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Exo1 independent DNA mismatch repair involves multiple compensatory nucleases

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Abstract

Functional DNA mismatch repair (MMR) is essential for maintaining the fidelity of DNA replication and genetic stability. In hematopoiesis, loss of MMR results in methylating agent resistance and a hematopoietic stem cell (HSC) repopulation defect. Additionally MMR failure is associated with a variety of human malignancies, notably Lynch syndrome. We focus on the 5′ → 3′ exonuclease Exo1, the primary enzyme excising the nicked strand during MMR, preceding polymerase synthesis. We found that nuclease dead Exo1 mutant cells are sensitive to the O6-methylguanine alkylating agent temozolomide when given with the MGMT inactivator, O6benzylguanine (BG). Additionally we used an MMR reporter plasmid to verify that Exo1^{mut} MEFs were able to repair G:T base mismatches *in vitro*. We showed that unlike other MMR deficient mouse models, Exo1^{mut} mouse HSC did not gain a competitive survival advantage post temozolomide/BG treatment *in vivo*. To determine potential nucleases implicated in MMR in the absence of Exo1 nuclease activity, but in the presence of the inactive protein, we performed gene expression analyses of several mammalian nucleases in WT and Exo1^{mut} MEFs before and after temozolomide treatment and identified upregulation of Artemis, Fan1, and Mre11. Partial shRNA mediated silencing of each of these in Exo1^{mut} cells resulted in decreased MMR capacity and increased resistance to temozolomide/BG. We propose that nuclease function is required for fully functional MMR, but a portfolio of nucleases is able to compensate for loss of Exo1 nuclease activity to maintain proficiency.

Keywords

Mismatch repair; Exonuclease 1; Temozolomide; DNA repair

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dnarep.2014.06.005.

1. Introduction

The DNA mismatch repair pathway (MMR) is a fundamental process in cells that functions to repair mispaired bases and insertion/deletion loops caused by errors in replication or recombination [1,2]. The pathway has been elucidated largely through studies in *Escherichia coli* and *Saccharomyces cerevisiae* although many of the required enzymes are conserved in higher organisms [3]. In eukaryotic cells the pathway is similar to that found in prokaryotes but the nature of the enzymes involved can vary based on the nature of the mismatch [4]. Several studies have shown that failure in MMR can lead to the accumulation of mutations and carcinogenesis, notably Lynch syndrome [5–7].

As is currently understood, MMR is initiated when a mispaired base or insertion/deletion loop is recognized and bound by either heterodimer of MSH2-MSH6 (MutSα) or MSH2-MSH3 (MutSβ). This binding triggers an ATP dependent reaction which recruits an additional heterodimer consisting of either MLH1-PMS2 (MutLα) or MLH1-PMS1 (MutLβ). The Mutlα complex has been characterized as containing latent endonuclease activity which serves as a discrimination signal for the nascent strand [8]. This results in the recruitment of a variety of other factors including the proliferating cell nuclear antigen (PCNA) which is thought to interact with the MutSα complex to enhance repair, Exonuclease 1 (Exo1) which functions to excise the mispaired base to allow for subsequent resynthesis, and replication protein A (RPA) which functions to stabilize the single stranded intermediate formed by Exo1 activity. DNA polymerase δ has been implicated in the resynthesis of the mismatched strand with DNA ligase functioning to reseal the break [9–12].

Exo1 is a $5' \rightarrow 3'$ exonuclease member of the Rad2 family of nucleases. It has been implicated in a variety of cellular pathways including homologous recombination, meiotic crossing over, telomere maintenance, and mismatch repair [13–17]. It is currently the only nuclease identified in MMR where the enzyme is recruited to the complex by the Mutlα discrimination signal. While it was previously unclear how the $5' \rightarrow 3'$ exonuclease could excise a $3'$ heteroduplex, the Mutlα endonuclease activity is proposed to be directed to the distal side of the mismatch, thus resulting in incision $5'$ to the mispair and allowing for Exo1 recruitment [18]. At that point it excises the mismatched strand of DNA for as long as 1000 nucleotides, thus generating a single stranded DNA gap which is bound by RPA to provide stability. Recently Liberti et al. [19] have described a more precise role in which Exo1 processes replication errors by demonstrating it is specific to lagging strand errors, where it is recruited to the DNA by the $5'$ ends of Okazaki fragments [19]. While MMR mutations in Mlh1, PMS2 and Msh2 have been implicated in the pathogenesis of Lynch syndrome (LS) [20,21], suggesting that a dysfunctional MMR contributes to cancer pathogenesis, mutations in Exo1 are not conclusively linked to patients with LS [22,23]. Additionally studies in yeast have shown that the mutator phenotype in Exo1 deficient *S. pombe* is less significant than that of Msh2 deficient strains. $Exo1^{mut}$ ES cells display an increase in mutation rates at the *Hprt* locus, although they were 5-fold lower than those observed in $MSH2^{-/-}$ cells. Microsatellite instability (MSI) studies in $Exo1^{mut}$ genomic DNA demonstrated increased MSI at mononucleotide markers but levels comparable to WT at dinucleotide markers. [24,25]. These data suggest that loss of Exo1 function does not

completely ablate MMR as occurs with loss of other MMR proteins, and that Exo1 nuclease independent MMR occurs which at least partially maintains the efficiency of the pathway. This complementation of nucleases has been characterized in *E. coli*, where at least 4 exonucleases contribute to MMR [26].

In this manuscript we examined the proficiency of the mismatch repair pathway in mice containing an inactivating mutation in the nuclease domain of Exo1. Of note, unlike the Exo1 null mouse recently described by Schaetzlein et al. [50], this nuclease-dead protein is stable and might contribute to the DNA repair processes, perhaps as a scaffold protein. We show that unlike MMR deficient models, cells derived from these mice were sensitive to the alkylating agent temozolomide and the MGMT inhibitor BG, and that MEFs displayed DNA repair capacity similar to WT mice. Additionally we show that unlike the MMR deficient $Msh2^{-/-}$ mice $Exo1^{mut}$ mice did not gain a hematopoietic competitive survival advantage *in vivo* post temozolomide/BG treatment [27]. Using a heteroduplex eGFP plasmid containing a G:T mismatch we show that the Exo1 mutant MEFs were able to repair G:T mismatches at a level comparable to WT MEFs, indicating a proficient mismatch repair pathway. We used gene expression studies after temozolomide/BG treatment in WT and $Exo1^{mut}$ MEFs and identified 3 upregulated nucleases: Artemis, Fan1, and Mre11. shRNA knockdown of these nucleases resulted in impaired repair of G:T mismatches and an increased resistance to temozolomide/BG, suggesting that complementary nucleases are able to partially maintain MMR capacity in the absence of Exo1. We have identified a potential compensatory mechanism cells utilize to ensure replicative fidelity and mutation avoidance in the absence of a functional Exo1.

2. Materials and methods

2.1. Animals

$Exo1^{mut}$ mice used in these studies were donated by Dr. Winfried Edelmann from the Albert Einstein College of Medicine. Their generation was described by Wei et al. [25]. Mice were used along with their WT littermates throughout. All mouse studies were approved by the institutional animal care and use committee at Case Western Reserve University.

2.2. Temozolomide survival assay

Temozolomide (Ochem Inc.) was prepared by dissolving in DMSO and diluting with serum free DMEM. Final DMSO levels were always <2%. Cells were plated at 5000 cells per well in 6-well tissue culture plates and treated with 10 μM 06-benzylguanine for 1 h to inactivate MGMT [28]. Cells were treated with a temozolomide dose range for 3 h after which media was replaced. 10 μM 06-benzyguanine was added every 24 h for 3 days after which MTT assay was performed to assess cell viability [29]. All experiments were performed in triplicate with identical controls for each replicate and student's *t*-tests were performed at each treatment dose for all cell types.

2.3. Heteroduplex eGFP plasmid

The heteroduplex eGFP plasmid was donated by Dr. Luzhe Sun from the University of Texas Health Science Center at San Antonio. The plasmid was prepared as outlined [30].

Cells were transfected using Lipofectamine 2000 (Invitrogen) with 1 μg of the reporter plasmid, and 24 h post transfection the levels of GFP in cells were measured using cytometric analysis on a BD LSRII instrument [31,32]. All experiments were performed in triplicate with identical controls for each replicate and student's *t*-tests were used to measure statistical significance.

