Cloning of the Human MMR genes- Primers specific to hPMS1 (5'- GCG GAT CCC GGA TCC CAT ATG AAA CAA TTG CCT GCG GCA - 3' and 5'- GGC ATA CGC GAA TTC TAA TCA TGT AGT TTC TGG AAG - 3') and hMLH3 (5'- TGG ATC CAT GAT CAA GTG CTT GTC AGT TGA AGT A - 3' and 5'- CTA AGT CGA CAG ACC AGT GAT TCT GTT CTC ATG GT - 3') amplified each gene from a HeLa cDNA library (hPMS1) or human kidney cDNA library (hMLH3) (Clontech). The PCR product was subcloned to cloning vector Bluescript SK- (Stratagene) at BamHI and EcoRI restriction sites (hPMS1) or BamHI and SalI restriction sites (hMLH3). hPMS1, hMLH3, hMSH4, and hMSH5 were subcloned into pET29a (Novagen) and pGEX-SG1 (1) using the following restriction sites: NdeI and XhoI (hPMS1 pET29a), NdeI and NotI (hPMS1 pGEX-SG1), NdeI and NotI (pET29a), BamHI and NotI (hMLH3 pGEX-SG1). The subcloning of hMSH2, hMSH3, hMSH6, hMSH4, hMSH5, hMLH1, hPMS2 into pGEX-SG1 and pET29a has already been described (1-3).

Quantitiative Real Time PCR Reagents - ABI Taqman Gene Expression Assays (Applied Biosystems) include the primers and probes for the human mismatch repair genes. The specific assays are listed for each gene as follows, hMLH1 Hs00179866_m1, hMLH3 Hs00271774_m1, hPMS1 Hs00922266_m1, hPMS2 Hs00241053_m1, hMSH2 Hs00179887, hMSH3 Hs00988999_m1, hMSH6 Hs00943000_m1, hMSH4 Hs01548106_m1, and hMSH5 Hs00159268_m1. The hRAD51 primers and probes were obtained via Custom Taqman Gene Expression Assays (Applied Biosystems). The sequence of the hRAD51 primers and probe are as follows; hRAD51 forward primer 5′ GGG AAT TAG TGA AGC CAA AGC TGA T 3′, hRAD51 reverse primer 5′ CAG TGG TGA AAC CCA TTG GAA CTA 3′ and the hRAD51 probe 5’ (FAM) CCT CAG CCA GAA TTT (NFQ) 3’. For luciferase the forward primer is 5′

GGA GAG CAA CTG CAT AAG GCT AT 3′, the reverse primer is 5′ CCT CGA TAT GTG CAT CTG TAA AAG C 3′ and the probe is 5' (FAM) CAG GGC GTA TCT CTT C (NFQ) 3'. Although both types of the Taqman Gene Expression Assays are pre-optimized we tested their efficiency and dynamic range.

Reverse Transcription - Reactions were incubated at 25˚C for 10 min. followed by 37˚C for 120 min. EDTA was then added to a final concentration of 6mM and the reactions were incubated at 90ºC for 4 min. 1µl of RNase cocktail (Ambion, Inc) was added and the reactions were incubated at 37ºC for 15 min.

Validation of Quantitative PCR – We used real-time fluorescent quantitative PCR (qPCR) to determine mRNA copy number in a variety of human tissues. This method was chosen because of its sensitivity (large dynamic range) and reproducibility (4, 5). Validation of the assay is critical for accurate quantitation. The total human tissue RNA included single and pooled tissue samples from males and females (Clontech). mRNA was quantified from three different lots and the mean and standard deviation was calculated.

Since the rate at which amplicons are produced may vary due to a variety of factors we measured the amplification efficiency for each primer set. The efficiency was within the range of 100% ± 10% for each gene: hMSH2 (101.5%), hMSH3 (100.3%), hMSH6 (99.6%), hMSH4 (90.0%), hMSH5 (90.1%), hMLH1 (96.2%), hMLH3 (101.3%), hPMS1 (94.5%), hPMS2 (98.6%), and Rad51 (90.3%). The precision of each experiment was calculated. The inter-assay variance for each gene was as follows, hMSH2 (1.4%), hMSH3 (1.6%), hMSH6 (0.7%), hMSH4 (3.8%), hMSH5 (4.2%), hMLH1 (1%), hMLH3 (0.9%), hPMS1 (2.9%), hPMS2 (1.2%), Rad51

(3.7%), and 18S (3.4%). The PCR products were sequenced to verify that the appropriate sequence was amplified.

