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Human MutL-complexes monitor homologous recombination independently of mismatch repair

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Abstract

The role of mismatch repair proteins has been well studied in the context of DNA repair following DNA polymerase errors. Particularly in yeast, MSH2 and MSH6 have also been implicated in the regulation of genetic recombination, whereas MutL homologs appeared to be less important. So far, little is known about the role of the human MutL homolog hMLH1 in recombination, but recently described molecular interactions suggest an involvement. To identify activities of hMLH1 in this process, we applied an *EGFP*-based assay for the analysis of different mechanisms of DNA repair, initiated by a targeted double-stranded DNA break. We analysed 12 human cellular systems, differing in the hMLH1 and concomitantly in the hPMS1 and hPMS2 status via inducible protein expression, genetic reconstitution, or RNA interference. We demonstrate that hMLH1 and its complex partners hPMS1 and hPMS2 downregulate conservative homologous recombination (HR), particularly when involving DNA sequences with only short stretches of uninterrupted homology. Unexpectedly, hMSH2 is dispensable for this effect. Moreover, the damage-signaling kinase ATM and its substrates BLM and BACH1 are not strictly required, but the combined effect of ATM/ATR-signaling components may mediate the anti-recombinogenic effect. Our data indicate a protective role of hMutL-complexes in a process which may lead to detrimental genome rearrangements, in a manner which does not depend on mismatch repair.

Keywords

ATM; ATR; BLM; DNA double-strand break repair; MLH1; MSH2

1. Introduction

hMSH2 and *hMLH1* are the most prevalent mutated genes in hereditary non-polyposis colorectal carcinoma (HNPCC), which is characterized by high microsatellite sequence instabilities reflecting frameshift changes due to a lack of mismatch repair during DNA replication [1]. Additionally, epigenetic silencing of *hMLH1* was observed in a significant proportion of sporadic cancers [2]. *hMSH2* and *hMLH1* are homologs of the *E. coli* proteins

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MutS and MutL, which together with MutH execute well-characterized functions in the pathway of post-replicative mismatch repair: MutS, as homo-oligomer, recognizes nucleotides that deviate from Watson-Crick base pairing. Homo-oligomeric MutL links the mismatch recognition complex and nucleolytic components. MutH cleaves the unmethylated strand in the presence of a mismatch. This excision requires helicase II and a single-stranded exonuclease [3]. hMSH2 forms a hetero-dimer with hMSH6 in the hMutS α -complex, and with hMSH3 in the MutS β -complex. hMutS α preferentially recognizes mismatches involving one or two unpaired nucleotides, while larger mispairings of two to ten nucleotides are recognized by hMutS β . hMLH1 hetero-dimerizes primarily with hPMS2 to form the complex hMutL α , which supports repair initiated by hMutS α or hMutS β . Minor complexes are formed between hMLH1 and hPMS1 (hMutL β) and hMLH1 and hMLH3 (hMutL γ) [4]. Interestingly, *in vitro* studies failed to identify a role of hMutL β in mismatch repair [5].

Base mispairings can arise not only after DNA replication, but also after pairing of divergent sequences, i.e. during homologous recombination. This can lead to mutagenic events, including translocations and deletions, potentially causing cancer [6]. The problem had already been recognized in bacteria, where RecA-promoted branch migration proceeds through regions of imperfect homologies [7]. Similarly, Rad51-dependent strand transfer allows the incorporation of short mispairings [8,9], so that mechanisms must exist to guarantee error-free recombinational repair. Indeed, homologous recombination (HR) is a very safe mechanism, in comparison to other repair pathways, which are initiated by a double-strand break (DSB) in the DNA, like non-homologous end joining (NHEJ) or single-strand annealing (SSA) [10,11]. Studies in prokaryotic and lower eukaryotic systems showed that MutS and MutL homologs are involved in a mechanism to reverse strand exchange in the presence of low levels of heterologies. Studies in yeast showed that MSH2, MSH3, MSH6, MLH1, and PMS1 (equivalent of PMS2 in mammals) each are important in this HR fidelity control mechanism, although mutating MSH2 always had a larger effect than deletion of MLH1 or PMS1. In combination with biochemical data, these results have led to a model, in which Rad51 initiates strand transfer, if a short region of homology is found, MutS homologs control heteroduplex extension through blockage of strand exchange involving mispairings, and MutL homologs stimulate MutS protein function and possibly destabilize blocked intermediates [6]. However, the picture emerging from studies on yeast meiosis is even more complex, because MSH2, PMS1, MLH1, and MLH3 prevent crossover between diverged sequences, but on the other hand MLH1 in complex with MLH3 promotes crossovers between homologous sequences, suggesting that MLH1/MLH3 controls recombination, possibly in response to specific protein interactions [6,12]. Similarly, in murine meiosis MLH1/MLH3-complexes are required for the selection of a subset of recombination intermediates marked by MSH4/MSH5 for reciprocal crossover events, i.e. for accurate chromosomal segregation, whereas localization of MLH3 to genomic repeat sequences at the centromere and on the Y chromosome is determined by MSH2/MSH3 as part of a surveillance mechanism [13,14].

In mammalian cells MSH2, MSH3, and MSH6 all prevent recombination between divergent sequences [15–17]. Tischfield and co-workers [18] demonstrated that the suppression of mitotic recombination in fibroblasts from hybrid mice is alleviated upon loss of MLH1. Wang et al. [19] observed that ionizing radiation treatment of a mouse kidney cell line null for *MLH1* induced mitotic recombination events, which was not observed in an MLH-positive line. To gain a better understanding of the role of hMLH1 in repair processes initiated by a DSB, we applied our cellular assay system designed for the analysis of the different DSB repair mechanisms [20] on a series of human cell pairs differing in the hMLH1 status. To distinguish direct influences of hMLH1 from those exerted indirectly via mismatch repair, we tested DSB repair in *hMSH2* null cells, when downregulating hMLH1,

hPMS1, or hPMS2. hMLH1 also participates in signaling cell cycle arrest via the phosphatidylinositol 3-kinase related kinases ataxia-telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), and it has been discussed whether the functions of hMLH1 in damage-signaling are separable from mismatch repair activities [21–26]. Therefore, we also examined the influence of this kinase and downstream targets. We demonstrate that hMLH1, as a component of hMutL-complexes, impedes hRad51-mediated and particularly error-prone HR processes in the absence of hMSH2, ATM, Bloom's syndrome protein (BLM), BACH1, and p53, but significantly reduced in the absence of both ATM and ATR. Our findings, thus, link hMutL to DNA recombination independently of mismatch repair.

