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Title: Phenotypic Analysis of Human MLH1 Variants for DNA Mismatch Repair

Abstract approved:_____

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DNA mismatch repair (MMR) is an evolutionarily conserved process used by cells to decrease mutation rates, thereby increasing genetic stability and reducing cancer risk. Germline mutations in the MMR gene *MLH1* have been identified in Lynch Syndrome patients, and in many cases are suspected but not confirmed as causative for cancer predisposition. This study compared wild-type and variant hMLH1 proteins in terms of MLH1 stability and the ability to complement a MMR-deficient phenotype in cell cultures lacking endogenous MLH1. The goal was to describe the possible mechanisms of pathogenicity for individual mutations in MLH1 by identifying functional deficiencies associated with each mutation. By measuring and comparing the degradation rates of wild-type and variant hMLH1 proteins, it was determined that variants that accumulated to low steady-state levels indeed had elevated degradation rates. By using a novel pooled complementation approach, the ability of wild-type and variant hMLH1 to restore a MMR-proficient phenotype to MLH1-deficient mouse embryonic fibroblast cultures was measured. Variants with

decreased MLH1 stability (eg. K616Δ and K618A) were incapable of inducing a MLH1-dependent cytotoxic response or decreasing spontaneous mutation frequencies to levels observed in wild-type expressing cultures. Variants with steady-state hMLH1 levels with similar to wild-type (eg. K751R and R755W) displayed variable phenotypes, depending on the individual variant's biochemical activity. In our pooled approach, the controversial and weakly penetrant variants examined (D132H and E578G) were capable of restoring a cytotoxic response and reducing spontaneous mutation frequencies to levels comparable to wild-type transfected cultures. The study shows that the pooled approach is an effective approach for determining the phenotypic behavior of hMLH1 variants in order to identify potential mechanisms of pathogenicity.

Phenotypic Analysis of Human MLH1 Variants for DNA Mismatch Repair

by

Karen Jean Hippchen

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Karen Jean Hippchen, Author

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LIST OF ABREVIATIONS

6-TG	6-Thioguanine
β-gal	β-galactosidase
CMV	Vector plasmid lacking cDNA insert
GST	Glutothione S-transferase
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
h-	Human
IHC	Immunohistochemistry
m-	Mouse
MEF	Mouse embryonic fibroblast
MF	Mutant frequency
MLH	MutL homolog
MMR	Mismatch repair
MSH	MutS homolog
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
WT	Wild-type

CHAPTER 1: INTRODUCTION

1.1 Introduction to DNA Mismatch Repair

DNA mismatch repair (MMR) is an evolutionarily conserved process used by cells to decrease mutation rates, thereby increasing genetic stability and reducing cancer risk. Loss of mismatch repair activity leads to an increased mutation rate and decreased cytotoxic responses to genotoxins, and is associated with increased cancer risk. MMR activity contributes as much as 1000-fold to maintenance of DNA integrity (Harfe and Jinks-Robertson 2000).

MMR primarily functions in error correction but is also used for the induction of a cytotoxic response to DNA damage and in controlling cell cycle checkpoints (Kunkel and Erie 2005). MMR prevents mutations by correcting errors that spontaneously arise from nucleotides that were misincorporated by DNA polymerase during replication, during recombination (Kolodner and Marsischky 1999), or that were induced as the result of DNA damaging agents. By correcting replication errors when possible and causing apoptosis in cells when the damage levels exceed repair capacity, the fidelity of DNA is maintained.

The general mechanism of DNA mismatch repair involves the identification of the DNA mismatch, excision of the DNA containing the error, re-synthesis of the excised patch and ligation of the adjacent DNA (Schofield and Hsieh 2003). While DNA mismatch repair is best understood in *E. coli*, the main protein components and functions are relatively conserved among a broad range of species in both prokaryotes to eukaryotes (Harfe and Jinks-Robertson 2000).

1.2 DNA Mismatch Repair Mechanism Overview

In the *E. coli* system, the mismatch in post-replicative DNA is recognized and bound by a complex composed of two MutS proteins. A homodimer consisting of two MutL proteins then interacts with the MutS:DNA complex in an ATP-dependent manner and activates the endonuclease MutH, which nicks the newly synthesized, unmethylated strand near the mismatch (Bocker et al 1999; Hall and Matson 1999). Exonucleases and other associated proteins excise the mismatch and a DNA polymerase re-synthesizes the missing fragment, generally with the correct nucleotides and no mismatch (Modrich and Lahue 1996).

While similar to prokaryotes, the DNA mismatch repair system in eukaryotes is more complex and not as well understood. MutS Homologs (MSH) form heterodimers consisting of MSH2-MSH6 or MSH2-MSH3 that recognize the mismatch and recruit MutL Homolog (MLH) heterodimers. MLH heterodimer complexes consisting of either MLH1-PMS2, MLH1-PMS1, or MLH1-MLH3 interact with the ATP-bound MSH:DNA complex. Exonuclease I is targeted to excise the region and replicative DNA polymerase resynthesizes the new strand (Boland and Fishel 2005) (Figure 1.1).

1.3 Lynch Syndrome

Lynch syndrome (also known as HNPCC – hereditary nonpolyposis colorectal cancer) is a condition that causes a predisposition to early onset cancers as the result of inherited mutations in post-replicative DNA mismatch repair genes. The lifetime cancer risk of individuals affected by Lynch Syndrome is estimated to be as much as

90% for men and 70% for women (Lynch and de la Chapelle 2003). The identification of at risk individuals is important so the affected individuals can undergo more frequent assessment and cancer screening compared to the general population.

The inheritance of variant forms of DNA mismatch repair genes has been identified as a cause of Lynch Syndrome. Variants have been identified by the sequencing of DNA from biopsied tumor samples taken from Lynch Syndrome patients and were screened for germline mutations (Barnetson et al 2007). Unfortunately, the existence of a variant, or multiple variants, in a tumor tissue does not directly identify the particular variant as the cause of increased cancer risk, but only as a potential cause due to the fact that many suspected polymorphisms have been identified in the general population.

1.4 Defects in hMLH1

Mutations in the gene for *MLH1* are estimated to account for about 40% of the more than 400 known MMR gene mutations (Peltomaki, 2003). Many identified mutations in MLH1 are missense mutations whose effect on protein activity is unclear and difficult to define. Complete loss of hMLH1 leads to an increased mutation rate and decreased cytotoxic responses to genotoxins. Buermeyer et al (1999) showed that complementation of the MLH1-deficient phenotype could be obtained by expressing a wild-type hMLH1 cDNA in MLH1-deficient mouse embryonic fibroblast (MEF) cells. Using the complementation approach, Mohd et al (2006) compared the ability of wild-type and variant hMLH1 proteins to stabilize PMS2 and restore a MMR-proficient

phenotype. Through their analysis, Mohd et al (2006) identified a particular C-terminal mutant which was unable to restore a wild-type phenotype, and demonstrated that the complementation of MLH1-deficient MEFs could be used to identify the consequences of mutations in hMLH1.

1.5 Research Goal

The goal of this study was to determine the phenotypic consequences of point mutations in hMLH1 by examining the ability of variants to complement a MMR-deficient phenotype. In particular, the effect of mutations on hMLH1 stability, interaction with and stabilization of PMS2, hMLH1-dependent mutation suppression and cytotoxic responses to genotoxins were determined. The specific hypothesis tested was that the presumptive pathogenic hMLH1 mutations would show measurable defects in critical mismatch repair functions. Through this research we hoped to identify cellular phenotypes consistently associated with pathogenic mutations, which could be used to determine the risks associated with individual variants as well as identify potential mechanisms of their pathogenicity. We also aimed to identify potentially non-pathogenic hMLH1 variants mistakenly included in the mutant databases.