Antibodies – Commercially available polyclonal antibodies included hMSH2 Ab-3 (Calbiochem), hMSH3 (BD Transduction Laboratories), hMLH1 (BD Biosciences), and hPMS2 C-20 (Santa Cruz Biotechnology). Polyclonal antibodies were raised against full-length hRAD51 and hMSH5 and the C-terminus of hMLH3 (amino acids 2425-4362). All antibodies except anti-actin were conjugated to HRP using the SureLINK HRP conjugation kit (KPL). Validation of protein specificity was performed by protein/peptide competition that included 1-1000 nM of protein/peptide with the antibody inclubation step. Specific competition was observed with the hMSH2, hMLH1, hPMS2, hMLH3, and hRAD51 antisera, which were included in subsequent Western analysis.

Analysis of protein expression - Human tissue Western blots (ProSci Inc.) with 15μg of protein from indicated tissues were blocked with 5% milk or BSA in 0.05% TBST. Tissues include: HeLa cells, breast, colon, ovary, skin, small intestine, spleen, and testis. Specific protein bands were detected with a 1:1000 dilution of the HRP labeled antibody and the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). For actin detection 1:30000 and 1:1000 dilutions of primary and secondary antibody were used, respectively.

GST-IVTT Interaction Assay - The GST interaction assay was performed to determine protein interactions. The reactions were performed as described (3) except that all Glutathione S-transferase (GST) fusion constructs were transformed into BLR(DE3)pLysS (Novagen) cells to

enhance protein expression. Briefly a 50ml overnight culture of hMSH(X)-pGEX-SG1, hMLH(Y)-pGEX-SG1 , hPMS(Z)-pGEX-SG1, or pGEX-SG1 alone was added to 1L LB, 100µg/ml ampicillin, 12.5µg/ml tetracycline, and 30µg/ml chloramphenicol and shaken at 37ºC. At 0.4 A_{600}, 0.5mM isopropyl-1-thio-β-D-galactopyranoside was added. After 2hr at 30˚C, cells were centrifuged at 2000*g* for 10 min at 4ºC. The bacterial pellet was resuspended in PBS containing 0.1mM EDTA, 5mM dithiothreitol (DTT), 0.2% Triton X-100, 100µg/ml lysozyme, 1mM phenylmethylsulfonyl fluoride, 2µg/ml leupeptin, and 2µg/ml pepstatin and snap frozen with liquid nitrogen in 1ml aliquots and stored at -80ºC. The cells were lysed by two freeze-thaw cycles, treated with 20µg/ml DNaseI, and incubated on ice for 30 min. The cellular debris was clarified by centrifugation at 14,000 rpm for 30 min. The supernatant was incubated with 5mg of glutathione-agarose beads (Sigma) on a rocking platform at 4°C. After 1 hr the sample was centrifuged at 1000 rpm for 30sec. The GST-fusion protein-containing beads were washed three times with 1ml of binding buffer (20mM Tris-HCl pH 7.5, 10% glycerol, 150mM NaCl, 5mM EDTA, 1mM DTT, 0.1% Tween 20, 0.75 mg bovine serum albumin, 0.5mM PMSF, 0.8mg/ml leupeptin and 0.8mg/ml pepstatin) and resuspended in 0.5ml of binding buffer.