2.4. Competitive repopulation assay

10 Boy J mice were lethally irradiated with 1100 rad Cs^{137}. Five mice were injected with 1:1 ratio of C57/B6:Boy J whole bone marrow and 5 mice were injected with 1:1 ratio of $Exo1^{mut}$:BoyJ whole bone marrow. After 4 weeks the mice were treated with 40 mg/kg 06-benzylguanine followed 1 h later by 80 mg/kg temozolomide for 3 consecutive days. 8 weeks post temozolomide treatment cytometric analysis was performed on peripheral eye blood of transplanted recipients to measure percent chimerism *via* the CD45.1 and CD45.2 cell surface markers [27]. Student's *t*-tests were used to measure statistical significance.

2.5. Gene expression studies

WT and $Exo1^{mut}$ MEFs were treated with 250 μg/mL temozolomide. 24 h post treatment RNA was extracted using the Trizol method and cDNA synthesized (Superscript III First Strand Kit-Invitrogen). Gene expression was measured using validated primers (Applied Biosystems) and quantitative real-time PCR.

2.6. Lentiviral gene silencing

Artemis, Fan1, and Mre11 were silenced *via* shRNA transduction with validated clones (Sigma–Aldrich). Artemis clone IDs (NM 146114.1-880s1c1 and NM 146114.1-1402s1c1). Fan1 clone IDs (XM 885802.2-1049s21c1 and XM 885802.2-1073s21c1). Mre11 clone IDs (NM 018736.2-602s1c1 and NM 018736.2-1594s1c1). Lentiviral particles were synthesized *via* HEK293 cells and target cells were infected, selected for with puromycin, and clones were assessed for verification of gene silencing.

3. Results

3.1. $Exo1^{mut}$ MEFs demonstrate temozolomide sensitivity and repair G/T mismatches in vitro

To determine whether $Exo1^{mut}$ MEFs would display the same methylating agent resistance observed in the MMR deficient $Msh2^{-/-}$ models [27,33] we treated WT, $Exo1^{mut}$ and $Msh2^{-/-}$ MEFs with a temozolomide dose range and measured cell viability *via* MTT assay three days post treatment. MGMT was inactivated by 06-benzylguanine (BG). Temozolomide is an alkylating agent that forms O6-methylguanine which base pairs with thymidine (T) invoking G:T mismatch recognition by MMR and induces cytotoxicity in cells *via* a cycle of futile repair [34,35]. We found that WT and $Exo1^{mut}$ MEFs were sensitive to the drug in a dose dependent fashion. $Exo1^{mut}$ MEFs were slightly more resistant to temozolomide/BG than WT at each dose, suggesting an MMR defect, but the difference was statistically significant ($p < 0.05$) only at the 50 μg/mL dose. $Msh2^{-/-}$ MEFs demonstrated a complete dose independent resistance relative to WT ($p < 0.05$), consistent with loss of MMR function (Fig. 1a).

We used a heteroduplex eGFP plasmid described by Zhou et al. [30] to show that Exo1^{mut} MEFs are capable of repairing base mismatches that would be processed by functional MMR [31,32]. The plasmid contains a G:T mismatch and a nick on the template strand (to serve as a strand discrimination signal) and repair of the G:T to the proper G:C pairing eliminates a premature stop codon in the eGFP gene. Those cells able to convert the G:T mispair display GFP fluorescence. Upon transfecting WT, Exo1^{mut} and Msh2$^{-/-}$ MEFs with the reporter plasmid and measuring GFP fluorescence *via* flow cytometry we found that Exo1^{mut} MEFs demonstrated a modestly decreased MMR capacity compared to WT MEFs that was not quite statistically significant ($p = 0.0523$). The Exo1^{mut} MMR capacity was significantly greater than the Msh2$^{-/-}$ MEFs, which were severely defective in their MMR response compared to WT ($p < 0.05$, Fig. 1b). This data suggests that Exo1^{mut} cells are able to process and repair mismatches *via* a functional, albeit somewhat impaired MMR.

3.2. Exo1^{mut} bone marrow does not exhibit a competitive repopulation defect nor does it gain a competitive survival advantage post temozolomide treatment

We performed a hematopoietic competitive repopulation assay to measure hematopoietic stem cell (HSC) proficiency of Exo1^{mut} bone marrow. Competitive repopulation measures HSC proficiency in engrafting into the HSC niche and performing long term multi-lineage reconstitution. Previous studies have shown that HSC from MMR deficient Msh2$^{-/-}$ mice display defects in competitive repopulation and additionally gain a competitive survival advantage over WT marrow when the chimeric mice are treated with temozolomide [27]. We used cytometric analysis to show that at both 8 and 16 weeks post marrow transplantation, the Exo1^{mut} marrow remained an approximately 1:1 ratio with WT marrow suggesting proficient hematopoietic function. We treated 1:1 chimeric mice with 40 mg/kg BG to inactivate MGMT followed by 80 mg/kg temozolomide (3×), measured the percent chimerism 8 weeks post treatment and found that the ratio remained approximately 1:1 (Fig. 2). This data demonstrates in an *in vivo* setting that HSC in Exo1^{mut} mice are sensitive to methylating agents due to proficiency in the MMR pathway. In contrast, we previously observed a profound selection advantage in favor of MSH2$^{-/-}$ HSC after temozolomide in similar hematopoietic competitive repopulation studies [27].

3.3. Gene expression changes of multiple nucleases following temozolomide treatment

To elucidate the mechanism of MMR in the absence of Exo1, we performed gene expression analysis of multiple nucleases in WT and Exo1^{mut} MEFs. We examined five mammalian nucleases also containing $5' \rightarrow 3'$ enzymatic activity (Artemis, Fan1, Fen1, Mre11 and XPF) and studied changes in expression after temozolomide/BG treatment. In WT MEFS we found that temozolomide induced strong expression of Artemis, Fan1, and Mre11 24 h after treatment. XPF and Fen1 levels were unchanged as was expression of Exo1, suggesting that basal levels of Exo1 are sufficient for MMR activity in normal settings (Fig. 3a). Transcript induction was followed by increases in protein concentrations detected by western blot (Supplemental Fig. 1). The upregulation of the three nucleases in WT MEFs suggested that these enzymes may complement each other in normal DNA damage response pathways, including mismatch repair.

In contrast to the pattern observed in WT MEFs, Exo1^{mut} MEFs demonstrated increased basal expression of Artemis, Fan1, and Mre11 but no induction of any nucleases was observed after temozolomide treatment (Fig. 3b and c). While the mechanism of post DNA damage induction is not yet elucidated, one interpretation is that levels of WT Exo1 are sufficient for MMR whereas the other three enzymes are upregulated in response to damage, and constitutively upregulated in the absence of Exo1 nuclease activity in Exo1^{mut} MEFs, perhaps in a compensatory manner.

3.4. Partial silencing of Artemis, Fan1, and Mre11 results in increased temozolomide resistance and decreased MMR proficiency in Exo1^{mut} MEFs

After identifying Artemis, Fan1, and Mre11 as potentially being involved in MMR proficiency after loss of Exo1 nuclease activity, we partially silenced these genes *via* lentiviral shRNA knockdown in WT and Exo1^{mut} MEFs to determine whether the loss of each nuclease singly and collectively would result in an impaired MMR phenotype. We additionally included a scrambled shRNA sequence in WT and Exo1^{mut} MEFs to demonstrate that the transduction had no effect on temozolomide sensitivity (Supplemental Fig. 2). As shown in Fig. 4a only moderate levels of gene silencing were achieved in surviving cells. A second shRNA vector to each gene was also used and the data shown in Supplemental Fig. 3. In addition it should be noted that the level of Artemis, Fan1, and Mre11 nuclease knockdown in Exo1^{mut} MEFs, which, as noted, displayed a higher basal level of each transcript than WT MEFs (Fig. 3c), results in remaining transcript levels that are similar to those of WT MEFs. We treated each cell type (WT, WT + shArtemis, WT + shFan1, WT + shMre11, Exo1^{mut}, Exo1^{mut} + shArtemis, Exo1^{mut} + shFan1 and Exo1^{mut} + shMre11) with BG and a temozolomide dose range and measured cell survival after three days *via* MTT assay. We showed that partial knockdown of each nuclease in both WT and Exo1^{mut} MEFs resulted only in a modest increase in temozolomide resistance. For shArtemis, only the 50 μg/mL dose in Exo^{mut} shArtemis cells was significantly different from WT MEFs ($p < 0.05$). WTshFan1 cells demonstrated a significant difference at the 50 μg/mL dose, while Exo^{mut} shFan1 cells were significant at both the 50 μg/mL and 125 μg/mL doses ($p < 0.05$). This was the same trend observed in shMre11 cells. When directly comparing the WT silenced *vs.* Exo^{mut} silenced cells we found that the Exo^{mut} curves were significantly more resistant than WT for shArtemis and shFan1 ($p < 0.05$). We used the MMR reporter assay to determine whether the increased temozolomide resistance correlated with a decreased MMR capacity and found that only Exo^{mut} shMre11 cells were statistically reduced compared to WT ($p < 0.05$), while Exo^{mut} shFan1 demonstrated a not quite significant reduction in GFP levels compared to WT ($p = 0.0572$, Fig. 4). Interestingly, partial knockdown of any single nuclease in WT MEFs did not result in statistically significant differences in MMR capacity.