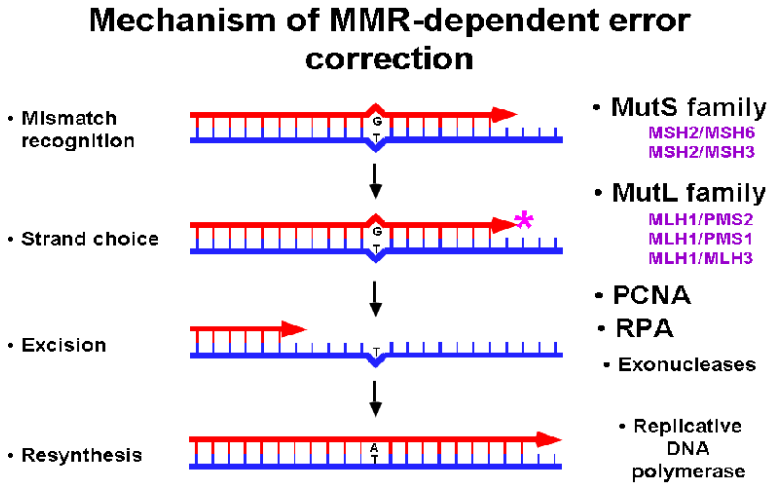


Figure 1.1: Mechanism of DNA mismatch repair

DNA mismatch repair mechanism and the associated proteins involved in error correction of eukaryotic cells.

CHAPTER 2: MATERIALS & METHODS

2.1 Maintenance of Cell Lines:

Cell lines were grown in either 10% or 15% complete medium (DMEM with 4.5g/L L-glutamine, 10% or 15% (v/v) bovine calf serum, 100 U/mL penicillin (Invitrogen), 100 U/mL streptomycin (Invitrogen) and 1X non-essential amino acids). Cellgro media and reagents (Mediatech Inc) were used unless otherwise noted. Cells were generally passed 1:10 when they reached 70-80% confluency and were stored in a 38º C incubator containing 5% CO_2.

2.2 Transient Transfection:

MP1 cells, a mouse embryonic fibroblast cell line deficient for both MLH1 and PMS2, were counted with a hemocytometer and plated at a density of $2.0x10^5$ cells per well in a six well plate containing 3 mL 10% complete medium. Plates were rocked back and forth to facilitate even cell distribution and incubated for 24 hours.

The following day, varying amounts of either wildtype or variant MLH1, 0.5 μg PMS2 and enough CMV empty vector to equal 6 μg total plasmid DNA were added to a 1.5 mL Eppendorf tube containing 125 μL non-sterile DMEM. Seven μL Plus Reagent (Invitrogen) was added, mixed gently and incubated at room temperature for 15 minutes. Following incubation, 5 μL Lipofectimine (Invitrogen) in 125 μL DMEM was added to each tube and incubated for an additional 15 minutes. During DNA/transfection mixture incubation, the 10% complete medium was aspirated from the six-well plates and each well was rinsed with 2 mL sterile DMEM. Following removal of the DMEM rinse, 1 mL

DMEM was added to each well. Following its 15 minutes incubation, the transfection mixtures were added to their corresponding well in the six-well plate, briefly rocked to mix and set in the incubator for 3 hours. After 3 hours, the transfection mixture was removed, the cells rinsed with 2 mL DMEM and then refed with 3 mL 10% complete medium. Cells were returned to the incubator for 18-20 hours after which whole cell lysates were prepared.

2.3 Preparation of whole cell lysates:

The medium was removed from the 6-well plates, the cells rinsed with PBS, trypsinized, stop media added and collected in a 15 mL conical tube. The wells were then rinsed with PBS to collect any remaining cells. A pellet was formed by centrifugation at 2000 rpm for 4 min. The supernatant was removed, the cells flicked and rinsed with 5 mL cold PBS. The cells were again collected by centrifugation and the supernatant removed. The pellet was flicked, resuspended in 1 mL PBS and transferred to a 1.5 mL eppendorf tube. The 1.5 mL tube was centrifuged and the supernatant carefully removed. The pellet was flicked and resuspended in 80 μL PBS. The cells were pipetted up and down to get a uniform cell suspension, 80 μL 2X lysis buffer (100mM Tris-Cl, pH 6.8, 2% w/v SDS, 20% v/v glycerol) added, the samples heated to 95º C for 5 minutes and placed in the -80º C freezer.

2.4 Protein Level Determination:

Protein levels were determined using SDS-PAGE and western blot analysis. Whole cell lysates were mixed with 2X SDS-PAGE loading buffer (100mM Tris-Cl, pH

6.8, 4% w/v SDS, 20% v/v glycerol, 20mM DDT, .2% bromophenol blue) and boiled in a thermocycler for 5 minutes. 12 μL of samples were loaded into a 4-12% Bis-Tris Criterion XT 26-comb precast gel (Bio-rad) and electrophoresed in 1X MOPS buffer (Bio-Rad) for 60 minutes. Gels were washed in transfer buffer (25mM Tris, 250mM glycine pH 7.4, 10%v/v methanol) for 15 minutes. Proteins were transferred to a pre-soaked PVDF membrane (splashed with methanol, soaked in nanopure water for 5 minutes and then soaked in transfer buffer for at least 5 minutes) using a Criterion Blotter (Bio-Rad) for 30 minutes at 100V. Following transfer, the membrane was immersed in 5% blotto blocking solution (w/v of powdered milk in 1X TBST (90mM NaCl, 17mM Tris, 0.1% Tween)) and placed on a shaker for 60 minutes at room temperature. Primary antibodies: anti-hMLH1 (554073, BD Pharmigen) 1:1000, anti-mPMS2 (556415, BD Pharmigen) 1:1000, and anti-MSH6/GTBP MAb (610919, BD Transduction Laboratories) 1:2500 were added and the membrane set on a rotator overnight at 4º C. The membrane was washed five times, for a minimum of 5 minutes each, with 1X TBST and placed in secondary antibody, HRP-conjugated goat anti-mouse IgG (31430, Pierce) in blotto and put on a shaker for 60 minutes at room temperature. Following secondary antibody, the membrane was washed five times with 1X TBST. Antibodies were detected by the addition of 10 mL Supersignal West Pico Chemiluminescent Substrate (Pierce) with agitation for 5 minutes and imaged using Chemi Genius Bio Imaging System and Gene Snap software (Synoptics LTD). Analysis was performed using Gene Tools software (Synoptics LTD).

2.5 hMLH1 Protein levels in the Absence of PMS2:

To determine the MLH1 protein levels in the absence of PMS2, 1, 3, and 5.5 μg of wild-type or variant MLH1 and empty-vector (CMV) were transfected as described above, but omitting the PMS2 cDNA. Whole cell lysates were prepared differently as well. The 6-well plate was rinsed with PBS and 400 μL 1X SDS-PAGE loading buffer (50mM Tris-Cl, pH 6.8, 2% w/v SDS, 10% v/v glycerol, 10mM DDT, 0.1% bromophenol blue) added and transferred to a 1.5 mL Eppendorf tube. The samples were heated to 95º C for 5 minutes and placed in the -80º C freezer.

2.6 Half-life Determination:

Wild-type and variant MLH1 half-life was determined in the absence of PMS2, based on the MLH1 protein levels determined in the absence of PMS2. Enough variant MLH1 cDNA (1, 3 or 5.5 μg) was transfected to give protein levels approximately equal to the transfection of 1 μg of wild-type cDNA. MP1 cells were transfected with MLH1 and CMV cDNA as described above. After the 18-20 hour incubation, 3 mL of 5 μg/mL cycloheximide in 10% complete medium was added to each well to stop protein synthesis. Whole cell lysates were prepared at time points of 0, 1, 2, 4 and 6 hours after the addition of cycloheximide.

2.7 DNA Linearization:

Plasmid DNA containing either wild-type or variant MLH1 cDNA, or empty vector, was linearized prior to electroporation. 25 μg of DNA was diluted into 110 μL

Y+/Tango buffer (Fermentas) in 0.5 mL Eppendorf tubes. 40 U of Xmn1 (Fermentas) was added to each and the tubes placed in a 37º C incubator for 2 hours. Following incubation, 1 μL was removed from each tube, diluted 1:10 and ran on a 0.8% agarose gel at 200V for 20 minutes to verify complete digestion. The DNA was precipitated by the addition of 11.4 μg NaOAC and 228 μL 100% ethanol and placed in a -20º C freezer for at least 90 minutes. Following precipitation, the tubes were spun at 14000 rpm for 15 minutes and the supernatant removed. The pellets were rinsed by the addition of 50 μL 70% ethanol and spun for an additional 3 minutes. The supernatant was removed and the pellets air-dried for 10 minutes. The pellets were resuspended in 25 μL sterile PBS. 0.5 μL was removed from each sample, diluted 1:20 and ran on a 0.8% agarose gel at 200V for 20 minutes to verify a similar DNA concentration in each tube. Tubes were store at -20º C until electroporation.

