PMID: 10886009.

606 59. Farrell A, Coleman BI, Benenati B, Brown KM, Blader IJ, Marth GT, et al.
607 Whole genome profiling of spontaneous and chemically induced mutations in
608 Toxoplasma gondii. BMC Genomics. 2014;15:1-15. doi: 10.1186/1471-2164-15-354.
609 PubMed PMID: 24885922; PubMed Central PMCID: PMC4035079.

610 60. Fossett NG, Arbour-Reily P, Kilroy G, McDaniel M, Mahmoud J, Tucker AB, et
611 al. Analysis of ENU-induced mutations at the Adh locus in Drosophila melanogaster.

612 Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis.
613 1990;231(1):73-85. doi: 10.1016/0027-5107(90)90178-7. PubMed PMID: 2114535.

614 61. Tosal L, Comendador MA, Sierra LM. N-ethyl-N-nitrosourea predominantly
615 induces mutations at AT base pairs in pre-meiotic germ cells of Drosophila males.
616 Mutagenesis. 1998;13(4):375-80. Epub 1998/08/26. PubMed PMID: 9717174.

617 62. Justice MJ, Noveroske JK, Weber JS, Zheng B, Bradley A. Mouse ENU
618 Mutagenesis. Human Molecular Genetics. 1999;8(10):1955-63. doi:
619 10.1093/hmg/8.10.1955. PubMed PMID: 10469849.

620 63. Preston BD, Singer B, Loeb LA. Comparison of the relative mutagenicities of
621 O-alkylthymines site-specifically incorporated into phi X174 DNA. The Journal of
622 biological chemistry. 1987;262(28):13821-7. PubMed PMID: 2958453.

623 64. Grevatt PC, Solomon JJ, Bhanot OS. In Vitro Mispairing Specificity of O2-
624 Ethylthymidinet. Biochemistry. 1992;31(17):4181-8. doi: 10.1021/bi00132a005.

625 65. Bronstein SM, Skopek TR, Swenberg JA. Efficient Repair of O6-Ethylguanine,
626 but not O4-Ethylthymine or O2-Ethylthymine, Is Dependent upon O6-Alkylguanine-
627 DNA Alkyltransferase and Nucleotide Excision Repair Activities in human cells.
628 Cancer Research. 1992;52:2008 LP - 11.

629 66. Lock A, Rutherford K, Harris MA, Hayles J, Oliver SG, Bähler J, et al.
630 PomBase 2018: user-driven reimplementation of the fission yeast database provides
631 rapid and intuitive access to diverse, interconnected information. Nucleic Acids
632 Research. 2018:1-7. doi: 10.1093/nar/gky961. PubMed PMID: 30321395.

633 # Supporting information

634 **S1 Fig. Evolutionary conservation of ADE10/ATIC gene.** A) Frequencies of residues
635 found in position 68 and 424 (*S. pombe* numbering) within all ortholog sequences
636 available in Ensembl database. B) Alignment of respective surrounding sequence
637 stretches around residues 68 and 424. Representative prokaryotic and eukaryotic model
638 organisms are included. Valine and Serine present in *S. pombe* are tightly conserved in
639 other yeasts and animals including human.

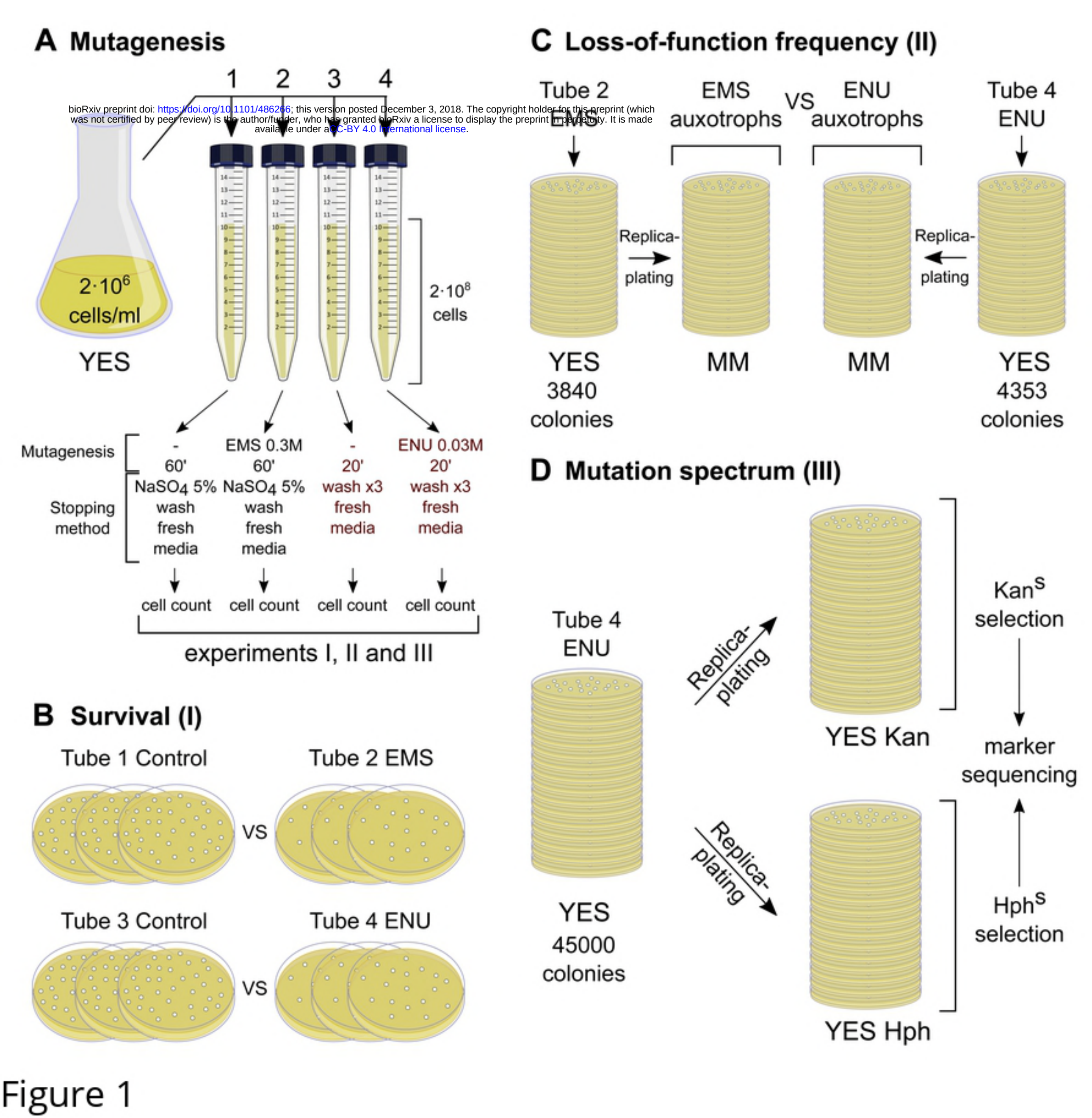

Figure 1