The GST-fusion protein was quatitated by comparison to a known quantity of BSA following separation by SDS-PAGE using a Gel-Doc Imager. The IVTT reaction used 1µg of the appropriate DNA construct in a TNT coupled reticulocyte lysate system (Promega) with ^{35}S-methionine. Labeled proteins were quantified as described (3), equivalent *molar* amounts of each IVTT reaction were added to a large molar excess of the GST-fusion protein, and binding reactions incubated on a rocking platform at 4ºC for 1 hr. The GST-fusion protein-containing beads with ^{35}S-IVTT protein were washed three times with binding buffer, resuspended in 35µl SDS loading buffer (25mM Tris-HCl [pH 7.0], 5% sucrose, 2% SDS, 5% 2-mercaptoethanol,

and 0.005% bromophenol blue), boiled for 10min, and resolved by 10% SDS-PAGE. The gel was dried and quantified by PhosphorImager (Molecular Dynamics). Semi-quantitative analysis (Int_{rel}) has been previously described and includes subtraction of non-specific binding with the GST moiety alone (3, 6).

PCR Truncation Mutagenesis - The truncation mutants of hMLH3, hMLH1, and hMSH3 were generated by PCR truncation mutagenesis. For hMLH3 forward primers were designed with the following first 13 nucleotides 5'- CGC GAG TCA TAT G -3' and reverse primers were designed with the following last 11 nucleotides 5'- CGC GGA TCC TCA -3'. PCR was performed using these primers and hMLH3 as template. The products and pET29a were digested with NdeI and BamHI, gel-purified, and ligated. The integrity of the clones were verified by sequencing. The generation of hMLH1 and hMSH3 truncation mutants has already been described (1, 3).

C-terminal GST constructs - pGEX-SG1 was modified to produce a C-terminal GST vector (pGEX-C). First, primers 5'- CTG GAG TGC GAT CTT CCT GA -3' and 5'- CCA GGA TCC TAC CAT GGA TAC TGT TTC CTG TGT GAA AT -3' amplified a fragment of pGEX-SG1. A second PCR of pGEX-SG1 with primers 5'- GGG AAT TCC CAT GTC CCC TAT ACT AGG TTA TTG GA -3' and 5'- ATC AGG TAG CGG CCG CCA GAT CCG ATT ATG GAG GAT G -3' amplified GST and introduced a stop codon at the end of the GST open reading frame. The first PCR product and pGEX-SG1 was digested with KasI and BamHI, gel purified, and ligated. The resulting vector, pGEX-R1, lacked the entire GST coding region. To reinsert GST into pGEX-R1, the second PCR product was digested with EcoRI and NotI and ligated to pGEX-R1. This new vector was termed pGEX-C and contained an inserted multicloning site

followed by a GST open reading frame with an introduced stop codon. After verifying the sequence, pGEX-C was used to generate C-terminal GST constructs of hMLH1, hMLH3, hPMS1, and hPMS2. Briefly, to clone hMLH1, hMLH3, hPMS1, and hPMS2 into pGEX-C, fragments of each gene were amplified with gene specific primers that mutated the stop codons (hMLH1 primers 5'- TGA GGT GAA TTG GGA CGA AGA AAA -3' and 5'- TTT TTT CCT TGC GGC CGC GAA TTC CTG AAC ACC TCT CAA AGA CTT TGT ATA G -3', hMLH3 primers 5'- GCG ACC TTG TTC TTC CTT TCC TTC -3' and 5'- TTT TTT CCT TGC GGC CGC GGT ACC CTC CTG GTG GCT CAC AGG GAG GCA T -3', hPMS1 primers 5'- GCG ACC TTG TTC TTC CTT TCC TTC -3' and 5'- TTT TTT CCT TGC GGC CGC GGT ACC CTC CTG GTG GCT CAC AGG GAG GCA T -3', hPMS2 primers 5'- GCG ACC TTG TTC TTC CTT TCC TTC -3' and 5'- TTT TTT CCT TGC GGC CGC GGT ACC CTC CTG GTG GCT CAC AGG GAG GCA T -3'). The PCR products and hMLH1 pGEX-SG1, hMLH3 pGEX-SG1, hPMS1 Bluescript SK-, hPMS2 pGEX-SG1 were digested with SalI and NotI, SacI and NotI, Hpa and NotI, and SpeI and NotI respectively, gel purified and ligated. The resulting products were subcloned into pGEX-C with NdeI and EcoRI (hMLH1 and hPMS2), NdeI and KpnI (hMLH3), and BamHI and EcoRI (hPMS1). Each clone was sequenced to verify its integrity and to ensure the coding sequences were in frame.

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