2.8 Electroporation and Selection:

MC2A cells, a mouse embryonic fibroblast cell line deficient for MLH1, were counted with a hemocytometer and the volume of medium containing $1x10^7$ cells transferred to a 15 mL conical tube. One tube was prepared for each culture to be electroporated. Tubes were spun at 2000 rpm for 4 minutes, the supernatant removed and the cells resuspended in 1 mL 15% complete medium. At the same time, the previously linearized plasmid DNA was thawed in a 37º C water bath. The 1 mL cell suspension and 20 μL linearized DNA were added to a 0.4 cM gap electroporation cuvette and electroporated at 350 volts for approximately 6.5 seconds using a Gene Pulser Xcell electroporator (Bio-rad). Transfected cells were quickly transferred to a conical tube

containing 5 mL 15% complete medium. 10 μL was removed from each tube and live cells counted using 0.4% trypan blue solution (Sigma). The remaining cells for each culture were divided into six 142-cm^2 plates containing 25 mL 15% complete medium and placed in a 38° incubator overnight. The following day, 5 mL of 15% complete medium containing 2.4 mg/ml G-418 sulfate (Mediatech Inc.) was added to each plate to reach a final G-418 concentration of 400 μg/ml. Subcultures were maintained under selection through subsequent passages at 400 μg/ml G-418 in 15% complete medium.

2.9 Forward mutation Assay:

The pooled cultures were grown to 70-80% confluency in a 75-cm^2 flask, counted and plated at a maximum density of $1.0x10^6$ cells per 142-cm^2 plate containing 30 mL 1mM ouabain (ICN Biomedicals) in 15% complete medium. At the same time, 300 cells were plated into 57-cm^2 plates containing 10 mL 15% complete media to determine growth efficiency. The 142-cm^2 plates were refed 5-7 days after plating with 25 mL 1mM Ouabain in 15% complete medium. The 57-cm^2 plates were fixed and stained with 1X methylene blue stain (4.5g methylene blue (Sigma) in 6.25% ethanol) 8-10 days after plating. The 142 cm^2 plates were fixed and stained with methylene blue 12-14 days after plating. Colonies were counted and mutations rates calculated based on survival of colonies exposed to Ouabain and the estimated cell survival determined from the efficiency plates.

2.10 Cytotoxic response Assay:

The pooled cultures were grown to 70-80% confluency in a 75-cm^2 flask, counted and 300 cells plated into four 57-cm^2 plates and 3000 cells into two 57-cm^2 plates. The following day, the 3000-cells plates and 2 of the 300-cell plates received a 7 ml dose of 1μM 6-Thioguanine (Sigma). 24 hours later, the four 300-cell and two 3000-cell plates were refed with 15% complete medium and returned to the incubator. Colonies were fixed and stained with 1X methylene blue stain 6-8 days following their refeed.

2.11 Immunohistochemical Staining:

Cells were counted and plated at a density of $1.0x10^5$ cells per well in a twelve-well plate containing 1 mL 15% complete medium. The following day the medium was aspirated and cells fixed with 10% formalin in buffered saline (VWR) for 10 minutes. Following fixation, 0.3% hydrogen peroxide (Sigma-Aldrich) was added for 10 minutes at room temperature to inactivate the endogenous peroxidases. The cells were then permeabilized and the non-specific binding sites blocked with 1.5% Normal Horse serum (Vector Labs) in 0.05% Triton X-100 (Sigma) for 30 minutes at room temperature with gentle rocking. Following blocking, 0.5 μg/ml anti-hMLH1 antibody (51-1327GR, BD Pharmingen) was added to blocking solution and incubated overnight at 4º C. Following incubation with the primary antibody, the cells were washed 3 times for 5 minutes each with 1X PBS with agitation. Horse anti-mouse secondary antibody (Vector Labs kit) was diluted 1:500 in PBS, added to the cells and incubated with agitation for 1 hour. During incubation, the ABC complex (Vector Labs) was created. Reagent A was diluted 1:50 in PBS and mixed, followed by the addition of reagent B at a 1:50 dilution. The ABC

complex was left at room temperature for a minimum of 30 minutes before use. Following incubation with the secondary antibody, the cells were washed 3 times for 5 minutes each with 1X PBS with agitation and incubated in ABC complex for 1 hour. Following ABC complex incubation, cells were again washed 3 times for 5 minutes with 1X PBS. Staining was developed with DAB/Nickel/Peroxide solution (Vector Labs, SK-4100) as directed. The staining was stopped with a 1X PBS rinse and counterstained using Eosin Y (J.T. Baker) in 70% ethanol.

2.12 Total Protein quantitation:

The quantitation of the total protein content in the whole cell lysates was determined using the BCA Protein Assay Kit (Pierce). Lysates were diluted 1:10 and in a 96-well plate, mixed with 200 μL working reagent (50 parts reagent A, 1 part reagent B) and placed in a 37º C incubator for 30 minutes. Plates were then cooled to room temperature and the UV absorbance at 562 nm read on a SpectraMax UV Plate reader and analyzed with Excel software (Microsoft).

2.13 hMLH1/PMS2 quantitation per μg total protein:

Western blotting was performed to determine the MLH1/PMS2 levels per μg total protein in the whole cell lysates. For each Western blot, a standard curve based on samples of known MLH1 and PMS2 concentration was plotted and the equation of that line used to quantify the unknown samples. Two wild-type sample were run on every blot to verify consistency among blots. For each blot, 25 μL total protein was mixed with 6X SDS-PAGE loading buffer and enough 1X SDS-PAGE loading buffer to bring the

total volume to 30 μL. 25 μL of samples were loaded into a 4-12% Bis-Tris Criterion XT 18-comb precast gel (Bio-rad) and electrophoresed in 1X MOPS buffer (Bio-Rad) for 60 minutes. Gels were washed in transfer buffer (25mM Tris, 250mM glycine pH 7.4, 10%v/v methanol) for 15 minutes. Proteins were transferred to a pre-soaked PVDF membrane (splashed with methanol, soaked in nanopure water for 5 minutes and then soaked in transfer buffer for at least 5 minutes) using a Criterion Blotter (Bio-Rad) for 30 minutes at 100V. Following transfer, the membrane was immersed in 5% blotto blocking solution (w/v of powdered milk in 1X TBST (90mM NaCl, 17mM Tris, 0.1% Tween)) and placed on a shaker for 60 minutes at room temperature. Primary antibodies: anti-hMLH1 (554073, BD Pharmigen) 1:1000 and anti-mPMS2 (556415, BD Pharmigen) 1:1000 were added and set on a rotator overnight at 4º C. The membrane was washed five times with 1X TBST and placed in secondary antibody, HRP-conjugated goat anti-mouse IgG (31430, Pierce) in blotto and put on a shaker for 60 minutes at room temperature. Following secondary antibody, the membrane was washed five times with 1X TBST. Antibodies were detected by the addition 10 mL Supersignal West Pico Chemiluminescent Substrate (Pierce) with agitation for 5 minutes and imaged using Chemi Genius Bio Imaging System and Gene Snap software (Synoptics LTD). Following imaging, the blot was washed four times with 1X TBST and placed in the third primary antibody, anti-GAPDH (CB1001, Calbiochem) 1:4000 and set on a rotator overnight at 4º C. The membrane was washed five times with 1X TBST and placed in secondary antibody, HRP-conjugated goat anti-mouse IgG (31430, Pierce) in blotto and put on a shaker for 60 minutes at room temperature. Following secondary antibody, the membrane was washed five times with 1X TBST. Antibodies were detected by the

addition of 10 mL Supersignal West Pico Chemiluminescent Substrate (Pierce) with agitation for 5 minutes and imaged using Chemi Genius Bio Imaging System and Gene Snap software (Synoptics LTD). Signal analysis was performed using Gene Tools software (Synoptics LTD) and excel (Microsoft).

