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RAD51D- and FANCG-dependent base substitution mutagenesis at the *ATP1A1* locus in mammalian cells

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Abstract

Elaborate processes act at the DNA replication fork to minimize the generation of chromatid discontinuity when lesions are encountered. To prevent collapse of stalled replication forks, mutagenic translesion synthesis (TLS) polymerases are recruited temporarily to bypass DNA lesions. When a replication-associated (one-ended) double strand break occurs, homologous recombination repair (HRR) can restore chromatid continuity in what has traditionally been regarded as an “error-free” process. Our previous mutagenesis studies show an important role for HRR in preventing deletions and rearrangements that would otherwise result from error-prone nonhomologous end joining (NHEJ) after fork breakage. An analogous, but distinct, role in minimizing mutations is attributed to the proteins defective in the cancer predisposition disease Fanconi anemia (FA). Cells from FA patients and model systems show an increased proportion of gene-disrupting deletions at the *hprt* locus as well as decreased mutation rates in the *hprt* assay, suggesting a role for the FANC proteins in promoting TLS, HRR, and possibly also NHEJ. It remains unclear whether HRR, like the FANC pathway, impacts the rate of base substitution mutagenesis. Therefore, we measured, in isogenic *rad51d* and *fancg* CHO mutants, mutation rates at the Na^+/K^+ -ATPase α -subunit (*ATP1A1*) locus using ouabain resistance, which specifically detects base substitution mutations. Surprisingly, we found that the spontaneous mutation rate was reduced ~2.5-fold in *rad51d* knockout cells, an even greater extent than observed in *fancg* cells, when compared with parental and isogenic gene-complemented control lines. A ~2-fold reduction in induced mutations in *rad51d* cells was seen after treatment with the DNA alkylating agent ethylnitrosourea while a lesser reduction occurred in *fancg* cells. Should the model *ATP1A1* locus be representative of the genome, we conclude that at least 50% of base substitution mutations in this mammalian system arise through error-prone polymerase(s) acting during HRR-mediated restart of broken replication forks.

Keywords

Fanconi anemia; homologous recombination; translesion synthesis; CHO cells; ouabain resistance

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Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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1. Introduction

Since mutations are a driving force in cancer, understanding their genesis and the processes used by the cell to minimize their occurrence is central to developing measures for disease prevention and treatment. Spontaneous mutations generally arise during DNA synthesis when replication forks encounter damage in the template strand, such as oxidized bases, abasic sites, bulky adducts, or breaks in the phosphate-sugar backbone. Translesion synthesis (TLS), which is mediated by specialized bypass polymerases, allows the replication fork to progress past a damaged site albeit with a high likelihood of producing a base substitution mutation [1,2]. If the replication fork encounters a nick in either strand, the resulting one-ended double-strand break (DSB) must be resolved by either homologous recombination repair (HRR) or, eventually, nonhomologous end joining (NHEJ). NHEJ is an efficient but error-prone process that directly rejoins broken DNA ends, generally resulting in the loss of genetic information and chromosomal rearrangement when the ends of different broken chromosomes are joined [3,4].

To prevent deletions and rearrangements, HRR acts at broken replication forks [5–9] to restart replication in a manner that has often been considered to be “error-free” repair [10–12]. HRR is also used during S and G2 phases of the cell cycle to repair a portion of direct (replication-independent) DSBs [9] such as those induced by ionizing radiation or bleomycin. The HRR process employs the Rad51 recombinase, an enzyme that catalyzes the search for homologous DNA sequence and subsequent strand exchange, and requires the activity of BRCA2 [13], and the five paralogs of Rad51 (XRCC2, XRCC3, RAD51B, RAD51C, RAD51D) [14,15]. Rodent cells having defects in the Rad51 paralogs, which have been used extensively to study HRR, show elevated spontaneous chromosomal aberrations and sensitivity to a wide variety of DNA damaging agents, particularly those that cause interstrand crosslinks [8,14–17]. Mutagenesis studies using the *hprt* gene have been valuable in elucidating a major role for HRR in preventing deletion mutations. For example, CHO cells (which are Tp53-defective [18]) with defects in either XRCC3 or Rad51D show a large increase (>10-fold) in the rate of occurrence of spontaneous *hprt* mutations, most of which are deletions encompassing one or more exons as a result of NHEJ events [19].