2. Materials and methods

2.1. Cell lines and cultivation

Cells from the human colorectal adenocarcinoma line Lovo, devoid of hMSH2 protein [27], were cultivated in DMEM (PAA Laboratories, Pasching, Germany) supplemented with 10% FCS (PAA). HCT116+chr3 (hMLH-positive) [28] and parental HCT116 human colon cancer cells (hMLH-negative) were maintained in McCoy's (Invitrogen Gibco, Karlsruhe, Germany) with 10% FCS. For the cultivation of 293T-Tet-Off-hMLH1 cells, derived from the human embryonic kidney 293T line by stable transfection with Tet-Off-hMLH1 expression plasmid [29], we utilized DMEM supplemented with 10% FCS (Tet system approved, BD Biosciences Clontech, Heidelberg, Germany), 300 µg/ml hygromycin (Roche, Mannheim, Germany), and 100 µg/ml zeocin (Invitrogen, Karlsruhe, Germany). To switch-off exogenous hMLH1 expression, cells were cultivated for at least eight days in the presence of 50 ng/ml doxycycline (Clontech). KMV(HR-EGFP/3'EGFP) and KMV(Δ-EGFP/3'EGFP) cells, originating from the human myeloid leukemia cell line K562 and carrying chromosomally integrated HR-EGFP/3'EGFP and Δ-EGFP/3'EGFP plasmids, respectively [20], were cultivated in RPMI 1640 without phenol red (Gibco), 12% charcoal stripped FCS, and 5 mM L-glutamine (Biochrom, Berlin, Germany). The following lymphoblastoid cell lines were obtained from Coriell Cell Repositories: GM08436A, derived from an A-T-patient (compound heterozygous *ATM* mutations 4642delGATA and E1978X), GM03403 from a Bloom's syndrome patient (homozygous deletion/insertion mutation causing novel LDSR codons after amino acid 736 and stop), and GM02253F, mutated in the XPD gene but ATM-proficient [30]. HA290 from a mammary carcinoma patient (homozygous *XRCC4* mutation IVS7-1G > A), HA187 and HA126 from A-T-patients (compound heterozygous *ATM* mutations E1978X and R250X and homozygous *ATM* mutation Y171X, respectively) were a gift from Thilo Doerk, Hannover. Lymphoblastoid cells were maintained in RPMI 1640 supplemented with 15% FBS (Gibco) and 2 mM L-glutamine (Biochrom), GM08505 fibroblast cell lines [31] in α-MEM (Gibco), 10% FCS, 2 mM L-glutamine, and 350 µg/ml G418 (Gibco). EUFA30-F skin fibroblasts from the F-A J patient AG565 (gift from Hans Joenje, Amsterdam) were cultivated in DMEM and 15% FBS. The cell cultures used in this work were free from *mycoplasma* contamination.

2.2. Cell cycle and apoptosis

Cell cycle and apoptosis profiles were obtained by FACS analysis on fixed and propidium iodide stained cells [32] processed exactly under the conditions of the DSB repair assay.

2.3. Recombination assay and gene silencing

DSB repair via different pathways was investigated by use of the *EGFP*-based test system as described in detail previously [20,33]. The DSB repair substrates used in this study were: EJ-EGFP Δ-EGFP/5'EGFP, HR-EGFP/5'EGFP, Δ-EGFP/3'EGFP, HR-EGFP/3'EGFP (Fig. 1a). HCT116, HCT116+chr3, and 293T-Tet-Off-hMLH1 were transfected with FuGENE

reagent (Roche) and a mixture of 2 µg plasmid DNA containing meganuclease expression plasmid (pCMV-I-SceI), DSB repair substrate, and pBS (control vector) or wildtype *EGFP* plasmid (for transfection efficiency). For assays with GM08436A cells, a corresponding plasmid mixture of 15 µg DNA was introduced via electroporation, with GM02253F, HA187, HA126, GM03403, and HA290 cells of 5 µg DNA via nucleofection (Amaxa, Cologne, Germany). To measure recombination on chromosomally integrated substrates, KMV(HR-EGFP/3'EGFP) or KMV(Δ-EGFP/3'EGFP) cells were electroporated with pCMV-I-SceI together with pBS or wildtype *EGFP* plasmid (10 µg total).

To downregulate expression of specific proteins during repair measurements in Lovo and 293T-Tet-Off-hMLH1 cells by RNA interference, we performed co-transfections with XtremeGene transfection reagent (Roche), pCMV-I-SceI, DSB repair substrate, and pBS/wildtype *EGFP* plasmid (1 µg total), and 10 nM siRNA. For hMLH1 knockdown in GM08505 and FA-J cells during the recombination assay we introduced pCMV-I-SceI (4 µg), DSB repair substrate Δ-EGFP/5'EGFP (4 µg), and 13 nM siRNA by nucleofection (Amaxa). The following siRNAs were designed, synthesized, and HPP-purified by Qiagen (Hilden, Germany): AllStars Negative Control siRNA, non-specific control siRNA (5'-AATTCTCCGAACGTGTCACGT-3'), MLH1-1 (5'-GTGGCTCATGTTACTATTACA-3'), MLH1-2 (5'-CCGATACAAAGTGTGTATCA-3'), PMS 1-1 (5'-CAGCGAATGGTTTCAAGATAA-3'), PMS1-2 (5'-TTGGAACGATACAATAGTCAA-3'), PMS2-1 (5'-CCGGATGTAAATTTTCGAGTTT-3'), PMS2-2 (5'-CAGACTCGTGAAT-GAGGTCTA-3'), MSH2-1 (5'-CCCATGGGCTATCAACTTAAT-3'), and MSH2-2 (5'-TCCAGGCATGCTTGTGTTGAA-3'). For hMLH1 knockdown experiments in KMV(HR-EGFP/3'EGFP), KMV(Δ-EGFP/3'EGFP), or GM08436A, we added 20 µg of the shRNA plasmids pRSMLH1F (5'-AGATCCAAGACAATGGCACCG-3') pRSMLH1G (5'-GTATTTCTACCTATGGCTTTC-3'), or of pRScontrol (Origene, Rockville, MD, USA) to the electroporation mixture, for hMLH1 silencing in GM02253F, GM03403, HA126, HA187, and HA290 cells 5 µg of the shRNA plasmid to the nucleofection mixture. For the analysis of hMSH2-deficient KMV(Δ-EGFP/3'EGFP) cells, we electroporated the cells with 15 µg each of the hMSH2-knockdown plasmids pRSMSH2-5 and pRSMSH2-8 (Origene, Rockville, MD, USA) followed by recultivation for 72 h until retransfection with pCMV-I-SceI and pRSMLH1F. For ATM and ATR knockdown in 293T-Tet-Off-hMLH1 cells, we included 2 µg of the shRNA constructs pSHAG-ATM plus pSHAG-ATR or pSHAG control vector [34].

Following cultivation for 24–48 h (K562 derivatives, GM02253F, A-T lines) or 24 h (all other cell lines), 50 000–400 000 living cells (50–100% of total cell population, selectively analysed by the gating procedure in the FSC/SSC plot) were examined each to distinguish between EGFP-positive and EGFP-negative cells by the diagonal gating method in the FI1/FI2 dot plot (FACS Calibur® FACSscan, Becton Dickinson) [20]. Each transfected sample was accompanied by a sample transfected with the same mixture, however, replacing pBS (control vector) by wildtype *EGFP* plasmid (for transfection efficiency). Each quantification of green fluorescent cells in repair assays (with pBS) was normalized by use of the individually determined transfection efficiency (with *EGFP* plasmid) to calculate the DSB repair frequency (absolute values). The statistical significance of differences was determined using Student's *t* test for unpaired samples.