2.14 Statistical Analysis

The statistical analyses of wild-type and variant half-lives were performed using an F-test comparison of one-phase exponential decay. The statistical analyses of the MF of wild-type and variant expressing cultures were done using the log transformation of measured MFs then one-way ANOVA after confirmation of equal variances using Bartlett's test. Pair-wise comparisons were performed as a post-test using Tukey's Multiple Comparison Test. The statistical analyses of the 6-TG response of wild-type and variant expressing cultures were done using the log transformation of the measured response and Kruskal-Wallis test with Dunn's multiple comparisons and the difference in rank sum as a post-test.

CHAPTER 3: METABOLIC STABILITY OF MUTANT hMLH1 PROTEINS

3.1 Introduction:

Missense mutations in hMLH1 have been identified in Lynch Syndrome patients and in patients suspected of Lynch Syndrome. In many cases the pathogenicity and the mechanism(s) by which the mutations affect hMLH1 function are not known. Previous studies in the Buermeyer lab have demonstrated that many hMLH1 mutations affect the steady-state accumulation of variants hMLH1 proteins (Mohd et al, 2006, and unpublished observations). The goal of experiments described in this chapter is to determine the effect of point mutations in human MLH1 on protein stability. The specific hypothesis to be addressed here is that mutants showing reduced steady-state hMLH1 accumulation are less stable and subjected to increased turnover compared to wild-type hMLH1.

3.2 Results & Discussion:

To determine whether the reduced levels of hMLH1 in variants observed previously were the result of decreased protein stability, the metabolic half-lives of the wild-type and variant proteins in mammalian cells were compared. To generate cells expressing hMLH1, mouse embryonic fibroblasts (MEFs) lacking both mMLH1 and mPMS2 (MP1 cells) were transfected with expression vectors for wild-type or variant hMLH1 generally as previously described (Mohd et al 2006). Following transfection, cells were

exposed to cycloheximide to stop new protein synthesis, cell lysates were prepared at various time points, and the protein levels were measured using quantitative western blot analyses to determine the rate of degradation. Previous experiments (Mohd et al, 2006) were done in the presence of hPMS2; however, wild-type hMLH1 generally is stable in the absence of hPMS2. Therefore, to simplify the analysis of hMLH1 variants, we chose to perform the half-life experiments in the absence of hPMS2. To confirm that the accumulation of hMLH1 variants relative to wild-type would be similar in both the presence and absence of hPMS2, representative variants were analyzed. The analysis included 3 previously described neutral polymorphic variants with no known association to human disease, and 21 variants previously identified in human cancer patients and listed in human gene mutation databases (Lucci-Cordisco et al, 2006). Initially the yield of hMLH1 protein produced in the transient transfection of 1 μg of expression vector for each variant relative to 1 μg of vector for wild-type hMLH1 was compared (Figure 3.1). Variant steady-state levels ranged from 6 to 150% of wild-type with the majority of variants affecting amino acid 616 onward showing protein levels below 18%.

The yield of hMLH1 variants observed generally was consistent with the results of the previous analysis in the presence of hPMS2, with several exceptions noted below. All polymorphic variants yielded protein levels similar to wild-type. Variants V506A, E578G, K618A and V716M each accumulated to lower levels than previously measured when co-expressed with

hPMS2. The previous analysis found accumulated levels of V506A to be equivalent, E578G to be ~80%, and K618A and V716M to be ~50% of wild-type. The difference in accumulation of variant hMLH1 proteins in the presence and absence of hPMS2 suggests that heterodimerization with hPMS2 may help stabilize some variants.

For the determination of protein half-lives, transfection conditions were adjusted to generate similar initial, steady-state levels of wild-type and variant hMLH1. With this approach, decreases in wild-type and variant hMLH1 levels over time would be measured by immunoblotting using a similar range of signal intensities. MP1 cells were transfected with various masses of the expression vectors for hMLH1 variants and the resulting hMLH1 levels compared (data not shown). The variant plasmid masses that yielded hMLH1 levels most similar to the transfection with 1 μg wild-type hMLH1 plasmid were used for further half-life determination.

To compare wild-type and variant degradation rates, a representative sample of two polymorphic variants with no known association to human disease and seven variants previously linked to human cancer that displayed reduced yields in the initial analysis were examined. Cells expressing either wild-type or variant hMLH1 were exposed to cycloheximide to stop new protein synthesis and whole cell protein lysates were prepared from cells harvested over a time course of 360 minutes. The hMLH1 protein level at each time point was determined by quantitative immunoblot analysis and was

compared to time zero. The protein degradation rate was then determined by nonlinear regression and used to calculate protein half-life (Figure 3.2).

The analysis showed decreasing hMLH1 levels over time in cells expressing either wild-type or variant hMLH1 plasmid following the addition of cycloheximide. The reduction in protein level varied among the mutants and wild-type hMLH1, thus confirming that some mutations in hMLH1 affect protein turnover more than others. Alterations nearer to the C-terminal region appeared to have the greatest effect on protein stability (Table 3.1).

Comparison of variant half-lives (Table 3.1) and yields (Figure 3.1) suggested a correlation. We reasoned that if increased protein turnover was the principal determinant of decreased yields for the hMLH1 variants, then there should be a simple quantitative relationship between half-life and yield. To determine that quantitative relationship, we plotted the relative steady-state levels as a function of degradation rates for each of the variants in Table 3.1. Indeed, decreased steady-state hMLH1 levels correlated with increased degradation rates among the tested variants (Figure 3.3). We further hypothesized that yield should be a simple function of a constant rate of production (all variants were expressed from identical plasmid constructs under the same transfection conditions) and a variable degradation rate, i.e. Yield = S / X, where X represents the variable degradation rate, and S represents the constant synthesis rate. We next fit the experimental data by non-linear regression using Yield = Synthesis / X (Figure 3.3). The results demonstrated a good fit ($r^2 = 0.99$) of the theoretical relationship (Y = S/X) to

experimentally-derived yields and degradation rates, consistent with the hypothesis. Therefore, yields as determined by measurements of the steady-state levels following transient transfection with 1 μg vector are reasonable predictors of the effect of hMLH1 mutations on protein stability.

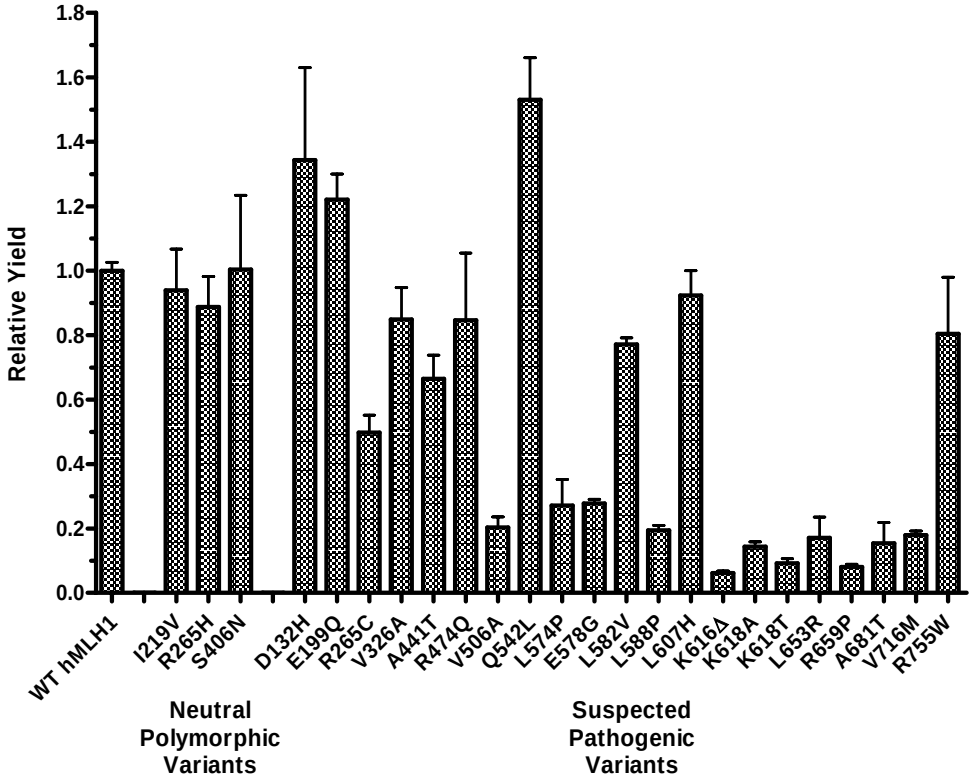


Figure 3.1: Steady-state accumulation of hMLH1 variants.