The role of HRR in preventing large-scale mutations is also becoming better understood through studies with cells from patients with the disease Fanconi anemia (FA) and its model systems. FA is a rare cancer predisposition disorder characterized by developmental abnormalities, progressive bone marrow failure, and increased risk of cancer [20,21]. The disease is caused by mutations in any of at least 13 *FANC* genes, and defects in any of these genes cause broadly similar clinical and cellular phenotypes [22–24]. The FANC proteins appear to maintain chromosome stability by coordinating events that are required to overcome blocked or broken replication forks [19,24–26], as we recently reviewed [27]. Though several complexes of FANC proteins have been identified [28–31], 10 FANC proteins (including those representing eight complementation groups: A, B, C, E, F, G, L, and M) form what can be referred to as the active nuclear “core complex” [24,25,30,32,33]. Two other core complex proteins, FAAP24 [34] and FAAP100 [35], have not been associated with FA patients. Full integrity of the complex is essential for the key step of monoubiquitylation of the FANCD2 and FANCI proteins, an event catalyzed specifically by FANCL, an E3 ubiquitin ligase [36]. In the absence of monoubiquitylation, the resultant cellular phenotype resembles that of HRR-defective cells (i.e. increased chromosomal aberrations and sensitivity to DNA interstrand crosslinks) [27,37–39]. The FANC damage response network also includes FANCI [40], FANCN [41], and the breast cancer susceptibility proteins BRCA1 and BRCA2/FANCD1 as reviewed [42].

Though the reasons for the clinical presentation of FA are not fully understood, mutagenesis studies have revealed that FANC proteins have genetic and functional links to the genomic maintenance processes of TLS, HRR, and NHEJ, as we have discussed in recent studies [19, 26]. Cells from FA patients have shown reduced mutant recovery (hypomutability) in certain mutagenesis studies such as those using the $Na^+/K^+-ATPase$ (α -subunit) (*ATP1A1*) locus, which is associated with ouabain resistance, [43] or *hprt* gene in the purine salvage pathway. Upon treatment with monofunctional or bifunctional DNA-adducting psoralens, decreased frequencies of mutants were recovered at both loci in FA-A fibroblasts relative to unrelated non-FA cells [44,45]. This curious reduction in mutant recovery can be explained by the finding that the spectrum of *hprt* mutations in FA cells, occurring spontaneously or after psoralen-treatment, shows a much higher percentage of deletions than in normal fibroblasts [45]. Moreover, a much higher proportion of *hprt* deletion mutations was found in circulating T-lymphocytes of FA patients relative to unrelated controls [46]. Though these earlier human FA mutagenesis studies employed non-isogenic control lines, they provided hypotheses for further study and, together with our findings utilizing the *fancg* mutant CHO cells, point toward a shift in the severity of the types of mutations occurring during DNA replication from base substitutions and gene-scale deletions (which allow survival of clones with mutations in selectable markers) to a higher proportion of lethal events: multigenic deletions and other chromosomal rearrangements [19,26].

We previously measured the rate of spontaneous mutagenesis at the *hprt* locus in the CHO cell model system in which we constructed a knockout mutation and gene-complemented derivative for *Fancg* [19]. This isogenic system allowed for the direct comparison of the *fancg* mutant to the *rad51d* knockout mutant in order to assess the relative contributions of the linked FANC and HRR “pathways” to spontaneous mutagenesis. Consistent with induced mutant frequencies in FA-patient cells, we found a >3-fold reduced spontaneous mutation rate and a shift in the mutation spectrum to more deletions at the *hprt* locus in the *fancg* cells [19]. The decrease in recoverable mutations of all classes supports a model of reduced TLS activity, as well as reduced HRR and NHEJ, in FANC-defective cells.