2.4. Western blotting and antibodies

For Western blot analysis total homogenates from 2×10^5 cells were loaded per lane [20]. Alternatively, cellular lysates were prepared by incubation of the cells in lysis buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 2 mM EGTA; 2 mM EDTA; 25 mM NaF; 25 mM β-glycerophosphate; 0.1 mM NaV; proteinase inhibitor, Roche). Protein concentrations were

determined with the BCA protein assay kit (Pierce/Perbio Science, Bonn, Germany) and 60 µg of total protein subjected to immunoblotting. Extracts were electrophoresed on 4.5–12% SDS-PAGE, and transferred to Hybond-C Extra (Amersham) or Immobilon-P (Millipore) membranes. Electrotransferred proteins were immunodetected by use of the following antibodies: MLH1 mAb G168-15 (Becton Dickinson), PMS1 rabbit serum C-20 (Santa Cruz, Heidelberg, Germany), PMS2 mAb A16-4 (Becton Dickinson), MSH2 rabbit serum N-20 (Santa Cruz, Heidelberg, Germany), ATM mAb 5C2 (Gene Tex), ATR rabbit ab2905 (Abcam), ERCC1 8F1 (Becton Dickinson), MRE11 rabbit NB 100–142 (Novus Biologicals), RAD50 mAb from clone 13 (Becton Dickinson), 3F10 HA-tag rabbit monoclonal antibody, peroxidase conjugated (Roche), actin goat I-19 (Santa Cruz), and tubulin mAb DM1A (Abcam, Cambridge, MA, USA). Western blot signals were visualized by SuperSignal West Pico Chemiluminescent Substrate or West Dura Extended Duration Substrate (Pierce). Densitometric quantification of band intensities was performed using a ChemImager 5500 with software (Alpha Imunotech Corporation, San Leonardo, California, USA), analyzing signals within the linear range. Protein expression levels were calculated by normalizing with actin-, tubulin-, or GAPDH-signals.

3. Results

3.1. Effect of hMLH1 on DSB repair mechanisms

To systematically investigate the involvement of hMLH1 in recombinative DNA repair pathways, we applied our *EGFP*-based model system [20] on isogenic human cellular systems, which differed in the *hMLH1* status. In this *EGFP*-based test system, recombinative DNA repair is initiated by targeted cleavage via meganuclease I-SceI and may occur via NHEJ or homology-directed mechanisms (HR), depending on the repair substrate chosen (Fig. 1a). Thus, EJ-*EGFP* was designed to detect NHEJ, Δ -*EGFP*/5'*EGFP* and HR-*EGFP*/5'*EGFP* for conservative HR (mostly gene conversion), and Δ -*EGFP*/3'*EGFP* and HR-*EGFP*/3'*EGFP* for all HR pathways (mostly gene conversion and SSA). Since *mlh1* yeast strains had been reported to increase aberrant exchange events far from the DSB [6], we also analysed HR after shortening the length of perfectly homologous sequences down to 164 bp by deleting 46 bp next to the I-SceI recognition site within the Δ -*EGFP* marker gene as compared to a 4 bp deletion in *HR-EGFP* (Fig. 1a). Genetic reconstitution of wildtype *EGFP* was monitored by FACS analysis through determination of the fraction of green fluorescent cells within the population of non-fluorescent cells. To rule out possible indirect effects on DSB repair related to differences in transfection, growth, cell lethality, transcriptional, and translational activities, we performed identical co-transfections including wildtype *EGFP* control plasmid in parallel. The resulting individual transfection efficiencies served to normalize each single DSB repair frequency.

For our studies on the role of hMLH1 in DSB repair, we first chose 293T-Tet-Off-hMLH1 cells, which had been generated from the parental hMLH1-deficient human embryonic kidney 293T line by stable transfection with a vector carrying the hMLH1 cDNA under the control of the inducible Tet-Off expression system [29]. When cultivating these cells in the presence of doxycycline (dox), hMLH1 expression was turned off (Fig. 1b). Concomitantly, levels of hPMS1 and hPMS2 dropped sharply, which is in agreement with earlier findings, which indicated that hMLH1 is required for the accumulation of its cognate partners [5,22,29]. By Western analysis (Fig. 1b) we additionally verified that dox-treatment did not influence hMSH2, hMRE11, or hRAD50 levels [35,36]. These cultures of hMLH1-positive and negative cells were, then, co-transfected with I-SceI expression plasmid and DSB repair substrates EJ-*EGFP*, Δ -*EGFP*/5'*EGFP*, HR-*EGFP*/5'*EGFP*, Δ -*EGFP*/3'*EGFP*, and HR-*EGFP*/3'*EGFP* (Fig. 1a), respectively. In samples without I-SceI expression, we measured 13–17× lower values for HR on Δ -*EGFP*/5'*EGFP* and HR-*EGFP*/5'*EGFP* and detected no green fluorescent cells with EJ-*EGFP*.

DSB repair measurements after 24 h of cell cultivation revealed that switching off exogenous hMLH1 expression caused a 26%, statistically insignificant increase in NHEJ ($P = 0.193$), a 68% and 41% stimulation of conservative HR with short (Δ -EGFP/5'EGFP: $P = 0.009$) versus long (HR-EGFP/5'EGFP: $P = 0.012$) homologies, and corresponding increments of 52% and 28%, when measuring HR on Δ -EGFP/3'EGFP ($P = 0.000$) and HR-EGFP/3'EGFP ($P = 0.052$), respectively (Fig. 1c). To exclude that cell cycle arrest or cell death indirectly caused HR changes, we performed DNA content analysis on propidium iodide stained cells, which had been processed parallel to repair measurements. This analysis revealed neither significant differences between the percentages of dox-treated and untreated 293T-Tet-Off-hMLH1 cells with a sub-G1 DNA content, which is indicative of apoptosis, nor between the fractions of G1-, S-, or G2-phase cells (Fig. 1d).

Next, we used the human colon cancer cell lines HCT116, which is deficient in wildtype *hMLH1*, and HCT116+chr3, in which an *hMLH1* copy had been re-introduced via chromosome 3 transfer [28]. Immunoblotting revealed that hMLH1 deficiency in HCT116 cells was again accompanied by a sharp decline of hPMS1 and hPMS2 levels (Fig. 2a). For comparison hMSH2, hMRE11, and hRAD50 protein levels were similar in HCT116 and HCT116+chr3 cells, even though the overall amount of hMRE11 protein was low with full-length and additional bands in the Western blot, which can be explained by mutations in *MRE11* [35,36]. In analogy to 293T-Tet-Off-hMLH1 cells with and without dox-treatment, HCT116 and HCT116+chr3 cells were subjected to DSB repair measurements with substrates EJ-EGFP, Δ -EGFP/5'EGFP, HR-EGFP/5'EGFP, and Δ -EGFP/3'EGFP (Fig. 1a). Interestingly, DSB repair frequencies were found to be altered for conservative HR with short homologies (Δ -EGFP/5'EGFP) exclusively (Fig. 2b). Thus, HCT116 cells, which are devoid of hMLH1, displayed an 86% ($P = 0.032$) higher HR frequency as compared to hMLH1-positive HCT116+chr3 cells. We also performed cell cycle analysis under the conditions of the assay. However, significant differences between HCT116 and HCT116+chr3 were neither found for the fractions of sub-G1-, G1-, S-, nor G2-phase cells (Fig. 2c). Taken together, both dox-treated 293T-Tet-Off-hMLH1 cells and HCT116 cells showed elevated HR activities as compared to their hMLH1-positive counterparts, particularly with respect to conservative HR involving short homologies. This suggested a role of hMLH1 in repressing homologous recombination.