The relative steady-state accumulation of hMLH1 variants was determined by quantitative immunoblotting following transient transfection of MLH1/PMS2-deficient MEFs.

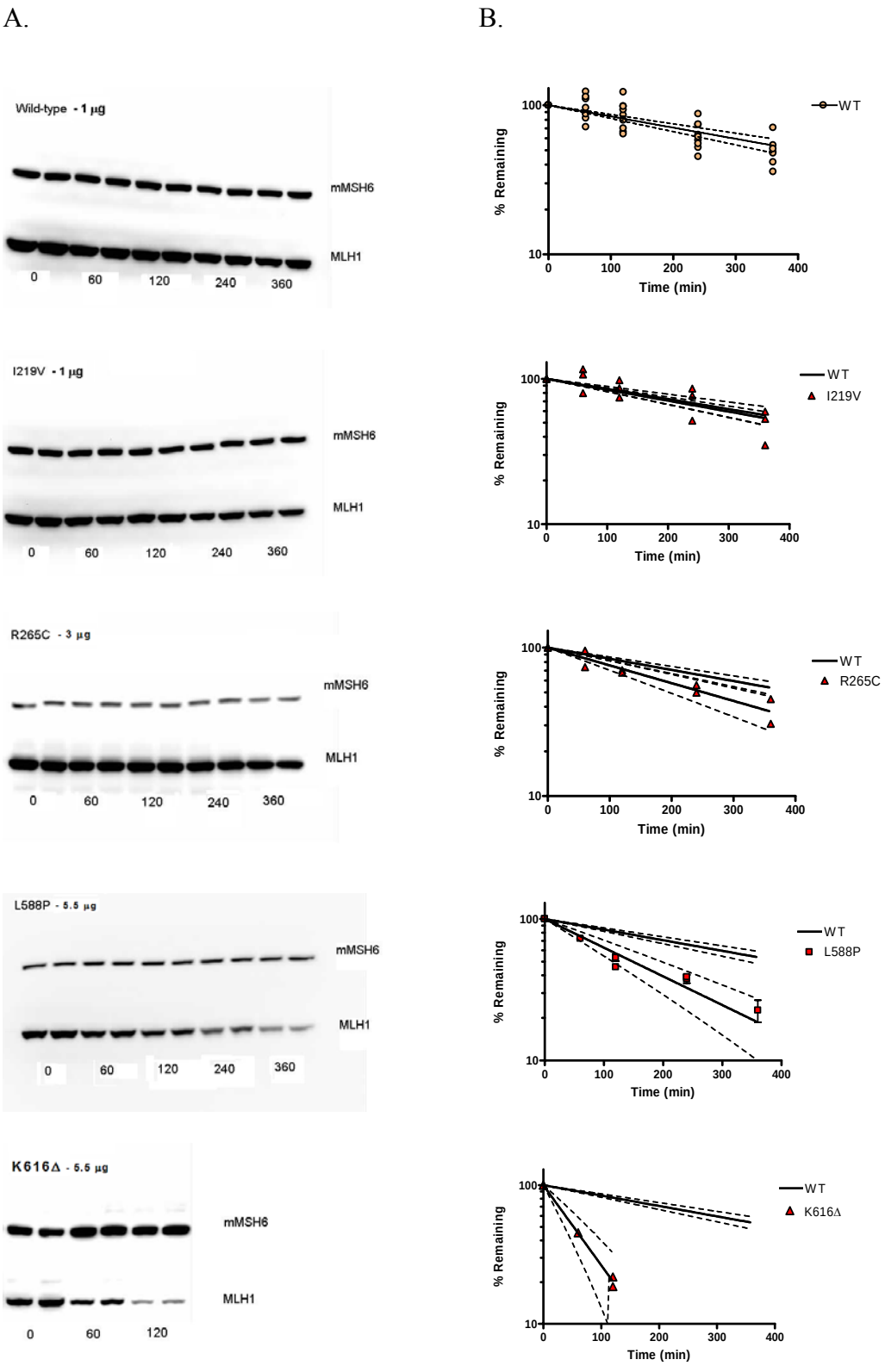


Figure 3.2: hMLH1 levels following the inhibition of new protein synthesis.

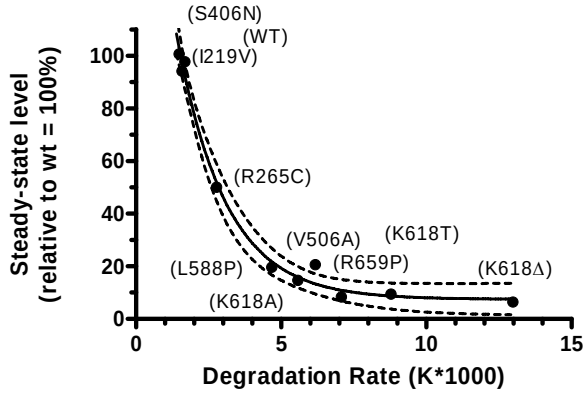
Figure 3.2: hMLH1 levels following the inhibition of new protein synthesis.

Half-life determination of wild-type and variant hMLH1 proteins when expressed in MLH1/PMS2-deficient MEFs. (A) Quantitative immunoblot analysis of lysates spanning 360 minutes following the addition of cycloheximide. (B) Percent of hMLH1 remaining as a function of time (min) after the addition of cycloheximide to transfection reactions. Solid lines indicate the non-linear regression lines fit to the equation [$Y = 100 \times (e^{-kx})$], where k = rate of decay. Dashed line represent the 95% CI.

Table 3.1. Degradation rates and half-lives of wild-type and variant hMLH1.

MLH1 Variant	Degradation Rate (K*1000; % reduction per min)	Half-life (95% Confidence Interval) (min)	Decreased half-life relative to wild-type (p-value)[a]
WT	1.7	401 (342 to 486)	no
I219V	1.6	433 (346 to 579)	no
S406N	1.5	465 (321 to 786)	no
R265C	2.8	252 (220 to 293)	yes (<0.0001)
V506A	6.2	113 (100 to 129)	yes (<0.0001)
L588P	4.7	149 (133 to 168)	yes (<0.0001)
K618A	5.6	124 (110 to 141)	yes (<0.0001)
K618T	8.8	78 (62 to 108)	yes (<0.0001)
K616Δ	13.0	52 (50 to 55)	yes (<0.0001)
R659P	7.1	97 (87 to 110)	yes (<0.0001)

[a] P-value determined by F-test comparison of one-phase exponential decay.



Curve fit: Y = S/X

Y = steady-state level of MLH1 (wt or variant)
S = constant rate of synthesis
X = variable rate of degradation of MLH1 (wt or variant)

Figure 3.3: Relationship of degradation rate and steady-state hMLH1 levels.

The relative steady-state level of hMLH1 protein in cells transfected with 1 μg expression vector plotted as a function of the calculated variant protein degradation rate. The solid line represents the non-linear regression ($r^2 = 0.99$) with the dashed lines representing the 95% CI.

CHAPTER 4: FUNCTIONAL ANALYSIS OF hMLH1 VARIANTS

4.1 Introduction:

To understand the relative contribution of different phenotypes of MMR-deficiencies to increased risk of cancer, one goal of the Buermeyer laboratory is to determine the phenotypic consequences of point mutations in the gene for *hMLH1*. Our approach is to use complementation of MLH1-deficient human or mouse cell lines, using transfection to introduce cDNA's for wild-type or variant hMLH1. Functional assays then compare the phenotype of wild-type or variant-expressing cells in order to assess mismatch repair capacity.