In parallel studies, the *rad51d* mutant behaved very differently from the *fancg* mutant, showing a 12-fold increased rate of spontaneous deletions in *hprt* [19]. Whereas the spectrum of the reduced *hprt* mutants in the *fancg* cells provided evidence for a reduction in both base substitutions and deletions, the very large increase in deletion mutations in the *rad51d* cells obscured the ability to assess any role for HRR in influencing base substitution mutation.

To test the possibility of a role for HRR in base-substitution mutagenesis, we used Luria Delbrück fluctuation analysis of *rad51d* and *fancg* mutants in this study to measure the spontaneous mutation rate at the *ATP1A1* locus, which detects base substitution mutations but not deletions [43,47]. In addition, we measured mutation induction by ethylnitrosourea (ENU), an alkylating agent and potent mutagen that efficiently produces base substitutions [48,49].

2. Materials and methods

2.1 Cell lines and culture conditions

CHO cell lines used in this study were the AA8 parental cells [50], the KO40 *fancg* knockout and its hamster *Fancg* gene-complemented derivative (40BP6) [51], and the 51D1 *rad51d* knockout and its hamster *Rad51d* gene-complemented derivative (51D1.3) [8]. V79-derived cell lines used were the *irs1 xrcc2* mutant [52,53] and its complemented derivative GT619 (*irs1* complemented with human *XRCC2* gene in genomic PAC clone) [54]. Cells were grown at 37°C in suspension or monolayer culture in α MEM supplemented with 10% fetal bovine serum, 100 μ g/ml streptomycin, and 100 U/ml penicillin.

2.2 Spontaneous mutagenesis measurements

Mutation rate was determined by fluctuation analysis [55]. 1000 cells (or 2000 for the V79-derived cell lines) were seeded in 24 replicas and expanded to $\sim 2 \times 10^6$ cells per replica (or $\sim 3 \times 10^6$ for the V79-derived cell lines). Cells from each replica were then counted and plated in 10-cm dishes at 2×10^6 cells per dish in medium containing 3.5 mM ouabain (CAS number 630-60-4) to select for cells with mutations at the *ATP1A1* locus. Cells were allowed to grow for eight days (AA8, 40BP6, 51D1.3, and V79-4 wild type) or nine days (KO40, 51D1, and V79-derived lines) for colony formation, then fixed with 95% ethanol, stained with crystal violet, and counted. Mutation rates were calculated by two statistical methods, P_0 [55] and the method of the mean [56].

2.3 Induced mutagenesis measurements

5×10^6 cells were placed in 50 ml growth medium for a 1-h exposure to ethylnitrosourea (ENU; CAS number 759-73-9) at 0, 40, and 80 $\mu\text{g/ml}$ at 37°C in suspension culture. After treatment, cells were transferred to 200-ml spinner flasks (2.5×10^4 cells/ml) and grown three days. Small numbers of cells were seeded for plating efficiency immediately after treatment and just before plating for mutant selection. Selection for *ATP1A1* mutants was performed by seeding 12 dishes at 2×10^6 cells per dish in 3.5 mM ouabain for each ENU dose.

3. Results and discussion

3.1 Reduced spontaneous mutation rates in *fancg* and *rad51d* cells at the *ATP1A1* locus

In this study we measured the rate of mutagenesis at the *ATP1A1* 1023-amino-acid α -subunit locus [57]. Selection for mutations was performed with the drug ouabain, which binds competitively to the K^+ ion-binding domain of the Na^+/K^+ pump in the plasma membrane [58], the function of which is necessary for cellular osmotic regulation. Since mutations at this locus must support a functional plasma membrane pump to be selected as viable colonies, only a limited set of base substitution mutations, those that provide protection from ouabain binding without disrupting pump function, are tolerated [47,59].