To assess the significance of these findings for repair processes in the chromatin context, we examined HR in the well-characterized KMV cell lines with stably integrated substrate HR-EGFP/3'EGFP and Δ -EGFP/3'EGFP, respectively [20]. Unfortunately, HR-EGFP/5'EGFP and Δ -EGFP/5'EGFP substrates are not suitable for comparative analysis of chromosomal HR under the conditions of hMLH1 measurements, because HR frequencies would be below the detection limit. However, through expression of the dominant-negative Rad51 protein SMRAD51 we recently demonstrated that Rad51-dependent HR represents a major fraction of the chromosomal DSB repair events leading to *EGFP* reconstitution in these cells [37].

KMV(HR-EGFP/3'EGFP) cells were electroporated with I-SceI expression plasmid together with pRSMLH1F and pRSMLH1G, which direct the synthesis of siRNAs targeting hMLH1 mRNA. pRSMLH1F and pRSMLH1G (transfection efficiency 80%) caused a similar decrease in hMLH1 protein levels, namely on average down to 50% as compared to the empty vector control and after normalization with the corresponding loading controls (Fig. 3b). Consistent with our findings in HCT116 and 293T-Tet-Off-hMLH1 cells, hPMS1 and hPMS2 levels were concomitantly reduced, although to a lesser extent. hMLH1 silencing in KMV(HR-EGFP/3'EGFP) cells caused a 2.5-fold HR enhancement for both hMLH1 knockdown plasmids tested (Fig. 3a). Transfection of KMV(Δ -EGFP/3'EGFP) cells with pRSMLH1F resulted in 3.9-fold higher HR frequencies as compared with pRScontrol ($P = 0.034$ for difference between increases in KMV(HR-EGFP/3'EGFP) and KMV(Δ -EGFP/

3'EGFP) cells). Indirect effects on HR stemming from cell death or cell cycle arrest in response to hMLH1 knockdown were again excluded by DNA content analysis on propidium iodide stained cells (Fig. 3c). To strengthen the argument for a physiological role of hMutL-complexes in HR, we additionally treated the cells with DSB-inducing agents like the topoisomerase II inhibitor etoposide and the radiomimetic drug bleomycin rather than triggering targeted cleavage by I-SceI meganuclease expression. Under both conditions HR was stimulated twofold (etoposide: $P = 0.035$; bleomycin: $P = 0.020$) through knockdown of hMLH1 (Fig. 3d). We further excluded an indirect effect of hMLH on HR via perturbation of NHEJ, since HR stimulation (fivefold, $P = 0.035$) was still observed in cells carrying mutated *XRCC4*, which encodes an essential NHEJ factor (Supplemental Fig. 1a and b). From the fact that hMLH1 downregulation is coupled to reduction of hPMS1 and hPMS2 levels we cannot discriminate between specific effects of hMLH1, hPMS1, or hPMS2. However, it can be concluded that hMutL-complexes downregulate HR in response to different DSB-inducing agents both on extrachromosomal and on chromosomal DNA substrates and that this effect is more pronounced when involving short homologies.

3.2. HR modulation by hMLH1 requires hPMS1 and hPMS2 but not hMSH2

Having established a modulatory role of hMutL-complexes in HR, we were interested in the functional links to hMSH2, i.e. the central molecule of hMutS mismatch repair complexes, in this process. For this purpose we performed DSB repair measurements in the human colorectal adenocarcinoma cell line Lovo, in which hMSH2 protein is absent due to a partial deletion of the *hMSH2* gene (Fig. 4b) [27]. To additionally downregulate hMLH1 levels, we introduced two hMLH1-specific siRNAs (MLH1-1 and MLH1-2) together with meganuclease I-SceI expression plasmid and the different DSB repair substrates. Control samples received non-specific siRNA or hMSH2-specific siRNAs (MSH2-1 and MSH2-2). Transfection with hMLH1-specific siRNAs caused minor, statistically not significant differences of DSB repair frequencies on substrates EJ-EGFP, HR-EGFP/5'EGFP, Δ -EGFP/3'EGFP, and HR-EGFP/3'EGFP (Fig. 4a, $P > 0.05$). However, in measurements with Δ -EGFP/5'EGFP, we observed a 2.5-fold, statistically significant enhancement of HR after treatment with hMLH1-specific siRNAs ($P = 0.011$). We verified downregulation of endogenous hMLH1 in Lovo cells by immunoblotting and densitometric quantification of band intensities, which indicated residual protein levels of 37–38% 24 h after transfection (corrected with loading controls, Fig. 4b). hPMS1 and hPMS2 levels were concomitantly reduced to 53–78% and 26–40%, respectively. FACS analysis of propidium iodide stained cells demonstrated that under the conditions of the assay, hMLH1 knockdown had no influence on cell cycle distribution or apoptosis induction (Fig. 4c). We excluded that the frequency with substrates other than Δ -EGFP/5'EGFP was too high to be further stimulated by hMLH1 silencing in experiments with decreasing I-SceI expression plasmid concentrations for substrate HR-EGFP/3'EGFP (Supplemental Fig. 2).

In parallel, we also examined, whether the two major hMLH1 interaction partners, hPMS1 and hPMS2 [38], are involved in this hMLH1-mediated regulation of HR. To this end we correspondingly introduced hPMS1- and hPMS2-specific siRNAs and performed DSB repair analysis. Strikingly, we observed the same pattern of DSB repair regulation as was noticed after hMLH1-specific siRNA treatment (Fig. 4a). Thus, hPMS1-knockdown resulted in a 2.5-fold ($P = 0.029$) and hPMS2-knockdown in a 2.4-fold ($P = 0.031$) enhancement of HR on Δ -EGFP/5'EGFP. Transfection with siRNAs PMS1-1 and PMS1-2 caused a downregulation of hPMS1 to 3–38% of the control protein level, with siRNAs PMS2-1 and PMS2-2 hPMS2 was reduced to 30–73% (Fig. 4b). After introduction of these siRNAs directed against hPMS1 or hPMS2, hMLH1 levels ranged between 56% and 87% of the controls (corrected with loading controls). Expression of I-SceI meganuclease, tubulin (Fig. 4b) and of the DNA repair protein hERCC1 (data not shown) were checked as controls for

non-specific effects. None of the protein levels was affected by siRNAs directed against hMLH1, hPMS1, or PMS2. As an additional control, we investigated the effect of siRNA-mediated depletion in hMLH-negative and hMLH-positive cells. While introduction of hMLH1-specific siRNAs in 293T-Tet-Off-hMLH1 without hMLH1 (+dox) did not influence HR, it stimulated HR after re-expression of hMLH1 (−dox) (Fig. 4d and e). Altogether these data indicated that hMSH2 is dispensable for the observed HR regulation. Moreover, the hPMS1 and hPMS2 knockdown experiments confirmed the involvement of hMutL α - and hMutL β -complexes in this regulation process.