Functional assays on a limited number of hMLH1 variants have previously been performed using cell lines derived from individual clones of transfected cells expressing either wild-type or variant hMLH1 (Mohd et al 2006, and unpublished observations). However, the use of clonal lines is time consuming and labor intensive, and therefore generally only examines one or a limited number of clones, the phenotype of which can be influenced by second site mutations. Using a pooled approach designed to examine the behavior of multiple clones simultaneously, we investigated the effect of hMLH1 mutations on protein function. Several specific questions were addressed: Is a pooled approach effective for evaluating mismatch repair activity? As previous studies in the Buermeyer lab (see Chapter 3 for example) showed that many hMLH1 mutations can affect hMLH1 protein stability and result in

lowered steady-state protein levels, does such a decrease in hMLH1 stability affect phenotype? Conversely, are variants previously shown not to exhibit reduced stability or poor interaction with PMS2 functional for mismatch repair? How sensitive is the assay for detection of mismatch repair deficiencies in previously described controversial or weakly penetrant variants?

4.2 Results and Discussion

MLH1-deficient cells grown in culture were transfected to stably express either wild-type or variant hMLH1; resulting clones were pooled and assessed for: (1) hMLH1 and mPMS2 levels, (2) the frequency of spontaneous mutations at the ouabain-resistance locus, a measure of MMR-dependent correction of spontaneous replication errors, and (3) relative survival following exposure to 6-thioguanine (6-TG), a measure of the MMR-dependent activation of a cytotoxic response to a genotoxin.

Previous work (Buermeyer et al 1999; Mohd et al 2006) demonstrated that hMLH1 expression in MLH1-deficient mouse embryonic fibroblast cells (MEFs) could restore MMR activity. In these experiments, individual clones of transfected cells were chosen for analysis based on a demonstrated steady-state level of expression of hMLH1. This approach was successful, but necessitated significant time and effort to establish and screen the clonal cultures. Initial experiments (e.g. Fig 1 in Buermeyer et al 1999, and unpublished observations) suggested that most clones transfected under our

standard conditions would express sufficient hMLH1 for MMR-complementation. Therefore, to determine the feasibility of using pooled transfected cultures to assess complementation of MMR-deficient cells, MLH1-deficient MEFs were electroporated with either an expression plasmid for wild-type hMLH1 or with the same vector lacking an hMLH1 cDNA insert. Transfected cells in which the vector DNA was stably integrated into the host genome were selected using G-418 sulfate; the resulting G418-resistant colonies were pooled, cultured and examined in functional studies. See Figure 4.1 for an overview of the experimental approach.

4.2.1 hMLH1 restores mismatch repair function to pooled transfected cultures.

To determine the level of accumulation of hMLH1 and mPMS2 in each pooled culture, whole cell lysates were prepared and analyzed by quantitative immunoblotting. Concentrations of hMLH1 and mPMS2 were determined by comparing the chemiluminescent signals for hMLH1 and mPMS2 for each lysate to that of a known standard. Lysates prepared from cultures transfected with wild-type hMLH1 generally showed detectable and variable hMLH1 levels and increased mPMS2 levels compared to cultures transfected with the empty vector which showed low mPMS2 levels and no hMLH1 protein (Figure 4.2). Similar results were seen with cultures transfected to express I219V, a previously described neutral (non-disease associated) polymorphic variant. Previous work with clonal lines and transiently transfected cultures

demonstrated that expression of hMLH1 in MLH1-deficient MEFs increased the steady-state accumulation of PMS2 by stabilizing the normally unstable PMS2 (Mohd et al 2006). Similarly here, as the concentration of hMLH1 (determined by comparison to chemiluminescent signals from similarly prepared lysates with known concentrations of hMLH1 and hPMS2) increased in lysates of different pooled cultures, so too did the mPMS2 concentrations (Figure 4.3). The resulting curve demonstrated a reasonably good fit (r^2 = 0.73) to the modified hyperbolic equation $Y = B_{min} + [(B_{max} - B_{min})(\chi/(K_{1/2} + \chi))]$, where B_{min} is the baseline level of mPMS2 detected in the absence of hMLH1, B_{max} the extrapolated plateau level and $K_{1/2}$ is the level of hMLH1 needed to increase mPMS2 abundance to the midpoint (apparent half-maximal yield) on the fitted hyperbolic curve (Figure 4.3). The apparent stoichiometry of hMLH1 and mPMS2 in lysates was approximately 1.2:1 (at accumulation of 0.05 fmol/μg hMLH1), similar to the 1:1 ratio reported previously using the transient transfection assay (Mohd et al, 2006), and consistent with the formation of heterodimers resulting in increased mPMS2 stability. The apparent modest excess of hMLH1 might be due to the presence of non-hMLH1 expressing cells in the population resulting in a dilution of the signal, or to a slight difference in the recognition of mouse and human PMS2 by the monoclonal antibody used.

To identify the variation in hMLH1 protein expression among individual cells within each pooled culture, immunohistochemistry was performed. Cells were fixed and stained to determine the location and

uniformity of hMLH1 staining among cells within the pooled culture. Cultures transfected with wild-type or I219V hMLH1 plasmid generally showed strong nuclear hMLH1 staining in most cells while cultures transfected with empty vector displayed no detectable staining for hMLH1 (Figure 4.4). Individual cells in wild-type transfected cultures with little or no hMLH1 staining were apparent, and generally accounted for <5% of cells examined. We conclude that expression of hMLH1 in pooled cultures of transfected MLH1-deficient MEFs generally is present in a sufficient percentage of cells, and at a sufficient steady-state level to demonstrate stabilization of endogenous mPMS2, and to warrant additional functional measures of MMR capacity.

Two functional measurements were performed to assess the capacity of hMLH1 expression to complement pooled cultures of MLH1-deficient MEFs. First, the ability to prevent spontaneous base substitution mutations was determined by measuring the frequency of ouabain resistant mutants within each pooled culture (Buermeyer et al 1999; Mohd et al 2006). Cells were plated into medium containing ouabain and the mutant frequency (MF) was calculated based on the number of resistant colonies and the total number of cells plated after correcting for plating efficiency. Secondly, the ability to trigger a MMR-dependent cytotoxic response to a genotoxin was assessed by measuring the relative survival of each culture to a 24 hour exposure to 6-TG.

Among cultures transfected with wild-type hMLH1 there was a range of responses observed in both measurements. Generally, transfection with 20 μg wild-type or I219V hMLH1 expression vectors restored the cultures' ability

to induce a cytotoxic response to 6-TG (median survival was 8.9% and 4.1% for wild-type and I219V, respectively; Figure 4.5A), and reduced the accumulation of ouabain^R mutants (median MF was 4.0 x 10^{-7} and 7.0 x 10^{-7} for wild-type and I219V, respectively; Figure 4.5B). In contrast, transfection with empty vector did not restore MMR function; cell survival remained high (nearly 100%; Figure 4.5A CMV) following exposure to 6-TG, and ouabain^R MF also remained high (median MF was 89.3 x 10^{-7}; Figure 4.5B CMV). For both the cytotoxic response and ouabain^R MF, the phenotype of the pooled cultures transfected to express hMLH1 generally was similar to previous reports of complementation of MLH1-deficient MEFs by expression of hMLH1 in clonal cultures. (Buermeyer et al 1999; Mohd et al 2006).

To determine if there was a correlation between the functional responses of transfected cultures and the steady-state levels of hMLH1, additional transfections were performed using decreased amounts of expression plasmid for hMLH1. The goal was to generate cultures with reduced accumulation of wild-type hMLH1 to determine if reduced hMLH1 levels would limit the functional responses. Transfection with half or one-tenth the amount of expression plasmid for hMLH1 decreased cytotoxic responses to 6-TG exposure relative to cultures transfected with 20 μg plasmid. Cultures transfected with 10 μg hMLH1 vector generally displayed an intermediate response to 6-TG, consistent with a partial MMR capacity, whereas cultures transfected with 2 μg hMLH1 vector were not significantly different from empty-vector transfected cultures (Figure 4.5A). In contrast to

the 6-TG responses, increased ouabain mutant frequencies, similar to empty vector cultures, were observed both in 10 μg and in 2 μg hMLH1 vector-transfected cultures (Figure 4.5B).