These results suggested that loss of a single nuclease, even in combination with Exo^{mut}, was not enough to yield a defective MMR phenotype. To elucidate whether multiple nucleases were responsible for Exo1 nuclease independent MMR, we silenced a combination of each nuclease (Artemis/Fan1, Artemis/Mre11, Fan1/Mre11) in both WT MEFs which contain functional Exo1, and Exo1^{mut} MEFs. This would help clarify whether functional Exo1 would be sufficient to maintain proficient MMR in the absence of the additional nucleases. It

would also elucidate the necessity of these nucleases in maintaining MMR in the absence of Exo1. Similar to what we observed with single gene silencing, only modest levels of silencing of nuclease pairs was achieved (Fig. 5a). However, even with the moderate silencing, cells with loss of these nuclease pairs demonstrated proliferation defects and spontaneous apoptosis. This suggests that these genes play important roles in multiple cellular pathways and are important for cell survival. We examined temozolomide sensitivity of each of the double knockdown cells. WTshArt/Fan cells were more resistant than WT MEFs at 50 μg/mL, while Exo1shArt/Fan cells were significantly different at both 50 μg/mL and 125 μg/mL ($p < 0.05$). WTshArt/Mre cells were also only more resistant than WT MEFs at the 50 μg/mL dose, while Exo^{mut} shArt/Mre cells were more resistant at all three drug doses ($p < 0.05$). Finally the WTshFan/Mre MEFs were significantly different from WT MEFs at both the 50 μg/mL and 125 μg/mL doses while the $Exo1^{mut}$ shFan/Mre cells were resistant at all three doses ($p < 0.05$). All three sets of Exo^{mut} dual knockdown cells were more temozolomide resistant than their WT counterparts, suggesting a higher degree of loss of MMR as indicated by tolerance to DNA methylation from temozolomide ($p < 0.05$).

The MMR capacity in these combination knockdown cells was measured using the heteroduplex plasmid. Compared to WT MEFs, partial combination silencing in WT MEFs yielded reduced MMR capacity only in the combination of WTshFan/Mre11, while WTshArt/Fan ($p = 0.051$) and WTshArt/Mre11 ($p = 0.11$) cells were not statistically reduced. Knockdown in $Exo1^{mut}$ MEFs however displayed reduced MMR capacity with each combination ($p < 0.05$). While neither complete temozolomide resistance nor loss of MMR proficiency was observed in the $Exo1^{mut}$ nuclease KD combination silenced cells, the data strongly suggests that significant MMR deficiency develops under conditions of loss of Exo1 nuclease activity combined with loss of the other nucleases. While these nucleases appear complementary, the data from WT MEFs suggests that Exo1 alone is sufficient to retain a functional level of MMR capacity.

Finally to determine whether complete MMR deficiency would be observed after loss of all three identified nucleases, we attempted to silence the combination of Artemis/Fan1/Mre11 in both WT and Exo^{mut} MEFs. As with previous experiments the level of gene silencing was modest in both cell types and was accompanied by significant proliferation defects and spontaneous apoptosis. Surviving cells were assessed for gene knockdown level and treated with the temozolomide dose range and MMR reporter plasmid. Temozolomide resistance data demonstrated that both WTshArtemis/Fan1/Mre11 cells and Exo^{mut} shArtemis/Fan1/Mre11 cells were significantly more resistant than WT MEFs at all doses ($p < 0.05$), but that the Exo^{mut} triple knockdown cells were also more resistant than WT triple knockdown cells.

The MMR reporter assay was performed on these cells and both the WT and $Exo1^{mut}$ knockdown cells demonstrated significantly reduced activity compared to WT MEFs ($p < 0.05$), and were comparable to the GFP levels of $Msh2^{-/-}$ cells (Fig. 6b). Given this level of MMR dysfunction compared to WT, the difference between the WT and $Exo1^{mut}$ triple knockdown cells was not quite statistically significant ($p = 0.08$). These data demonstrated that the combination loss of these three nucleases resulted in increased temozolomide resistance and significant loss of MMR capacity, closely resembling the phenotype of

Msh2$^{-/-}$ cells. Thus, these complementary nucleases contribute to Exo1 nuclease independent MMR function.

4. Discussion

Our work demonstrates that while Exo1 nuclease activity is important for normal MMR function, its loss can be partially compensated for by the nucleases Artemis, Fan1, and Mre11. While these nucleases are induced in response to temozolomide in WT cells, perhaps implicating them in the normal repair of methylating agent induced DNA damage, they are basally upregulated in Exo1^{mut} MEFs which suggests that they serve as primary nucleases in the absence of Exo1 to maintain partial MMR function. This differs from MMR proteins Mlh1 and Msh2 for which there are no redundant proteins. In this paper we show that Exo1^{mut} MEFs were relatively sensitive to the O6-methylguanine alkylating agent temozolomide combined with O6BG to inactivate the MGMT repair protein, that they were able to repair G:T mismatches *in vitro*, that HSC from Exo1^{mut} mice did not demonstrate a hematopoietic competitive advantage *in vivo* after temozolomide treatment, that Exo1^{mut} MEFs displayed increased expression of nucleases potentially involved in MMR, and that partial silencing of these nucleases in Exo1^{mut} MEFs resulted in increased temozolomide resistance and decreased MMR function.

The nucleases Artemis, Fan1, and Mre11, which we identified as being upregulated after temozolomide treatment in Exo1^{mut} MEFs are the likely lead candidates for a complementation function in MMR. Mre11 has a role in MMR based on the following observations by others: Mre11 has been shown to be frequently mutated in MMR deficient cancers [36], its physical interactions with Mlh1 have been characterized [37], and its loss has been shown to result in increases in microsatellite instability (a marker for loss of MMR function) and impaired MMR proficiency [38,39]. Additionally the Fanconi Anemia nuclease Fan1, which contains $5' \rightarrow 3'$ exonuclease activity and endonuclease activity, was identified by a genetic screen as having interactions with MMR proteins Mlh1 and PMS2 [40,41]. Fan1, while possibly not critical to MMR in normal settings, may become actively involved in MMR after loss of Exo1. Finally the nuclease Artemis, which has been implicated in the DNA double strand break repair pathway non-homologous end joining, also has single strand specific $5' \rightarrow 3'$ exonuclease activity although it has not been found to interact with critical MMR proteins. However, Katsube et al. have described Artemis deficient MEFs to demonstrate increased resistance to MMS and propose that it may play a role in multiple DNA repair pathways [42]. The three candidate genes however all demonstrate similar $5' \rightarrow 3'$ exonuclease function as Exo1, with Mre11 also containing $3' \rightarrow 5'$ activity. Whether both are operant in MMR remains to be explored.