To verify this observation in another cell type, we pre-transfected KMV(Δ -EGFP/3'EGFP) cells with two hMSH2-knockdown plasmids (pRSMH2-5, pRSMH2-8) 72 h before retransfection with I-SceI expression plasmid and pRSMH1F to initiate DSB repair and to downregulate hMLH1 protein levels (down to 41–45%), respectively (Fig. 4g). Under these conditions we noticed a twofold HR increase in samples after hMLH1-knockdown both in cells without ($P = 0.000$) and with hMSH2 deficiency ($P = 0.027$) (Fig. 4f). As demonstrated for hMSH2-positive cells in Fig. 3c, the cell cycle distribution remained unaltered in cells with and without hMLH1 knockdown also in hMSH2-deficient cells (not shown). Thus, hMutL-complexes regulate HR independently of hMSH2 both in Lovo and KMV(Δ -EGFP/3'EGFP) cells.

3.3. hMutL-mediated HR-regulation in the absence of ATM, BLM, or BACH1

Since hMLH1, hPMS1, and hPMS2 have previously been described to stimulate the ATM-signaling cascade [21–23], the impact of ATM on hMutL-dependent HR regulation was examined. Thus, we subjected cells from the A-T lines GM08436A, HA187, and HA126 to HR analysis. For that purpose, the cells were transfected with I-SceI expression plasmid, the HR substrate Δ -EGFP/5'EGFP, and hMLH1 knockdown plasmid (pRSMH1F). Silencing hMLH1 expression down to 22–57% of the control level (corrected with tubulin level, Fig. 5b) caused a twofold ($P = 0.002$), 2.7-fold ($P = 0.041$), and 1.7-fold ($P = 0.031$) increase in the HR frequencies in GM08436A, HA187, and HA126 cells, respectively, compared to a 1.8-fold ($P = 0.008$) stimulation in the ATM-proficient [30] control cells (Fig. 5a).

It had been reported that ATR displays a substrate spectrum very similar to ATM and may compensate for loss of ATM function [39]. Consequently, we studied HR after silencing both ATM and ATR by use of the established knockdown plasmids pSHAG-ATM and pSHAG-ATR [34]. Using 293T-Tet-Off-hMLH1 cells, ATM and ATR levels (corrected with loading controls, $n = 2$) were reduced to 50–60% in the absence and to 0–40% in the presence of dox as compared to empty vector controls (Fig. 5d). As expected from Fig. 1b, hMLH1 switch-off (+dox) caused 1.8-fold ($P = 0.001$) elevated frequencies of HR on Δ -EGFP/5'EGFP in the controls. After ATM/ATR silencing, loss of hMLH1 expression led to a 1.3-fold HR activity rise, however, without statistical significance ($P = 0.227$). Interestingly, knockdown of ATM and ATR itself caused 1.9-fold HR stimulation in hMLH1-positive (−dox; $P = 0.002$) and 1.4-fold in hMLH1-negative (+dox; $P = 0.004$) cells. These observations suggest that ATM/ATR-signaling could be involved in the anti-recombinogenic effect of hMutL-complexes.

The ATM phosphorylation substrate BLM plays a role in regulating HR and was identified as a physical interaction partner of hMLH1 [31]. Consequently, we silenced hMLH1 in the Bloom's syndrome lymphoblastoid cell line GM03403 and measured HR. After transfection with plasmids pCMV-I-SceI, Δ -EGFP/5'EGFP, and pRSMH1F we scored 62% residual hMLH1 protein (corrected with the tubulin level, Fig. 5f) and a 12-fold ($P = 0.048$) stimulation of HR (Fig. 5e). Additionally, we made use of the immortalized Bloom's syndrome fibroblast cell line GM08505 and its BLM-reconstituted counterpart [31]. Silencing hMLH1 with hMLH1-specific siRNAs down to 37–45% of control levels (Fig. 5h)

was followed by a 1.9-fold ($P = 0.009$) and 2.7-fold ($P = 0.013$) HR stimulation in BLM-deficient and BLM-proficient cells, respectively (Fig. 5g). Significant changes in cell cycle profiles or apoptosis induction were neither detected in A-T, nor Bloom's syndrome, nor 293T-Tet-Off-hMLH1 cells (data not shown). In recent work, the BRCA1 associated helicase BACH1/BRIP1/FANCD1 was shown to bind hMLH1 [40]. To assess whether this interaction mediates the HR suppressive function of hMutL-complexes, we investigated DSB repair in *BACH1*-null Fanconi anemia J (F-A J) fibroblasts. hMLH1 knockdown in F-A J cells (19% residual level corrected with tubulin) caused a 2.7-fold ($P = 0.039$) HR rise (Fig. 5i and j). These results suggested that neither BLM nor BACH1 are strictly required for hMutL-dependent recombination repression.

4. Discussion

In this study we characterized the specific effect of hMutL-complexes on DSB repair in a series of human cell lines originating from colon, kidney, the lymphoid or myeloid lineage, or fibroblasts. The hMLH1 status was altered either by reconstitution or silencing of hMLH1 expression, thereby reducing the probability of indirect effects from frameshift mutations in recombinative repair genes [41]. Dissection of different DSB repair mechanisms revealed that altering the hMLH1 status, as well as the status of the hMutL homologs hPMS1 and hPMS2, (i) does not affect NHEJ, (ii) modulates conservative rather than non-conservative HR mechanisms, and (iii) had the most pronounced influence on HR, involving short stretches of sequence homology.

So far, two studies addressed a potential involvement of hMLH1 in NHEJ. In agreement with our observations, Jacob et al. [42] did not find evidence for changes in end joining of linearized plasmids in hMLH1-deficient cell lines, neither in terms of efficiency nor accuracy. At first sight somewhat contradictory, Lin et al. [43] reported that hMLH1-deficient cells enhanced illegitimate recombination, i.e. random integration of linearized DNA into the genome, a process which is related to NHEJ rather than HR mechanisms. It should be noted, however, that the assay was based on the reconstitution of a hygromycin B resistance marker and, therefore, on the survival and clonal outgrowth of cells under selection pressure. In view of the fact that hMLH1 has a direct signaling role towards cell cycle arrest and cell death [23], it cannot be excluded that growth-regulatory activities of MLH1 indirectly influenced their scorings. In this study, we generally measured HR at the earliest time point possible after the DSB trigger, when EGFP signals from successful repair events were already detectable, but cell cycle profiles of hMLH1-proficient cells were still indistinguishable from hMLH1-deficient cells. Indeed, in our experiments with HCT116/HCT116+chr3 cells we noticed that the extent of HR downregulation by hMutL-complexes decreased after 48 h as compared to 24 h, which was accompanied by an altered cell cycle distribution (see Supplemental Fig. 3). Considering that cells are highly active in HR during G2 phase, the increase in G2 phase cells with HCT116+chr3 compared to HCT116 may explain, why the HR downregulatory effect was not as readily detected at later time points.