To investigate the quantitative relationships between hMLH1 levels and the functional responses, results from all transfections with wild-type hMLH1 vector were combined and analyzed together. The goal was to define dose-response relationships useful for the subsequent analysis of cultures transfected to express hMLH1 variants. For example, the response of cultures expressing relatively unstable hMLH1 variants that fail to accumulate to levels as high as wild-type could be compared specifically to cultures with similar levels of wild-type hMLH1 and/or mPMS2. Variants for which most of the cultures plot above the dose-response line likely would reflect biochemical deficiencies in either the error correction or cytotoxic signaling capabilities of the variant. In contrast, variants for which most of the cultures plot on the dose response (within 95% confidence limits) likely represent variants with biochemical capabilities similar to wild-type. We plotted the relative survival following 6-TG and the measured ouabain MF for each culture versus the average concentration of hMLH1 in the whole cell lysate (Figure 4.6A and Figure 4.6B, respectively). Empty vector transfected cultures were included to establish baseline responses in the absence of hMLH1. As PMS2 stability is dependent on MLH1 (Mohd et al 2006), mPMS2 levels might more accurately reflect the level of MutLα heterodimer in our transfected cultures. Therefore

we performed a similar analysis of mPMS2 concentrations and responses of the pooled cultures (Figures 4.7A and Figure 4.7B, respectively).

As suggested by the initial analysis (Figure 4.5) cell survival in the presence of 6-TG decreased in an apparent dose-dependent manner as the average level of hMLH1 or mPMS2 increased. The resulting curves demonstrated reasonably good fits (r^2= 0.80 and 0.76 for hMLH1 and mPMS2, respectively) to a sigmoidal dose response (with a variable slope) suggesting that the capacity of each pooled culture to trigger a MMR-dependent cytotoxic response to 6-TG is directly related to the level of functional protein present.

In contrast, there was not an obvious dose-response relationship of ouabain^R MFs to average hMLH1 or mPMS2 protein concentrations in lysates of transfected cultures. Although cultures associated with high hMLH1 and mPMS2 concentrations generally displayed low MFs relative to vector-transfected cultures (also see Figure 4.5B) no specific hMLH1 or mPMS2 concentration range that identifies an intermediate MF range could be defined. The data were not well described by a linear, exponential, or sigmoidal dose response (r^2<.05 in each case). It is likely that very high MFs (>1000 x 10^{-7}) that can be generated during the growth of the clones of MLH1-deficient cells (Fig 4.5B and data not shown) interfere with detection of an intermediate phenotype in these mixed cultures. This property of the pooled assay therefore limits the ability to make mechanistic inferences about the error correction capabilities of relatively unstable hMLH1 variants that accumulate to lower levels than wild-type. However, the lack of an obvious intermediate phenotype

also suggests the pooled approach will be very sensitive for detection of partial deficiencies caused by a failure to accumulate sufficient MutLα.

In summary, analysis of pooled cultures transfected to express hMLH1 demonstrate: (1) significant stabilization of mPMS2 and accumulation of MutLα, (2) restoration of a MMR-dependent cytotoxic response to 6-TG, and (3) reduced accumulation of base substitutions leading to ouabain resistance. For the stabilization of mPMS2 and the cytotoxic response to 6-TG, simple quantitative relationships dependent on steady-state hMLH1 levels could be described. For the ouabain resistant MFs, a qualitative assay apparently sensitive to reduced hMLH1 levels was described. We conclude the pooled assay should prove useful for the determination of functional consequences of point mutations in hMLH1.

4.2.2 Does a decrease in hMLH1 stability affect phenotype?

To determine the degree of instability in hMLH1 necessary to cause observable defects in MMR, we analyzed two variants that displayed reduced half-lives in the transient transfection analysis of hMLH1 stability (Chapter 3). Relative to wild-type hMLH1, K618A and K616Δ demonstrated 3-fold and 8-fold increased degradation rates, respectively, resulting in steady-state levels of hMLH1 that were 50-90% reduced, depending on the presence or absence of hPMS2 (Figure 3.1 and Table 3.1).

In pooled cultures transfected to express either 20 μg K618A or K616Δ, hMLH1 staining detected using immunohistochemistry was nuclear in

localization but generally reduced and with greater variability in staining intensity among individual cells compared to wild-type transfected cultures (compare Figure 4.8 with 4.4). The reduced hMLH1 staining seen with K618A and K616Δ cultures was similar to the staining pattern seen with cultures transfected with reduced amounts of wild-type hMLH1 vector (data not shown). Similarly, lysates prepared from K618A and K616Δ cultures showed reduced accumulation of both hMLH1 and mPMS2 relative to wild-type hMLH1 transfected cultures (Figure 4.9).

The reduction in hMLH1 accumulation in K618A and K616Δ cultures (median levels were reduced by approximately 70% and 80% of wild-type, respectively, Figure 4.10A) were similar to reductions seen previously in transiently transfected cultures (50% and 80% reductions in the presence of hPMS2, data not shown). Although both variants demonstrated significant capacity to stabilize hPMS2 in the transient assay, mPMS2 levels in the pooled cultures were similar to vector-transfected cultures for most of the K618A and K616Δ cultures (Figure 4.10B). The apparent difference in PMS2 stabilization in the transient versus the stably transfected pooled cultures likely reflects an increased sensitivity to detect PMS2 stabilization in the transient assay due to the much higher levels of expression possible. We conclude that the relatively reduced stability of hMLH1 variants compared to wild-type hMLH1 as determined in the analysis of transiently transfected cells (Figure 3.1, Table 3.1) is associated with similarly reduced accumulation of hMLH1 and mPMS2 in pooled, stably tranfected cultures.

The effect of reduced hMLH1 and mPMS2 accumulation due to the K618A and K616Δ mutations on response to 6-TG and on the accumulation of ouabain^R mutants was assessed in the pooled cultures as described above and compared to cultures transfected with empty vector or with wild-type hMLH1 vector. Cultures expressing K616Δ displayed 6-TG resistance and ouabain MF's similar to vector-transfected cultures, and significantly elevated relative to wild-type expressing cultures (p-value <0.001) (Figure 4.11). Previously, Raevaara and colleagues (Raevaara et al 2003) demonstrated that MutLα heterodimers constituted with K616Δ and wild-type hPMS2 were functional in an in vitro biochemical assay of MMR-dependent error correction. Similar to our findings here, they also demonstrated reduced steady-state accumulation of K616Δ in human cells and suggested that apparent pathogenicity of the K616Δ mutation was due primarily to insufficient steady-state levels of an otherwise functional protein. We were unable to assess the apparent biochemical capacity in K616Δ by comparison to the concentration-dependent cytotoxic response to 6-TG of wild-type hMLH1 due to the very low levels of K616Δ and mPMS2 in the pooled cultures (Fig 4.12). However, our data do show conclusively that the greatly reduced steady-state accumulation of MutLα due to the instability of the K616Δ mutant is associated with MMR-deficiencies in the pooled cultures, consistent with the conclusions of Raevaara et al.

Results with the K618A cultures were somewhat different. Ouabain^R MF's were significantly elevated relative to wild-type cultures (p-value <0.001), but slightly reduced relative to vector-transfected cultures (p-value

<.001)(Figure 4.11B), suggesting that K618A expressing cultures retain some residual capacity to suppress the accumulation of ouabain^R mutants. Surprisingly, K618A cultures displayed cytotoxic responses to 6-TG similar to wild-type hMLH1 expressing cultures (p-value > 0.05)(Figure 4.11A), despite the significantly reduced levels of K618A and mPMS2 present. Comparison of 6-TG responses in K618A cultures with the sigmoidal dose response of wild-type transfected cultures suggests that K618A expressing cultures have an enhanced ability to induce a cytotoxic response to 6-TG relative to wild-type protein. Thus, K618A may represent a "gain-of-function" mutation, the biochemical activities of which result in greater than expected cytotoxic signaling following genotoxin exposure.