While our studies demonstrated that a modest knockdown of these nucleases resulted in changes in both temozolomide sensitivity and MMR activity, the effects may be more striking when these enzymes are completely inactivated. Of interest is the fact that basal Exo1^{mut} MEFs contained significantly higher transcript levels of all three genes than WT MEFs, thus the partial shRNA knockdown achieved in Exo1^{mut} MEFs resulted in nuclease gene expression levels that were similar to that found in unperturbed WT MEFs. That a phenotype was observed under these conditions suggests that their upregulation in the

Exo1^{mut} MEFs may be compensatory to provide partial Exo1 independent MMR activity. This predicts that complete gene inactivation of multiple nucleases could produce a phenotype that mimics complete MMR failure. Additionally the spontaneous apoptosis and proliferation defects we observed after silencing of 2 or 3 of these genes confirm that the nucleases play crucial roles in DNA repair processes beyond MMR, and it will be interesting to study how the loss of those additional pathways (such as potential defects in double strand break repair) would affect MMR capacity.

Our work extends the pioneering work with the cells and mice by the Edelmann and Samson groups [13,25]. We re-derived MEFs from mice received from the Edelmann lab and pursued studies with temozolomide whereas Klapacz reports studies with MNNG in Exo1^{mut} MEFs. There are some distinct differences between the studies. Whereas in Klapacz et al, MNNG sensitivity of Exo1^{mut} mice was similar to WT and not that different than with MSH6$^{-/-}$, the bone marrow CFU from these mice did show modest resistance to MNU that is less than that of the MSH6$^{-/-}$. The structure, and methyl group donor reactivity of MNU and temozolomide are closer than to that of MNNG [43,44], and the observation that our data with temozolomide is more similar to that of the MNU data with bone marrow is perhaps to be expected. Further, the protective effect of Exo1^{mut} on loss of MGMT observed by Klapacz, is not replicated in our experiments adding O6BG to cell cultures to block MGMT, given the well recognized ability of low levels of newly synthesized MGMT to repair O6mG lesions, explaining often seen differences between MGMT− cells and MGMT+ cells treated with O6BG [45]. The studies of Wei et al. [11], indicate in Exo1^{mut} cells that there is a template dependency of the loss of function of MMR, as to be expected given the function of Exo1. Our suggestion that there is some degree of complementation utilized by up regulation of other nucleases, may also be template dependent. It is also likely that the expression levels of these DNA repair enzymes are different in ES cells and MEFs. Lee et al. measured Exo1 levels in multiple organs and found that it was highest in testis, lung, and spleen [46], suggesting that different cell types may have altered reliance on Exo1 mediated MMR.

Previous work have demonstrated that loss of Exo1 function has a more modest phenotype in terms of cancer predisposition and mutability when compared to loss of critical MMR proteins Msh2 and Mlh1. However work from our group and recently Schaetzlein et al. [50] have demonstrated its activity is crucial for DNA double strand break (DSB) processing [45]. Our studies have shown that loss of Exo1 nuclease function results in increased sensitivity to DSB inducing agents due to impaired end processing. Like the studies by Klapacz et al. [25] who showed a physiologic impact of loss of Exo1 on the lymphoid system, we have shown that loss of Exo1 results in hypersensitivity of hematopoietic stem cells to homologous recombination mediated repair of double strand breaks after radiation, particularly when these stem cells are forced into cell cycle in response to 5-FU treatment ([47]). Thus its enzymatic function is critical to DNA end resection of double strand breaks, while in MMR its exonuclease activity appears somewhat dispensable due to compensatory nucleases. However a mechanism for MMR in the absence of Exo1 has yet to be fully elucidated. Kadyrov et al. [48] described a possible mechanism for Exo1 independent MMR by utilizing a purified system containing MutSα, MutLα, replication factor C, PCNA, RPA, and DNA polymerase δ and observing MMR function *in vitro* dependent on the

endonuclease activity of MutLα and strand displacement *via* polymerase δ [48]. While our work suggests that additional nucleases may also be involved, the fact that total MMR function is not completely ablated and complete temozolomide resistance is not achieved when silencing these enzymes suggests that additional mechanisms such as the strand displacement may also be occurring. Additionally the discrepancy between the complete loss of GT repair capacity but lack of complete temozolomide resistance in the triple knockdown cell lines (Fig. 6) may be explained by altered BER activity (responsible for resolving N-7mG and N-3 mA lesions induced by temozolomide) after partial loss of these nucleases.

Recently published work has shown that the structural function of Exo1 protein may be completely distinct from its catalytic one, very notably in MMR. Izumchenko et al. [49] showed *in vitro* and most recently Schaetzlein et al. [50] showed *in vivo* that the catalytic nuclease function of Exo1 may not be necessary for its role in MMR [49,50]. In fact it has been proposed that the structural component of Exo1 may serve as a docking site or play a scaffolding role in the formation of protein complexes including additional nucleases in MMR. Our work with the $Exo1^{mut}$ mouse, which contains a slightly truncated Exo1 protein, may corroborate these studies which would explain the modest phenotype observed after loss of Exo1 function, because the structural component could still participate to dock the additional nucleases.

Linking four nucleases to MMR suggests that cells prioritize genomic stability. We might next ask how this compensatory pathway affects the response to other types of DNA damage which requires these identified enzymes. In human disease this story raises some interesting questions such as whether syndromes associated with microsatellite instability and cancer predisposition, such as Lynch Syndrome may involve mutations in Artemis, Fan1, or Mre11, and be linked to spontaneous malignancies with loss of MMR from promoter methylation of these genes, or other diseases associated with dysfunctional MMR. The complementary nature of these nucleases also provides data linking seemingly independent DNA repair pathways. It appears cells may use multiple compensatory pathways to maintain MMR function and avoid genomic instability. This work further characterizes the multifaceted nature of mismatch repair and the implications that its loss has on genomic stability and other DNA repair pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Modrich P, Lahue R. Mismatch repair in replication fidelity, genetic recombination, and cancer biology. Annu. Rev. Biochem. 1996; 65:101–133. [PubMed: 8811176]
2. Li GM. Mechanisms and functions of DNA mismatch repair. Cell Res. 2008; 18(1):85–98. [PubMed: 18157157]
3. Jiricny J. The multifaceted mismatch-repair system. Nat. Rev. Mol. Cell Biol. 2006; 7(5):335–346. [PubMed: 16612326]
4. Kunkel TA, Erie DA. DNA mismatch repair. Annu. Rev. Biochem. 2005; 74:681–710. [PubMed: 15952900]
5. Peltomaki P. Role of DNA mismatch repair defects in the pathogenesis of human cancer. J. Clin. Oncol. 2003; 21(6):1174–1179. [PubMed: 12637487]
6. Kolodner RD. Mismatch repair: mechanisms and relationship to cancer susceptibility. Trends Biochem. Sci. 1995; 20(10):397–401. [PubMed: 8533151]
7. Marra G, Boland CR. Hereditary nonpolyposis colorectal cancer: the syndrome, the genes, and historical perspectives. J. Natl. Cancer Inst. 1995; 87(15):1114–1125. [PubMed: 7674315]
8. Kadyrov FA, et al. Endonucleolytic function of MutLalpha in human mismatch repair. Cell. 2006; 126(2):297–308. [PubMed: 16873062]
9. Jascur T, Boland CR. Structure and function of the components of the human DNA mismatch repair system. Int. J. Cancer. 2006; 119(9):2030–2035. [PubMed: 16804905]
10. Iyer RR, et al. The MutSalpha-proliferating cell nuclear antigen interaction in human DNA mismatch repair. J. Biol. Chem. 2008; 283(19):13310–13319. [PubMed: 18326858]
11. Kolodner R. Biochemistry and genetics of eukaryotic mismatch repair. Genes Dev. 1996; 10(12): 1433–1442. [PubMed: 8666228]
12. Schofield MJ, Hsieh P. DNA mismatch repair: molecular mechanisms and biological function. Annu. Rev. Microbiol. 2003; 57:579–608. [PubMed: 14527292]
13. Klapacz J, et al. O6-methylguanine-induced cell death involves exonuclease 1 as well as DNA mismatch recognition in vivo. Proc. Natl. Acad. Sci U. S. A. 2009; 106(2):576–581. [PubMed: 19124772]
14. Schaetzlein S, et al. Exonuclease-1 deletion impairs DNA damage signaling and prolongs lifespan of telomere-dysfunctional mice. Cell. 2007; 130(5):863–877. [PubMed: 17803909]
15. Maringele L, Lydall D. EXO1-dependent single-stranded DNA at telomeres activates subsets of DNA damage and spindle checkpoint pathways in budding yeast yku70Delta mutants. Genes Dev. 2002; 16(15):1919–1933. [PubMed: 12154123]
16. Genschel J, Bazemore LR, Modrich P. Human exonuclease I is required for 5′ and 3′ mismatch repair. J. Biol. Chem. 2002; 277(15):13302–13311. [PubMed: 11809771]
17. Tran PT, et al. EXO1-A multi-tasking eukaryotic nuclease. DNA Repair (Amst.). 2004; 3(12): 1549–1559. [PubMed: 15474417]
18. Pluciennik A, et al. PCNA function in the activation and strand direction of MutLalpha endonuclease in mismatch repair. Proc. Natl. Acad. Sci. U. S. A. 2010; 107(37):16066–16071. [PubMed: 20713735]
19. Liberti SE, Larrea AA, Kunkel TA. Exonuclease 1 preferentially repairs mismatches generated by DNA polymerase alpha. DNA Repair (Amst.). 2013; 12(2):92–96. [PubMed: 23245696]
20. Kuismanen SA, et al. Genetic and epigenetic modification of MLH1 accounts for a major share of microsatellite-unstable colorectal cancers. Am. J. Pathol. 2000; 156(5):1773–1779. [PubMed: 10793088]
21. Russo A, et al. Prognostic relevance of MLH1 and MSH2 mutations in hereditary non-polyposis colorectal cancer patients. Tumori. 2009; 95(6):731–738. [PubMed: 20210238]
22. Jagmohan-Changur S, et al. EXO1 variants occur commonly in normal population: evidence against a role in hereditary nonpolyposis colorectal cancer. Cancer Res. 2003; 63(1):154–158. [PubMed: 12517792]
23. Thompson E, et al. Hereditary non-polyposis colorectal cancer and the role of hPMS2 and hEXO1 mutations. Clin. Genet. 2004; 65(3):215–225. [PubMed: 14756672]