Rubnitz and Subramani [44] noticed the sharpest drop in the recombination frequency, when the homology was reduced from 197 to 163 bp, suggesting that in mammalian cells a minimal region of ~200 bp is required for fully efficient HR, whereas anti-recombinogenic mechanisms become active below this homology length limit. Within the recombination substrates used in this study, uninterrupted homologies of 164–168 bp (Δ -EGFP substrates) and 191–195 bp (HR-EGFP substrates) were present 5' to the I-SceI recognition sequence [20]. Consistently, in this study we measured lower DSB repair frequencies with Δ -EGFP substrates, when directly compared with the corresponding HR-EGFP substrates with the exception of Δ -EGFP/5'EGFP and HR-EGFP/5'EGFP in 293T-Tet-Off-hMLH1 cells. DSB repair measurements showed that hMLH1 deficiency was accompanied by HR

enhancement, which was more pronounced for Δ -EGFP as compared to HR-EGFP substrates. This indicated that hMLH1 is involved in the fidelity control pathway, which counteracts HR between divergent DNA sequences with only short stretches of uninterrupted homology. Comparison of HR on Δ -EGFP/5'EGFP and HR-EGFP/5'EGFP versus Δ -EGFP/3'EGFP and HR-EGFP/3'EGFP revealed the strongest anti-recombinogenic influence of hMutL-complexes on 5'EGFP substrates. 5'EGFP substrates are specific for the conservative, Rad51-dependent HR mechanisms (mostly gene conversion), whereas 3'EGFP substrates additionally allow to monitor Rad51-independent SSA [20]. Therefore, from our results we can conclude that Rad51-dependent gene conversion, rather than SSA, is under the control of hMutL-complexes. These observations are compatible with those made by Sugawara et al. [45] in *S. cerevisiae*, where SSA between divergent sequences is repressed via heteroduplex rejection through MSH2 and MSH6, while mutation of MutL homologs had little or no effect.

This is the first time, to our knowledge, that HR regulation by hMutL-complexes was studied in human cells. Our results are compatible with a previous report on increased gene amplification frequencies in human HCT116 versus HCT116+chr3 cells [43], because recombination coupled to overreplication is thought to underly gene amplification processes [46]. Our findings are also in line with studies in bacteria and yeast, indicating that mismatch repair proteins prevent recombination between divergent sequences [6]. Moreover, in fibroblasts from hybrids of distantly related mouse strains, interchromosomal, mitotic recombination was found to be suppressed by DNA sequence heterologies, and *MLH1* knockout alleviated this anti-recombinogenic effect [18].

In bacteria and yeast, MutL protein complexes alone did not interfere with homologous strand transfer but required MutS-complexes to recognize mismatches [6]. *In vitro* studies further indicated that *E. coli* MutS blocked strand exchange upon mismatch recognition. This effect of MutS was enhanced by MutL [47]. In contrast to these findings in bacteria and yeast, we discovered that downregulation of hMutL components is sufficient to alleviate recombination repression, even in an hMSH2-negative background. In further support of a mismatch repair-independent function, both loss and reduction of hMLH1 protein levels caused loss of HR-repression, whereas residual hMLH1 protein is sufficient to confer mismatch repair function. Interestingly, hMLH1-dependent damage-signaling also requires a full complement of hMLH1 [26].

Notably, an endonucleolytic function of hMutL α was recently discovered. This activity allows the endo- and exonucleolytic degradation of the error-containing strand during mismatch repair, independently of the position with respect to the nick [48]. It is conceivable that the endonuclease activity of hMutL α contributes to HR regulation through destabilization of heteroduplex intermediates. In eukaryotic cells, DSBs are processed to generate 3' single-stranded ends, which then invade the homologous donor sequence during gene conversion [49–51]. Extended non-homologous 3'-ends are removed by the excision repair endonuclease Rad1/Rad10 after recruitment by MSH2 and MSH3 [52]. During mismatch repair hMutL α was shown to be able to act as an endonuclease on the discontinuous strand 5' to the DNA strand break, preferentially distal to the mispairing [48]. Therefore, it will be extremely interesting to see whether hMutL α , possibly in the presence of recombination-related co-factors, can also cause oligonucleotide removal from the invading strand during recombination, and thereby lead to the destabilization of the strand invasion structure. Interestingly, the endonuclease metal binding site motif was detected on hPMS2 but not on hMLH1 or hPMS1 [48]. Here, we observed the same HR stimulatory response after silencing expression of endogenous hMLH1, hPMS1, or hPMS2, arguing against a hPMS2-specific function. However, in view of the fact that MLH1 is necessary for the stability of both hPMS1 and hPMS2 [5,22,29] and to a lesser degree also *vice versa* [53],

it is difficult to fully discriminate between distinctive roles of these hMutL homologs in HR regulation, i.e. an activity which appears to be influenced by even minor changes in protein expression.

Since MutL-complexes were shown to be important components in the stimulation of ATM [21–23], we examined whether the anti-recombinogenic role of hMutL-complexes is linked with this kinase. ATM triggers checkpoint-signaling in response to DSBs [39], which in our experiments were created by the meganuclease I-SceI. So far, contradictory results have been published on the role of mammalian ATM in DSB repair [54–57]. Nevertheless, ATM is believed to minimize error-prone repair events, and, therefore, is a potential target of hMLH1 in mediating the HR regulatory effect observed in this study. However, upon hMLH1 knockdown in A-T cells we still detected alleviation of HR-repression. From this result it is unlikely that hMLH1 activates HR fidelity control mechanisms via stimulation of ATM-mediated phosphorylation of other DSB repair factors. p53, a phosphorylation target of ATM, also counteracts recombination between divergent sequences [20,58], and this function is separable from its activities in transcription and checkpoint control [11]. Colocalization of p53 phosphorylated on serine 15 with DSB repair proteins like hRad51, hRad54, hMre11, and BLM as well as diminished HR regulation by p53(15A) were previously demonstrated, suggesting an HR-related function particularly for the ATM/ATR-modified subpopulation of p53 [37,59,60]. However, we ruled out that p53 is required for HR-repression by hMLH1, as we used cells derived from the cell line K562, which is p53-negative [20] and from 293T and GM08505 cells, in which p53 is inactivated by SV40 large T antigen [26]. Therefore, hMutL-complexes and p53 may act in complementary pathways rather than epistatically to guarantee high-fidelity DNA repair by HR.

hMLH1 was also found to physically interact with the helicases FANCD1, which was first linked to hereditary breast cancer through its direct interaction with BRCA1 [40], and BLM [31], which is mutated in patients with Bloom's syndrome associated with cancer-proneness and an unusually high frequency of sister chromatid exchanges [61]. BLM recognizes many different DNA structures, such as forked duplexes, Holliday junctions, and duplexes with internal bubbles, i.e. dsDNA comprising non-complementary sequences, but not fully paired dsDNA, and associates with hRad51, hMSH2/hMSH6, and p53 [61]. It promotes branch migration of Holliday junctions and can disrupt DNA displacement loops, which represent the initial strand invasion step of HR [62]. In a multienzyme complex together with topoisomerase III α , BLM mediates resolution of excess or aberrant recombination intermediates [61]. Despite these interesting connections to recombination control, use of defective cells in this work demonstrated that both BACH1 and BLM are dispensable for the HR-regulatory effect of hMutL-complexes. It should be noted, however, that in BLM-deficient versus BLM-proficient cells the degree of hMLH1-mediated HR-repression was decreased (see Fig. 5g). Importantly, significant reduction (Fig. 5c) of this effect was also observed after concomitant knockdown of ATM and ATR. Further candidates, providing mechanistic links to DSB repair and ATM/ATR-signaling, have come from a recent study, in which hMLH1, hPMS2, and hPMS1 interactors were identified by mass spectrometry [38]. Therefore, it is conceivable that hMutL-complexes downregulate HR through the combined effect of multiple components of ATM/ATR-signaling pathways.