The *fancg* KO40 cells showed a significantly reduced spontaneous mutation rate to approximately 50% that of the parental AA8 and gene-complemented control cells (40BP6), based on either of two methods for mutation rate calculation (Table 1). This decrease indicates reduced base substitutions and is consistent with our results at the *hprt* locus in these cells [19], as well as with induced mutagenesis studies in human FA lymphoblasts [44,45].

Surprisingly, the *rad51d* (51D1) cells also showed a significant reduction in spontaneous ouabain resistance mutations compared to parental and gene-complemented control cells (51D1.3) (Table 1). This result stands in sharp contrast to the 12-fold increased rate of mutagenesis in these cells at the *hprt* locus where there is a dramatic shift in the spectrum of *hprt* mutagenic events towards deletions (mediated by NHEJ), which result from broken replication forks [19]. Deletions within *ATP1A1* eliminate protein function and, therefore, do not confer resistance to ouabain. Thus, the reduced recovery of ouabain resistance mutants in *rad51d* cells implies that appreciable base substitution mutagenesis occurs in wild-type cells when HRR efficiently restores broken replication forks.

To test the possibility that reduced base substitution mutagenesis is detectable in another Rad51 paralog mutant, limited measurements of spontaneous mutation rate at the *ATP1A1* locus were made in the V79-derived hamster cell line *irs1* (*xrcc2*) and its corresponding gene-complemented control line GT619. This *XRCC2* Rad51 paralog is of particular interest because Rad51D is the only paralog that interacts with *XRCC2*, and the pair form a distinct heterodimeric complex of unknown function in cells [60–62]. Unlike the CHO system, which

showed close measurements between the parental and gene-complemented control cells, the V79-4 parental cells (in which mutants were created by random mutagenesis) showed a much lower mutation rate ($P_0 = 4 \times 10^{-8}$) than the gene-complemented control cells ($P_0 = 50 [\pm 10] \times 10^{-8}$). This discordance is likely due to the non-isogenic relationship between the parental and mutant cells. Although the rate for *irs1* ($P_0 = 33 [\pm 1] \times 10^{-8}$) was slightly lower than of GT619, the difference was not significant ($P = 0.09$). Thus, this preliminary result in the V79 system lacks the internal consistency of the CHO cells and highlights the importance of using an isogenic system for accurately evaluating mutation rate.

3.2 Reduced mutagenesis induced by ENU at the *ATP1A1* locus in *fancg* and *rad51d* cells

Since spontaneous mutations are thought to be primarily due to oxidative DNA lesions [63], we tested whether alkylation mutagenesis would show a similar pattern of *ATP1A1* mutagenesis in the repair-defective cell lines. We treated cells with the potent mutagen ENU, a monofunctional ethylating agent that is known to cause increased mutations in the *ATP1A1* gene in CHO cells [48,49]. The strong induction of base substitution mutations by ENU [64], along with its efficient induction of sister-chromatid exchange [65], makes this an appropriate agent for our purposes. Sister-chromatid exchange occurs when broken replication forks are restarted by HRR [66] and are presumed to be induced by ENU when replication forks encounter breaks induced by base excision repair.

At two doses of ENU we found significantly reduced mutation induction at the *ATP1A1* locus in both *fancg* and *rad51d* cells relative to parental and gene-complemented control cells (Fig. 1). Reduced mutation induction was seen in both *rad51d* and *fancg* cells, compared with the three control cell lines, at both doses of ENU. At 80 $\mu\text{g/ml}$ ENU, *rad51d* cells showed a significantly greater reduction than did the *fancg* cells ($P < 0.05$). Curiously, whereas AA8 and *fancg* cells show a significant increase in mutations between 40 and 80 $\mu\text{g/ml}$ ($P < 0.05$), *rad51d* cells do not ($P = 0.2$). This apparent saturation of mutagenesis in *rad51d* cells suggests that any residual HRR activity present in these cells is operating at maximum capacity at the level of toxicity caused by 40 $\mu\text{g/ml}$ (58% survival average in the two experiments), such that doubling the level of DNA damage to 80 $\mu\text{g/ml}$ does not produce a corresponding increase in HRR-associated base substitution mutagenesis. The small increase in mutations that occurs with this doubling in dose may be due to the TLS component of replicative synthesis, which seems to play a relatively small role in mutagenesis relative to HRR at the higher level of DNA damage.