24. Liberti SE, Rasmussen LJ. Is hEXO1 a cancer predisposing gene? Mol. Cancer Res. 2004; 2(8): 427–432. [PubMed: 15328369]
25. Wei K, et al. Inactivation of Exonuclease 1 in mice results in DNA mismatch repair defects, increased cancer susceptibility, and male and female sterility. Genes Dev. 2003; 17(5):603–614. [PubMed: 12629043]
26. Burdett V, et al. In vivo requirement for RecJ, ExoVII, ExoI, and ExoX in methyl-directed mismatch repair. Proc. Natl. Acad. Sci. U. S. A. 2001; 98(12):6765–6770. [PubMed: 11381137]
27. Reese JS, Liu L, Gerson SL. Repopulating defect of mismatch repair-deficient hematopoietic stem cells. Blood. 2003; 102(5):1626–1633. [PubMed: 12730104]
28. Vlachostergios PJ, Hatzidaki E, Papandreou CN. MGMT repletion after treatment of glioblastoma cells with temozolomide and O6-benzylguanine implicates NFkappaB and mutant p53. Neurol. Res. 2013; 35(8):879–882. [PubMed: 23561593]
29. van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol. Biol. 2011; 731:237–245. [PubMed: 21516412]
30. Zhou B, et al. Preparation of heteroduplex enhanced green fluorescent protein plasmid for in vivo mismatch repair activity assay. Anal. Biochem. 2009; 388(1):167–169. [PubMed: 19248754]
31. Tichy ED, et al. Mismatch and base excision repair proficiency in murine embryonic stem cells. DNA Repair (Amst.). 2011; 10(4):445–451. [PubMed: 21315663]
32. Mohni KN, et al. DNA mismatch repair proteins are required for efficient herpes simplex virus 1 replication. J. Virol. 2011; 85(23):12241–12253. [PubMed: 21957315]
33. Claij N, Te Riele H. Methylation tolerance in mismatch repair proficient cells with low MSH2 protein level. Oncogene. 2002; 21(18):2873–2879. [PubMed: 11973647]
34. Fu D, Calvo JA, Samson LD. Balancing repair and tolerance of DNA damage caused by alkylating agents. Nat. Rev. Cancer. 2012; 12(2):104–120. [PubMed: 22237395]
35. Newlands ES, et al. Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat. Rev. 1997; 23(1):35–61. [PubMed: 9189180]
36. Giannini G, et al. Human MRE11 is inactivated in mismatch repair-deficient cancers. EMBO Rep. 2002; 3(3):248–254. [PubMed: 11850399]
37. Her C, Vo AT, Wu X. Evidence for a direct association of hMRE11 with the human mismatch repair protein hMLH1. DNA Repair (Amst.). 2002; 1(9):719–729. [PubMed: 12509276]
38. Vo AT, et al. hMRE11 deficiency leads to microsatellite instability and defective DNA mismatch repair. EMBO Rep. 2005; 6(5):438–444. [PubMed: 15864295]
39. Gaymes TJ, et al. Microsatellite instability induced mutations in DNA repair genes CtIp and MRE11 confer hypersensitivity to poly (ADP-ribose) polymerase (PARP) inhibitors in myeloid malignancies. Haematologica. 2013; 98(9):1397–1406. [PubMed: 23349304]
40. Yoshikiyo K, et al. KIAA1018/FAN1 nuclease protects cells against genomic instability induced by interstrand cross-linking agents. Proc. Natl. Acad. Sci. U. S. A. 2010; 107(50):21553–21557. [PubMed: 21115814]
41. Smogorzewska A, et al. A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. Mol. Cell. 2010; 39(1):36–47. [PubMed: 20603073]
42. Katsube T, et al. Differences in sensitivity to DNA-damaging Agents between XRCC4- and Artemis-deficient human cells. J. Radiat. Res. 2011; 52(4):415–424. [PubMed: 21785230]
43. Wyatt MD, Pittman DL. Methylating agents and DNA repair responses: methylated bases and sources of strand breaks. Chem. Res. Toxicol. 2006; 19(12):1580–1594. [PubMed: 17173371]
44. Beranek DT. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat. Res. 1990; 231(1):11–30. [PubMed: 2195323]
45. Weller M, et al. MGMT promoter methylation in malignant gliomas: ready for personalized medicine? Nat. Rev. Neurol. 2010; 6(1):39–51. [PubMed: 19997073]
46. Lee BI, et al. Expression specificity of the mouse exonuclease 1 (mExo1) gene. Nucleic Acids Res. 1999; 27(20):4114–4120. [PubMed: 10497278]

47. Desai A, Qing Y, Gerson SL. Exonuclease 1 is a critical mediator of survival during DNA double strand break repair in nonquiescent hematopoietic stem and progenitor cells. Stem Cells. 2014; 32(2):582–593. [PubMed: 24420907]

48. Kadyrov FA, et al. A possible mechanism for exonuclease 1-independent eukaryotic mismatch repair. Proc. Natl. Acad. Sci. U. S. A. 2009; 106(21):8495–8500. [PubMed: 19420220]

49. Izumchenko E, Saydi J, Brown KD. Exonuclease 1 (Exo1) is required for activating response to S(N)1 DNA methylating agents. DNA Repair (Amst.). 2011; 11(12):951–964.

50. Schaetzlein S, et al. Mammalian Exo1 encodes both structural and catalytic functions that play distinct roles in essential biological processes. Proc. Natl. Acad. Sci. U. S. A. 2013; 110(27):E2470–E2479. [PubMed: 23754438]