The barrier to recombination between divergent sequences is not completely overcome in MSH2-deficient cell, indicating that alternative pathways contribute to HR fidelity control [17]. The role of hMutL proteins in restricting recombination between DNA sequences with only short stretches of uninterrupted homology identified in this work provides new clues to the nature of these alternative mechanisms. Thus, we propose that in human cells there are multiple pathways for interference with recombination, some requiring hMutS-complexes, some hMutL-complexes. Interestingly, mitotic recombination was shown not to be reduced

in T cells from *MLH1*^{-/-} mice, although point mutations increased in both fibroblasts and T cells, suggesting that functions of MLH1 in mismatch repair and HR control are activated in a tissue-specific manner [18]. In HNPCC families with mutated *MLH1*, colon tumors are the most common type of tumor, and this tumor type has been linked to defective post-replicative mismatch repair. However, *MLH1* alterations also cause a variety of other tumor types, including endometrial and ovarian malignancies [1]. In analogy to recent findings indicating that the mechanism responsible for tumor suppression by p53 is dependent on the tumor type [63], MutL-complex functions in post-replicative mismatch repair, HR control, and checkpoint-signaling may, therefore, differentially contribute to tumor formation in a tissue-specific manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.dnarep.2008.10.011.

Fig. 1.

DSB repair in 293T-Tet-Off-hMLH1 cells inducibly expressing exogenous hMLH1. (a) Substrate design for the analysis of different DSB repair pathways. The fluorescence-based DSB repair assay monitors reconstitution of wildtype *EGFP* after I-SceI meganuclease-mediated cleavage of the 5' positioned mutant *EGFP* gene (*EJ-EGFP*, Δ -*EGFP* or *HR-EGFP*), which is under the control of a CMV promoter (kinked arrow). In plasmid substrate *EJ-EGFP*, the I-SceI site (triangle) is flanked by 5 bp microhomologies (m), enabling quantification of NHEJ. The 3' positioned mutant *EGFP* genes *5'EGFP* and *3'EGFP* represent 3' and 5' truncated versions of *EGFP*, respectively, and provide sequence information for HR repair of the cleaved, 5' positioned *EGFP* gene. Substrates Δ -*EGFP*/5'EGFP and *HR-EGFP*/5'EGFP serve the analysis of conservative HR. Δ -*EGFP*/3'EGFP and

HR-EGFP/3'EGFP allow to monitor conservative and non-conservative HR. Substrates Δ -EGFP/5'EGFP and Δ -EGFP/3'EGFP enable analysis of HR between short, HR-EGFP/5'EGFP and HR-EGFP/3'EGFP between long homologies. (b) Protein expression analysis. 293T-Tet-Off-hMLH1 cells were cultivated in the absence or presence of dox to express exogenous hMLH1 and to completely switch-off hMLH1 expression, respectively. After Western blotting of cell lysates, hMLH1, hPMS1, hPMS2, hMRE11, hRAD50, and hMSH2 were immunodetected. Equal loading was verified by reincubation of the Western blot with anti-actin or anti-tubulin antibodies. (c) DSB repair changes. 293T-Tet-Off-hMLH1 cells, cultivated in the presence or absence of dox, were co-transfected with meganuclease expression plasmid pCMV-I-SceI and DSB repair substrates EJ-EGFP (EJ), Δ -EGFP/5'EGFP ($\Delta/5'$), HR-EGFP/5'EGFP (HR/5'), Δ -EGFP/3'EGFP ($\Delta/3'$), or HR-EGFP/3'EGFP (HR/3'). After cultivation for 24 h DSB repair frequencies were determined as detailed in Materials and Methods. For direct comparison of DSB repair without versus with hMLH1, the resulting mean frequencies in cultures without dox are shown as 100% (absolute values: 0.9×10^{-4} for EJ-EGFP, 1.8×10^{-3} for Δ -EGFP/5'EGFP, 1.5×10^{-3} for HR-EGFP/5'EGFP, 7.4×10^{-3} for Δ -EGFP/3'EGFP, 10.5×10^{-3} for HR-EGFP/3'EGFP). Mean values and S.E.M. from 15 measurements are shown (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). (d) DNA content analysis. Cell cycle distribution and apoptosis were analysed flow cytometrically for propidium iodide stained cells. The percentage of cells with sub-G1 DNA content was determined, and viable cells were subdivided into populations in the cell cycle phases G1, S, and G2 (given in %). Columns, mean values of five measurements; bars, S.D.

Fig. 2.

Comparison of hMLH1-deficient HCT116 and reconstituted HCT116+chr3 cells. (a) Western blot analysis. Lysates of HCT116 and HCT116+chr3 cells were immunoblotted with antibodies against hMLH1, hPMS1, hPMS2, hMRE11, hRAD50, and hMSH2. Bands additional to full-length hMRE11 are marked by asterisks. Tubulin immunostaining served as loading control. (b) Determination of DSB repair frequencies. HCT116 and HCT116+chr3 were transfected with pCMV-I-SceI together with EJ-EGFP (EJ), Δ-EGFP/5'EGFP (Δ/5'), HR-EGFP/5'EGFP (HR/5'), and Δ-EGFP/3'EGFP (Δ/3'), respectively. For better comparison mean DSB repair frequencies in HCT116+chr3 cells expressing hMLH1 are depicted as 100% (absolute values: 0.9×10^{-3} for EJ-EGFP, 1.2×10^{-3} for Δ-EGFP/5'EGFP, 1.6×10^{-3} for HR-EGFP/5'EGFP, 17.7×10^{-3} for Δ-EGFP/3'EGFP). Columns, mean values from three to 15 measurements; bars, S.E.M.; * $P < 0.05$. (c) Apoptosis and cell cycle distribution. DNA content analysis was performed as described in legend to Fig. 1d. Columns, mean values of two to three measurements; bars, S.D.

Fig. 3. Homology-directed DSB repair on cellular chromosomes after hMLH1 knockdown. (a) Chromosomal recombination. KMV(HR-EGFP/3'EGFP) and KMV(Δ-EGFP/3'EGFP) cells were electroporated with pCMV-I-SceI together with plasmids pRSMH1F or pRSMH1G, mediating RNA interference directed against hMLH1, or empty vector (pRScontrol). Mean frequencies in cells with pRScontrol plasmid are shown as 100% (absolute values: HR-EGFP/3'EGFP: 2×10^{-4} ; Δ-EGFP/3'EGFP: 1×10^{-4}). Columns, mean values from 4 to 11 measurements; bars, S.E.M.; * $P < 0.05$; *** $P < 0.001$. (b) Downregulation of hMLH1. Total homogenates were immunoblotted utilizing antibodies against hMLH1, hPMS1, and hPMS2. Equal loading was verified by reincubation of the Western blot with anti-tubulin antibody. (c) DNA content analysis. The percentage of cells with sub-G1 DNA content, of G1, S, and G2 phase cells was analysed under the conditions of the repair assay. Columns, mean values of four measurements; bars, S.D. (d) Effect of hMLH1 on recombination after etoposide and bleomycin treatment. KMV(Δ-EGFP/3'EGFP) cells were electroporated with pRScontrol or pRSMH1F and treated with 50 μ M etoposide (eto) or 50 mU/ml bleomycin (bleo). Mean DSB repair frequencies with empty pRScontrol vector were set as 100% (absolute values for both eto and bleo: 2×10^{-5}). Columns, mean values of six measurements; bars, S.E.M.; * $P < 0.05$.