The 1.5- to 2-fold reduction in ENU mutagenesis seen in the *fancg* and *rad51d* cells at both doses of ENU, together with the spontaneous mutagenesis data, suggest that there is a generalized reduction in base substitution mutagenesis in both mutant cell lines. We previously reported reduced mutation induction at the *hprt* locus in the *fancg* cells after exposure to ENU [26], which is consistent with the reduction in Fig. 1 for ouabain resistance. The reduction in base substitution mutations in the *fancg* cells treated with ENU also confirms induced mutagenesis studies performed with psoralens on cells from FA patients [44,45]. As we previously discussed in detail for additional loci [8,19], many mutagenic lesions that would normally cause base substitution mutations by TLS polymerases likely become lethal events (e.g. multigenic deletions and translocations) in FANC mutant cells.

3.3 Role of HRR in base substitution mutagenesis

Our initial characterization of the *rad51d* mutant implies a major role for HRR in restarting broken DNA replication forks [8]. The conceptually simplest model to explain our *Rad51D* results is that the repair synthesis occurring during HRR-mediated restart of broken replication forks is inherently error-prone via the involvement of low-fidelity polymerase(s). Studies in *S. cerevisiae* have shown greatly increased point mutations occurring during synthesis

associated with recombinational repair of HO-endonuclease-induced DSBs in a reporter substrate [67,68], and the REV3 subunit of the translesion polymerase pol ζ plays a key role in this repair-associated mutagenesis [68,69]. In avian DT40 cells pol η is required for 90% of the gene conversion events occurring at DSBs created enzymatically in a *SCneo* HRR chromosomal reporter substrate carrying an I-*SceI* recognition site [70]. Consistent with this findings, pol η has also been shown to efficiently perform DNA synthesis in a D-loop structure, in which the invading DNA strand served as a primer for synthesis in cell extracts [71].

The loss of TLS polymerase pol ζ , composed of Rev3 and Rev7 subunits [10], has severe cellular consequences in higher eukaryotes. The interesting phenotype of the pol ζ *Rev3* knockout mutant in DT40 cells includes several features akin to reduced efficiency of HRR [72]: a) elevated spontaneous chromosomal aberrations; b) modest reduction in gene targeting; c) hypersensitivity to diverse DNA damages, especially crosslinking [73]; and d) large increase (~15-fold) in chromosomal aberrations in cells irradiated with γ -rays in G2 phase. Similarly, mouse *Rev3L* null cells (*rev3l*) show chromosomal instability and broad-spectrum sensitivity to DNA-damaging agents, a phenotype characteristic of vertebrate HRR mutants [74,75]. Mouse *rev3l* null cells are more sensitive to mitomycin C than to UV-C or methyl methanesulfonate [75]. The high sensitivity of these vertebrate *rev3* mutants to crosslinking might be explained by a dual requirement for Rev3 in HRR-associated polymerization as well as in TLS during lesion bypass (see model in [27]).

In conclusion, we present evidence for reduced base substitution mutagenesis at the *ATP1A1* locus in *rad51d* cells for both spontaneous and ethylation-induced DNA base damage. These reductions may reflect the normal involvement of one or more mutagenic TLS polymerases, such as pol ζ or pol η , in HRR-associated DNA synthesis. Thus, our findings, along with the studies of vertebrate TLS mutants, are inconsistent with the concept that HRR in proliferating cells is an entirely error-free repair process [11]. Based on the *ATP1A1* locus, we conclude that at least 50% of base substitution mutations in our model system arise during HRR-mediated restart of broken replication forks while the remainder are caused by TLS polymerases operating during normal DNA replication to overcome fork blockage at lesions. Moreover, since Rad51 is essential for cell viability [76] and *rad51d* cells presumably retain a basal level of HRR activity, our measurements may underestimate the contribution of HRR to base substitution mutagenesis. While our initial results in the CHO model system are provocative, further studies are needed (ideally in a Tp53-normal isogenic, euploid human cell system) to understand whether our *Rad51D* findings apply more generally to other cell types and deficiencies in other HRR proteins.