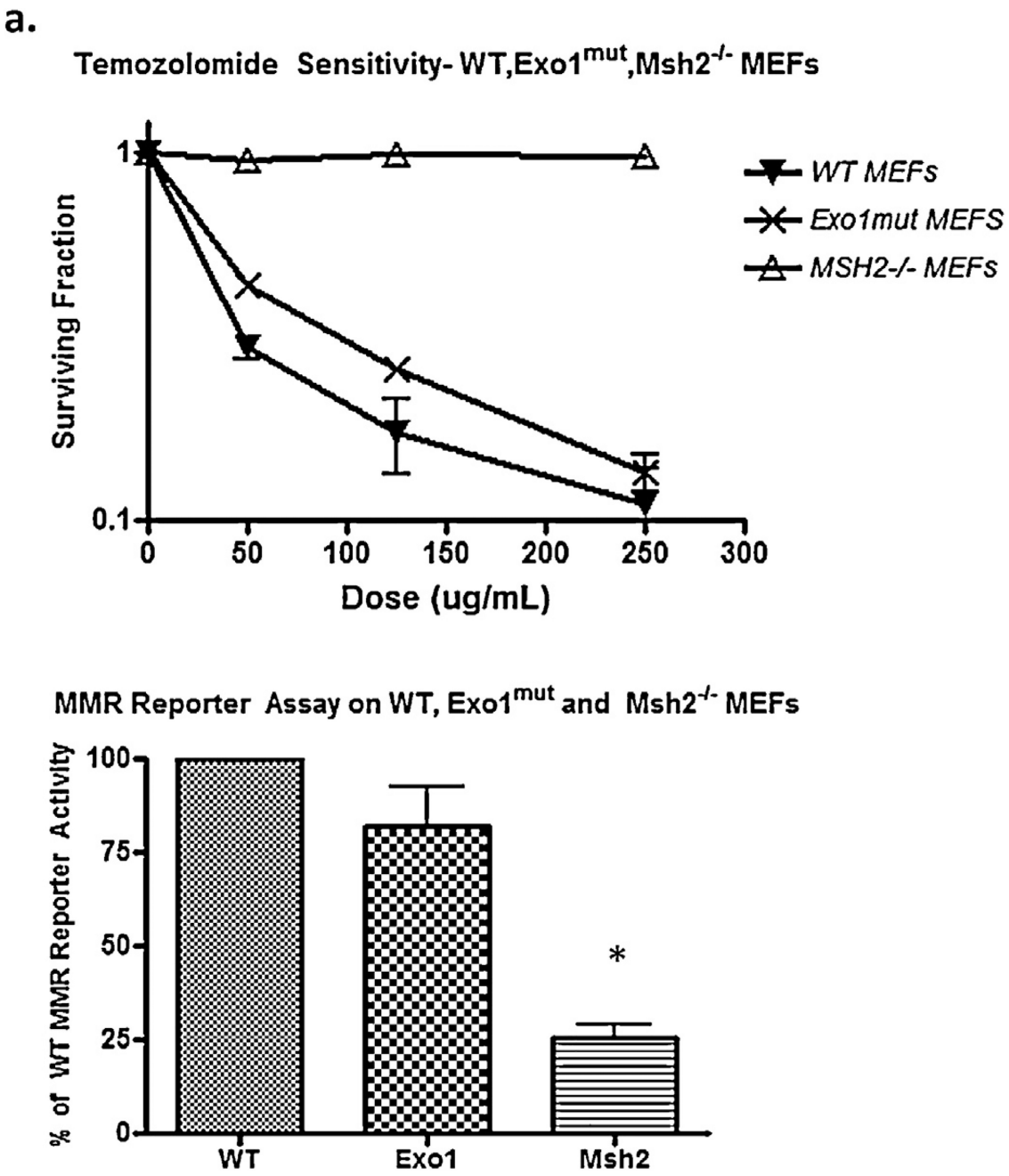

Fig. 1.

Exo1^{mut} MEFs demonstrate temozolomide sensitivity and repair G/T mismatches *in vitro*. (A) WT, Exo1^{mut}, and Msh2$^{-/-}$ MEFs were treated with a temozolomide dose range and survival was monitored three days post treatment *via* MTT assay. Error bars indicate SEM from 3 independent experiments. (B) The heteroduplex eGFP plasmid was used on WT, Exo1^{mut}, and Msh2$^{-/-}$ MEFs and GFP fluorescence was measured 24 h post transfection *via* cytometric analysis. Error bars indicate SEM from 3 independent experiments.

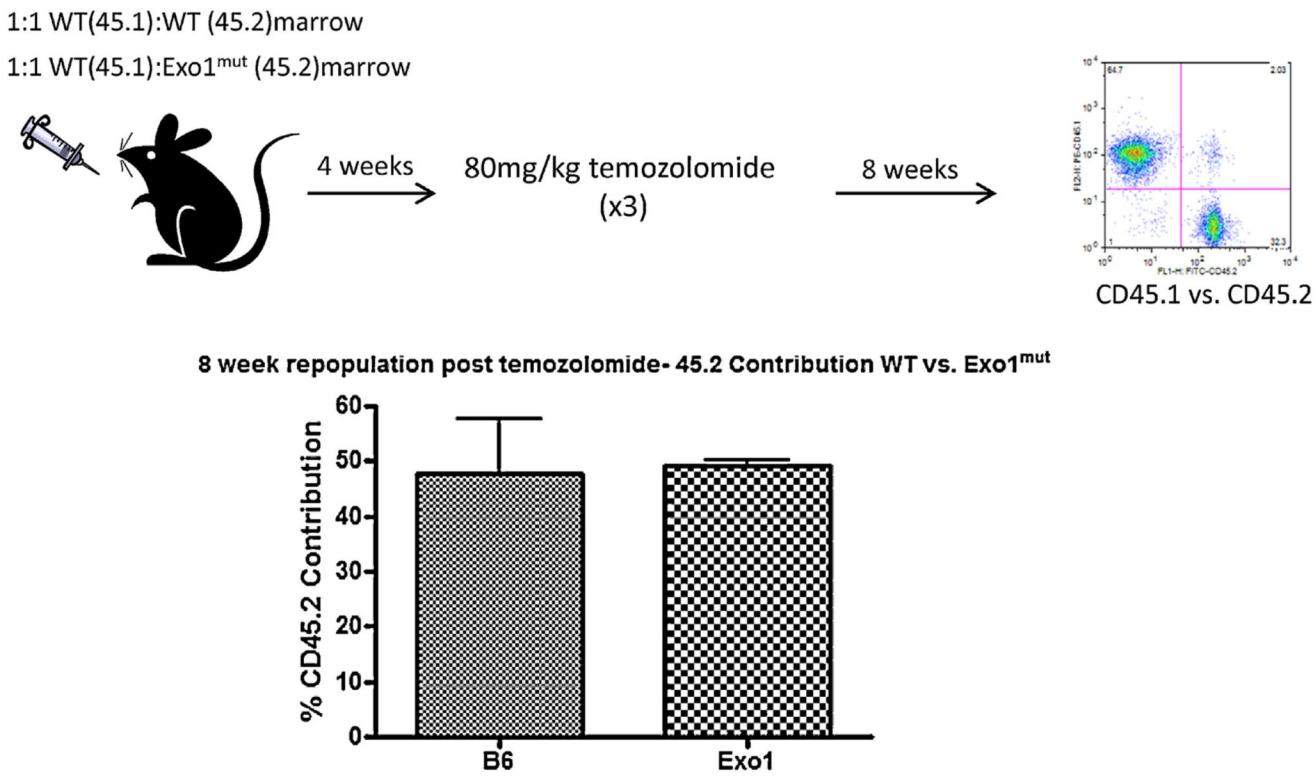

Fig. 2.

Exo1^{mut} mice do not gain a hematopoietic competitive advantage after temozolomide treatment *in vivo*. WT and Exo1^{mut} whole bone marrow was mixed 1:1 and transplanted into lethally irradiated WT recipients. 4 weeks post transplants chimeric mice were treated with 40 mg/kg BG followed by 80 mg/kg temozolomide (3× days) and 8 weeks post treatment chimerism was measured *via* cytometric analysis of peripheral eye blood.