Fig. 4. Knockdown of hMLH1, hPMS1, and hPMS2 in mismatch repair-deficient cells. (a) DSB repair in hMSH2-negative Lovo cells. Lovo cells were co-transfected with pCMV-I-SceI together with EJ-EGFP (EJ), Δ -EGFP/5'EGFP ($\Delta/5'$), HR-EGFP/5'EGFP (HR/5'), Δ -EGFP/3'EGFP ($\Delta/3'$), or HR-EGFP/3'EGFP (HR/3') as well as together with two siRNAs directed against hMLH1, hPMS1, hPMS2, hMSH2, or with non-specific control siRNA. For better comparison mean values for control siRNA are depicted as 100% each (absolute values: 0.7×10^{-2} for EJ-EGFP, 1.8×10^{-2} for Δ -EGFP/5'EGFP, 4.3×10^{-2} for HR-EGFP/5'EGFP, 8.1×10^{-2} for Δ -EGFP/3'EGFP, 9.2×10^{-2} for HR-EGFP/3'EGFP). Columns, mean values from 6 to 12 measurements; bars, S.E.M.; * $P < 0.05$. (b) Protein expression analysis for Lovo cells. Lovo cells were co-transfected with EJ-EGFP (EJ) and Δ -EGFP/5'EGFP ($\Delta/5'$) as in (a). Endogenous protein levels were visualized via immunodetection with antibodies against hMLH1, hPMS1, hPMS2, and HA-tagged I-SceI meganuclease followed by reprobing with anti-tubulin antibody. Loss of MSH2 protein expression in Lovo as compared to HCT116 cells was verified by Western blotting. (c) DNA content analysis. Cells, harvested along with DSB repair measurements, were propidium iodide stained for flow cytometric analysis. Columns, mean values of two measurements; bars, S.D. (d) Influence of siRNA directed against hMLH1 on DSB repair in 293T-Tet-Off-hMLH1 cells. 293T-Tet-Off-hMLH1 cells were cultivated in the presence or absence of dox and co-transfected with pCMV-I-SceI together with Δ -EGFP/5'EGFP and two siRNAs directed against hMLH1 or non-specific control siRNA. Mean values and S.E.M. from six measurements are shown, setting the mean frequency for dox+/control siRNA as 100% (absolute value: 1×10^{-3} ; ** $P < 0.01$). (e) Immunoblotting of 293T-Tet-Off-hMLH1 cells. 293T-Tet-Off-hMLH1 cells were processed as in (d) and lysates subjected to Western blot analysis of hMLH1 and tubulin. (f) DSB repair in hMSH2-deficient KMV(Δ -EGFP/3'EGFP) cells. KMV(Δ -EGFP/3'EGFP) cells were electroporated with pRSMSH2-5 and pRSMSH2-8, mediating RNA interference directed against hMSH2, or empty vector

pRScontrol followed by retransfection with pCMV-I-SceI and hMLH1 knockdown plasmid (pRSMLH1F) or pRScontrol 72 h later. The mean frequency in cells with pRScontrol plasmid is shown as 100% (absolute value: 2×10^{-4}). Columns, mean values from six measurements; bars, S.E.M.; * $P < 0.05$; *** $P < 0.001$. (g) Protein expression analysis for KMV(Δ -EGFP/3'EGFP) cells. Cells were processed as in (f) and lysates subjected to Western blot analysis of hMSH2, hMLH1, and tubulin.

Fig. 5.

DSB repair as a function of ATM, BLM, FANCI, and hMLH1. (a) HR after hMLH1 knockdown in A-T cells. GM02253F (control) and the AT-cells GM08436A, HA187, and HA126 were subjected to electroporation or nucleofection with pCMV-I-SceI, repair substrate Δ -EGFP/5'EGFP, and pRScntrl or pRSMH1F plasmid. Mean DSB repair frequencies with empty pRScntrl vector were set as 100% (absolute values: 1×10^{-4} for GM02253F, HA187, and HA126 cells, 6×10^{-4} for GM08436A). Columns, mean values of six to nine measurements; bars, S.E.M.; * $P < 0.05$; ** $P < 0.01$. (b) Downregulation of hMLH1 in A-T cells. Cells were processed as in (a) and total homogenates immunoblotted with anti-hMLH1 and anti-hMLH1-tubulin antibodies. (c) HR after ATM and ATR knockdown. 293T-Tet-Off-hMLH1 cells were cultivated without or with dox, co-transfected with pCMV-I-SceI, repair substrate Δ -EGFP/5'EGFP, and plasmids pSHAG-ATM plus pSHAG-ATR for concomitant knockdown of ATM and ATR or empty vector (pSHAG). DSB repair frequencies are shown graphically, taking the mean frequency in the control (-dox, pSHAG) as 100% (absolute value: 1×10^{-3}). Columns, mean values of 15–21 measurements; bars, S.E.M.; ** $P < 0.01$. (d) Downregulation of ATM and ATR. Cells were processed exactly as in (c) and cellular lysates prepared 12 h after transfection for Western analysis of ATM, ATR, hMLH1, and GAPDH. (e) HR after hMLH1 knockdown in Bloom's syndrome cells. GM03403 cells were processed as cells in (a). Columns, mean values of six measurements (absolute value for 100%: 2×10^{-5}); bars, S.E.M.; * $P < 0.05$. (f) Downregulation of hMLH1 in GM03403 cells. Immunoblotting was performed with total homogenates from cells transfected as in (e). (g) HR after hMLH1 knockdown in GM08505 cells without and with BLM. The GM08505 fibroblast cell line from a Bloom's syndrome patient (-BLM) and stably BLM-reconstituted GM08505 (+BLM) cells were nucleofected with pCMV-I-SceI together with Δ -EGFP/5'EGFP as well as with AllStars Negative Control siRNA or with two siRNAs directed against hMLH1. Columns, mean values of three measurements (absolute value for 100%: 2×10^{-3}); bars, S.E.M.; * $P < 0.05$; ** $P < 0.01$. (h)

Downregulation of hMLH1 in GM08505 cells without and with BLM. Immunoblotting was performed with total homogenates from cells nucleofected as in (g). (i) HR after knockdown of hMLH1 in *FANCI* mutant cells. F-A J cells were co-transfected with pCMV-I-SceI, Δ -EGFP/5'EGFP, and two siRNAs directed against hMLH1/non-specific control siRNA. The mean value for control siRNA is given as 100% (absolute value: 1×10^{-4}). Columns, mean values of six measurements; bars, S.E.M.; * $P < 0.05$. (j) Downregulation of hMLH1 in F-A J cells. Immunoblotting was performed with cellular lysates from cells processed as in (i).