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Abbreviations

CHO	Chinese hamster ovary
DSB	double-strand break
ENU	ethylnitrosourea

hprt	hypoxanthine phosphoribosyltransferase
HRR	homologous recombination repair
IR	ionizing radiation
NHEJ	nonhomologous end joining
TLS	translesion synthesis

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Fig. 1.

ENU-induced *ATP1A1* mutagenesis. The columns represent: AA8 (parental), white; KO40 (*fancg*), diagonal lines; 40BP6 (*FancG* gene-complemented KO40), horizontal lines; 51D1 (*rad51d*), gray; 51D1.3 (*Rad51D* gene-complemented 51D1), black bars. Induced mutant frequency is the average of two experiments for AA8 and the mutant lines, and the gene-complemented control cell lines were measured once; error bars represent standard deviations. At the time of mutant selection, the surviving fraction for each cell line at the 40 μg/ml and 80 μg/ml ENU doses, respectively, was as follows with results of repeat experiments are listed separately: AA8, Expt. 1: 1.03 and 0.99, Expt. 2: 0.97 and 0.94; *fancg* KO40, Expt. 1: 0.66 and 0.42, Expt. 2: 0.58 and 0.32; 40BP6, 0.97 and 0.81; *rad51d* 51D1, Expt. 1: 0.57 and 0.27, Expt. 2: 0.59 and 0.33; 51D1.3, 0.87 and 0.76. These plating efficiencies were used to correct the yield of mutant colonies in treated cultures within each individual experiment. The reduced mutation induction seen in both KO40 and 51D1 cells is statistically different ($P < 0.05$) from that of the parental AA8 cells and from the mean of the two gene-complemented cell lines at both doses of ENU, based on the Student's *t*-test. Asterisks mark significant reductions; the double asterisk indicates a significant difference between the *rad51d* mutant line and the *fancg* mutant line as well as both gene-complemented lines.

Table 1Spontaneous ouabain resistance mutation rate for CHO parental, *rad51d* mutant, and gene-complemented mutants

Cell line	Spontaneous mutation rate $\times 10^{-8} \pm$ std. dev.		P_O ratio WT ^a : mutant	MM ratio WT ^a : mutant
	P ₀ method	Method of mean (MM)		
Parental line, AA8 (3)	12 $\pm$ 2	30 $\pm$ 8		
BP6 (KO40 + <i>Fancg</i> gene) (2)	15 $\pm$ 2	24 $\pm$ 9		
51D1.3 (51D1 + <i>Rad51d</i> gene) (3)	10 $\pm$ 2	22 $\pm$ 4		
KO40 <i>fancg</i> (2)	7 $\pm$ 0.1* ^b	13 $\pm$ 0.9*	1.7	2.0
51D1 <i>rad51d</i> (3)	4 $\pm$ 1** ^b	10 $\pm$ 2*	3.0	2.6

^a In the two right-hand columns “WT” refers to the average of AA8, BP6, and 51D1.3

^b One or two asterisks denote significant ($P < 0.05$) or highly significant ($P < 0.01$) differences between the mutant and corresponding control cells (AA8 parental and specific gene-complemented controls) as determined by students *t*-test. 51D1: P_O ($P < 0.01$), MM ($P < 0.05$); KO40: P_O ($P < 0.05$), MM ($P < 0.05$). Rate measurement differences between KO40 and 51D1 are significant for the P_O calculation ($P < 0.01$) but not for the MM ($P = 0.08$). The number of experiments is given in parentheses.