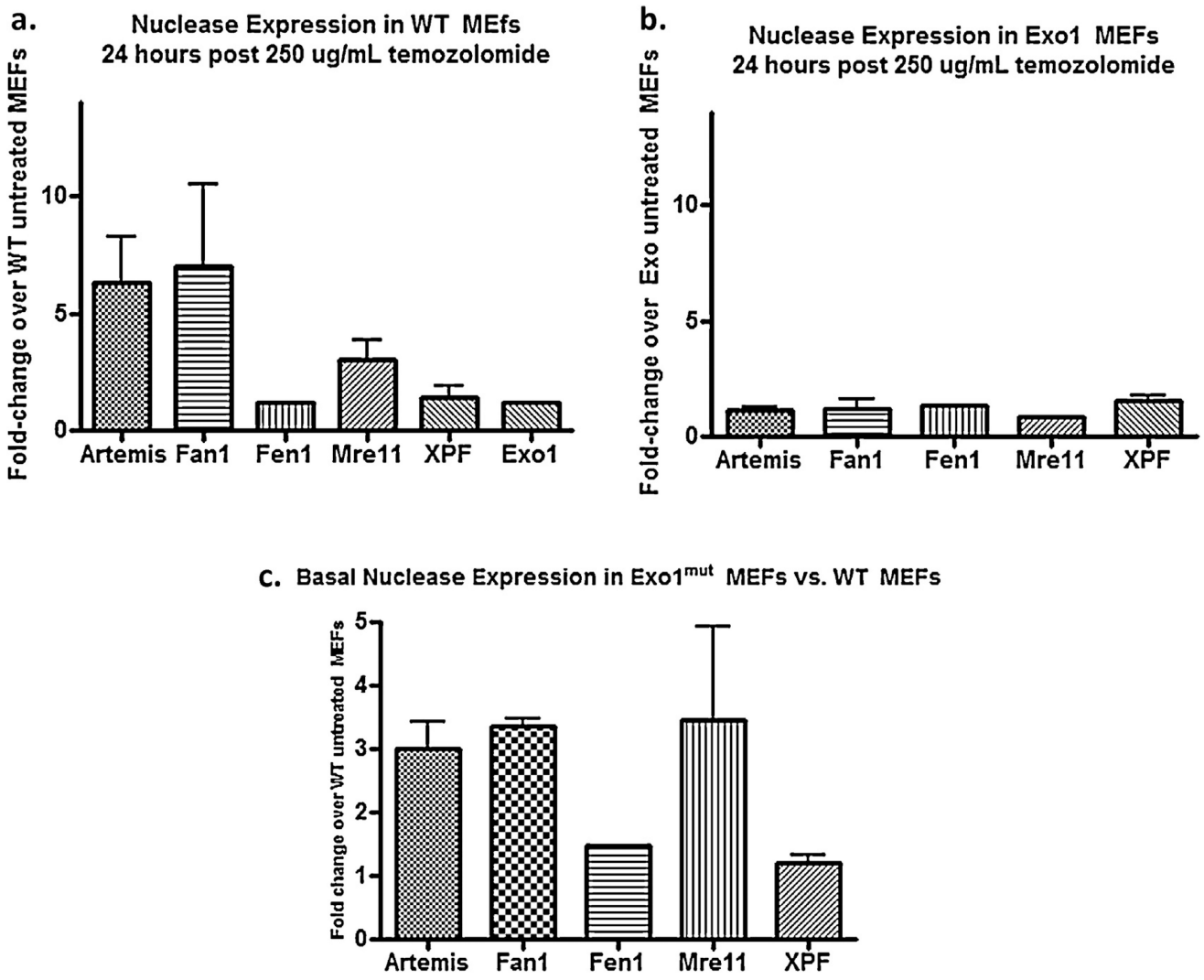

Fig. 3.

Exo1^{mut} MEFs demonstrate upregulated gene expression of multiple nucleases after temozolomide treatment. WT and Exo1^{mut} MEFs were treated with 250 μg/mL temozolomide and 24 h later RNA was extracted from cells and cDNA synthesized for real-time PCR analysis. Data shown is the fold change of expression of each nuclease compared to WT or Exo1^{mut} untreated MEFs.

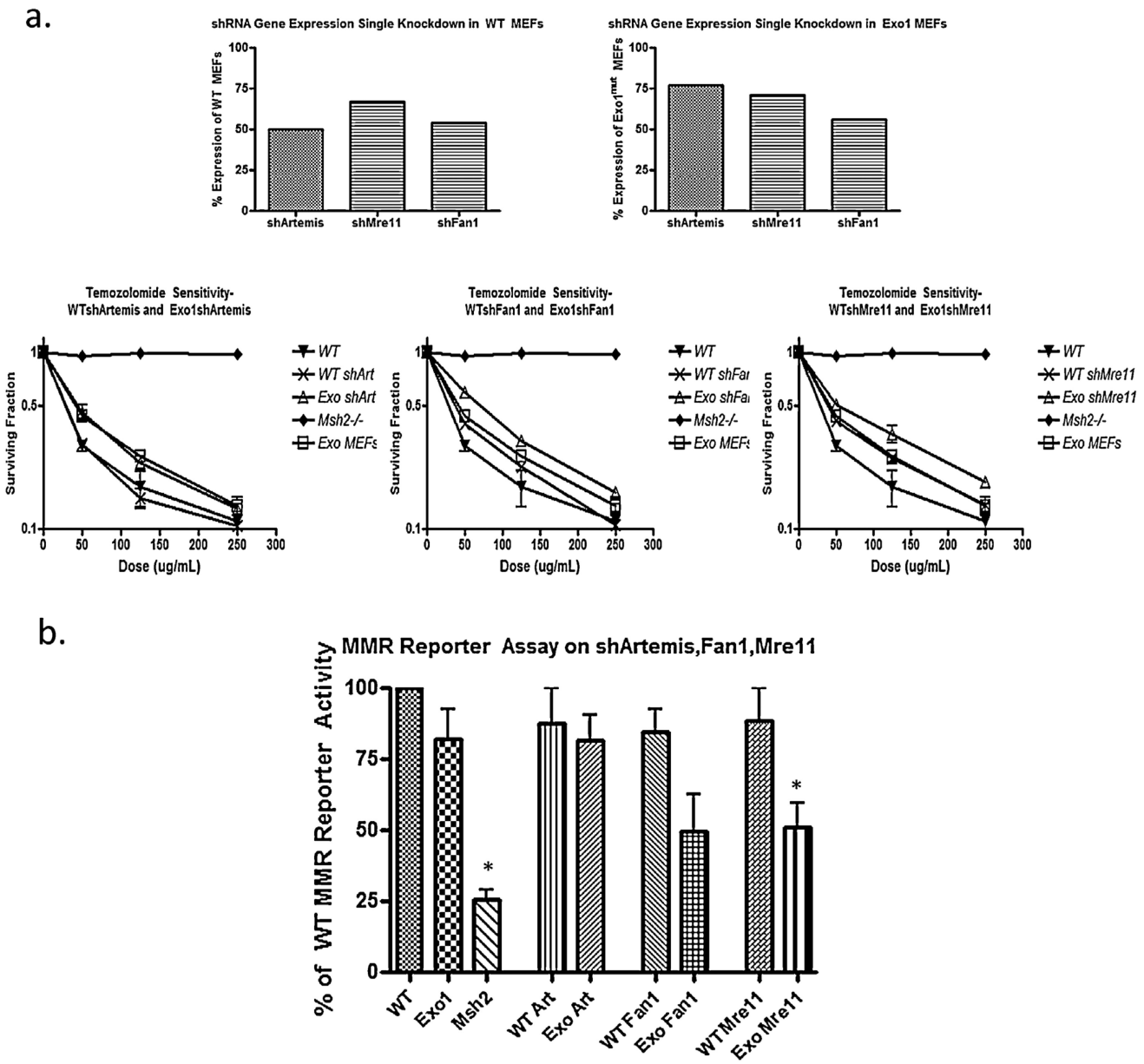

Fig. 4.

shRNA mediated silencing of Artemis, Fan1, and Mre11 in Exo1^{mut} MEFs results in mild temozolomide resistance and decreased MMR capacity. (A) Artemis, Fan1 and Mre11 were silenced *via* lentiviral shRNA in WT and Exo1^{mut} MEFs. Silenced cells were subsequently treated with a temozolomide dose range and survival measured three days post treatment *via* MTT assay. Error bars indicate SEM from 3 independent experiments. (B) MMR reporter heteroduplex eGFP plasmid was used on each silenced cell type and GFP fluorescence was measured 24 h post transfection *via* cytometric analysis. Error bars indicate SEM from 3 independent experiments.

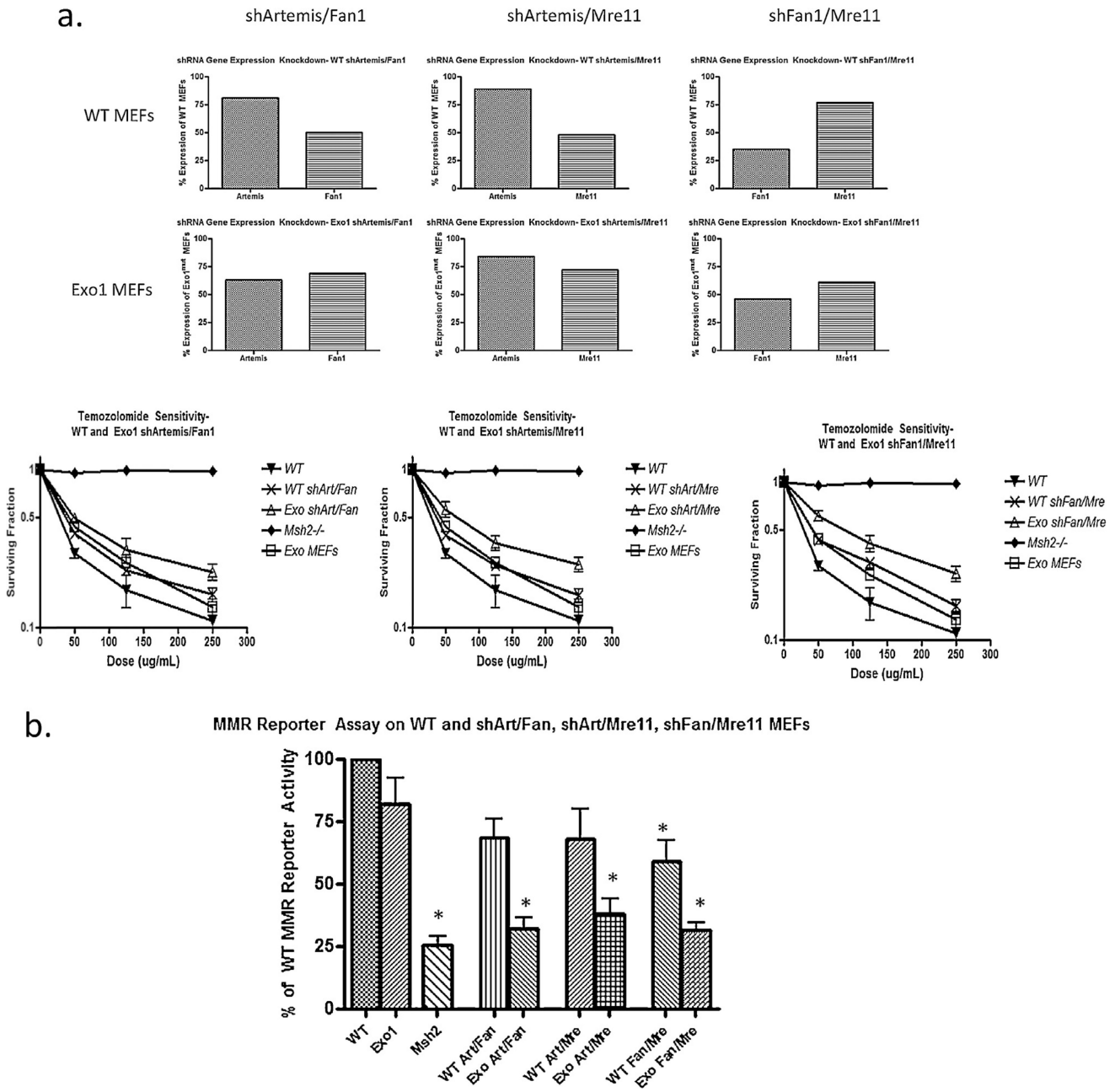

Fig. 5.

Combination silencing of Artemis/Fan1, Artemis/Mre11 and Fan1/Mre11 in Exo1^{mut} MEFs demonstrate increased temozolomide resistance and decreased MMR capacity. (A) Artemis, Fan1 and Mre11 were silenced in combination *via* lentiviral shRNA in WT MEFs. Silenced cells were subsequently treated with a temozolomide dose range and survival measured three days post treatment *via* MTT assay. Error bars indicate SEM from 3 independent experiments. (B) MMR reporter heteroduplex eGFP plasmid was used on each silenced cell type and GFP fluorescence was measured 24 h post transfection *via* cytometric analysis. Error bars indicate SEM from 3 independent experiments.

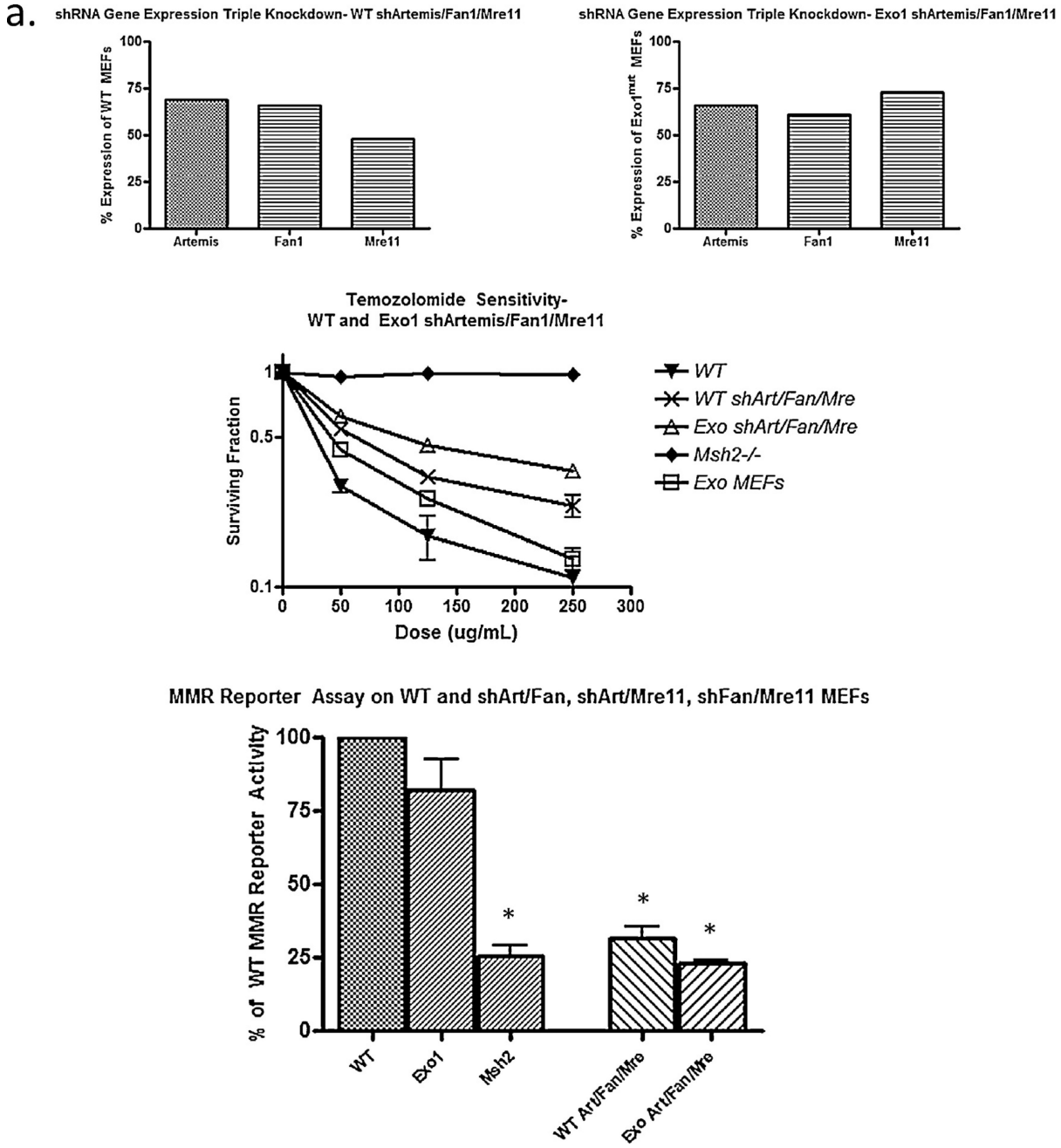

Fig. 6.

Triple silencing of Artemis/Fan1/Mre11 demonstrates significant MMR loss in both WT and Exo1 MEFs. (A) Artemis, Fan1 and Mre11 were silenced *via* lentiviral shRNA in WT and Exo1 MEFs. Silenced cells were subsequently treated with a temozolomide dose range and survival measured three days post treatment *via* MTT assay. Error bars indicate SEM from 3 independent experiments. (B) MMR reporter heteroduplex eGFP plasmid was used on each silenced cell type and GFP fluorescence was measured 24 h post transfection *via* cytometric analysis. Error bars indicate SEM from 3 independent experiments.