4.2.3 Are variants that appear wild-type in the transient assay functional for MMR?

To investigate the MMR capacity of hMLH1 variants that expressed hMLH1 and stabilized hPMS2 similarly to wild-type in the transient transfection assay, variants K751R and R755W were assessed using the pooled approach. Transfected cultures for both variants produced cultures with uniform, nuclear hMLH1 staining similar to wild-type transfected cells (Figure 4.13). Similarly, lysates of K751R cultures contained hMLH1 and mPMS2 concentrations similar wild-type cultures (Figure 4.14), whereas lysates from R755W transfected cultures generally contained reduced levels of hMLH1 and mPMS2. Reduction in hMLH1 levels in R755W-expressing cultures (median

and mean concentrations were approximately 40% of wild-type hMLH1-expressing cultures, Figure 4.15A) was somewhat surprising, given that our analysis using transient transfections did not detect a significant reduction in stability due to the R755W mutation. It is possible that the stable transfection approach is a more stringent assessment of the stability of hMLH1 variants, possibly due to the reduced and more physiologically relevant levels of expression achieved. Alternately, reductions in R755W levels in the pooled cultures might be due to reduced interaction with mPMS2 versus the hPMS2 expressed in the transient transfections. However, although mPMS2 levels were reduced in R755W-expressing cultures (mean and median mPMS2 concentrations were approximately 60% of wild-type hMLH1 expressing cultures, Figure 4.15B) stabilization of mPMS2 by R755W was similar to stabilization by wild-type hMLH1 (Figure 4.15C), suggesting that the interaction of mPMS2 and R755W is similar to the interaction of the human heterodimer partners and indeed similar to wild-type hMLH1 interaction with mPMS2.

In the functional assays, transfection of the K751R plasmid restored a 6-TG response and reduced the ouabain^{R} MF similar to wild-type-expressing cultures (p-value > .05). In contrast, transfection of the R755W plasmid was unable to restore mismatch repair activity; R755W cultures displayed both 6-TG responses and MFs similar to vector transfected cells (p-value >.05) (Figure 4.16). Comparison of 6-TG survival in R755W-expressing cultures to the hMLH1 and mPMS2 concentration dependent dose responses of wild-type

expressing cultures suggests that levels of MutLα in R755W cultures should have been high enough to detect a partial capacity to trigger a cytotoxic response (Figure 4.17). Thus it appears that R755W is biochemically deficient in triggering the apoptotic response to 6-TG. A similar biochemical deficiency in error correction is suggested by preliminary data using an in vitro MMR assay (unpublished observation). We were unable to quantitatively assess the biochemical capacity of K751R for the 6-TG response, as too few cultures were obtained with intermediate levels of hMLH1 and mPMS2 within the linear portion of the wild-type dose response. Nonetheless, K751R-expressing cultures were well complemented for MMR-deficiencies. In summary, we conclude that variants with similar to wild-type protein levels in the transient assay do not necessarily retain wild-type activity in the functional assays.

4.2.4 How sensitive is the pooled assay for detection of mismatch repair deficiencies?

To determine the sensitivity of the pooled approach to detect potentially modest MMR deficiencies, the functional activity of two previously described controversial or weakly penetrant variants were examined. Both D132H and E578G are suspected pathogenic mutations. However, unlike most identified Lynch Syndrome variants, they are not associated with microsatellite instability (MSI) (Lipkin et al 2004; Liu et al 1999). D132H was identified in Israeli families and showed reduced MLH1 ATPase activity measured using a fusion protein consisting of glutathione S-transferase (GST) fused to the N-

terminus of hMLH1 (Lipkin et al 2004). E578G showed reduced binding to PMS2 in a GST-IVTT assay (Guerrette et al 1999) but was also reported to bind to hPMS2 as well as wild-type in a yeast two-hybrid assay (Kondo et al 2003). When examined by immunohistochemistry, both D132H-expressing and E578G-expressing cultures appeared similar to wild-type cultures, producing fairly uniform nuclear hMLH1 staining (Figure 4.18). Lysates analyzed by quantitative immunobloting for hMLH1 and mPMS2 levels revealed that hMLH1 and mPMS2 accumulation differed slightly from wild-type in one of the two variants examined (Figure 4.19). While D132H-transfected cultures produced hMLH1 and mPMS2 levels similar to wild-type cultures (p-value >.05), E578G-transfected cultures accumulated modestly decreased levels of hMLH1 and PMS2 (p-value <.05), ~60% of the level observed with the transfection of a similar mass of wild-type plasmid. Stabilization of mPMS2 was similar to wild-type capacity for both D132H (not shown) and E578G (Figure 4.20C).

The relative accumulation of hMLH1 and mPMS2 in the pooled cultures for both D132H and E578G was consistent with previous analyses using the transient transfection assay. For example, E578G levels were approximately 70% of wild-type hMLH1 when co-expressed with hPMS2 in the transient assay (unpublished observation) similar to the 60% reported here. In the absence of hPMS2, E578G showed more dramatic reductions in steady-state levels (only 28 % of wild-type), suggesting that interaction with hPMS2 stabilizes E578G (discussed in Chapter 3). The accumulation of E578G and

the stabilization of mPMS2 seen with the pooled cultures, suggests that co-expression with mPMS2 similarly stabilizes E578G.

Functional responses of pooled cultures expressing the variants D132H and E578G generally were similar to wild-type cultures. Both variants clearly were able to support a cytotoxic response to 6-TG (Figure 4.21A) with no evidence from the dose response analysis (Figure 4.22) to suggest reduced activity relative to wild-type hMLH1. Both variants also were capable of suppressing the accumulation of spontaneous ouabain^{R} mutations (Figure 4.21B). Although the median ouabain^{R} MFs (13.3×10^{-7} and 15.8×10^{-7} for D132H and E578G cultures, respectively) were slightly elevated versus wild-type cultures (4.0×10^{-7}) these differences were not statistically significant. It is possible that the analysis of additional cultures would increase the statistical power to a level sufficient to demonstrate whether the modestly elevated MF's with D132H and/or E578G-expressing cultures were significantly different for wild-type hMLH1 cultures. In the absence of such additional data, we conclude the D132H and E578G are similar in MMR capacity to wild-type hMLH1. There remains the possibility that the pooled approach as performed here (with generally <20 cultures per hMLH1 variant) has insufficient sensitivity to detect modest decreases in MMR capacity associated with D132H and E578G.

4.3 Conclusion:

The pooled approach is reasonably effective for analyzing the mismatch repair activity of hMLH1 variants. The transfection of 20 μg wild-type hMLH1 expression vector into MLH1-deficient MEFs generally restored both sensitivity to 6-TG and reduced the spontaneous ouabain^R MF of the pooled population to a level previously described in hMLH1 complemented clonal cultures.

A reduction in the stability of hMLH1 can reduce the cultures' apparent mismatch repair capacity. While K618A was capable of supporting the activation of a cytotoxic response to 6-TG, K616Δ was not. Both K616Δ and K618A were unable to reduce accumulation of ouabain^R mutations observed in MMR-deficient cultures. However, the biochemical capacity of these variants to engage in error correction could not be evaluated with the pooled culture approach.

Variants that appeared wild-type in the transient assay were not necessarily wild-type in terms of protein function. R755W-transfected cultures produced both hMLH1 and mPMS2 at significant levels, and yet the cultures displayed MMR-deficient phenotypes in both functional assays. In contrast, K751R appeared wild-type in both the transient and pooled assays.

The controversial and weakly penetrant variants tested behaved relatively similar to wild-type hMLH1. E578G cultures showed modestly decreased protein levels compared to wild-type, but cytotoxic responses and ouabain^R MFs were similar to wild-type cultures. D132H cultures were not

significantly different than wild-type expressing cultures in any of the measurements. We conclude that analysis of additional pooled cultures would be necessary to detect with confidence any decreased functional activity associated with these or similar potentially weakly penetrant variants.

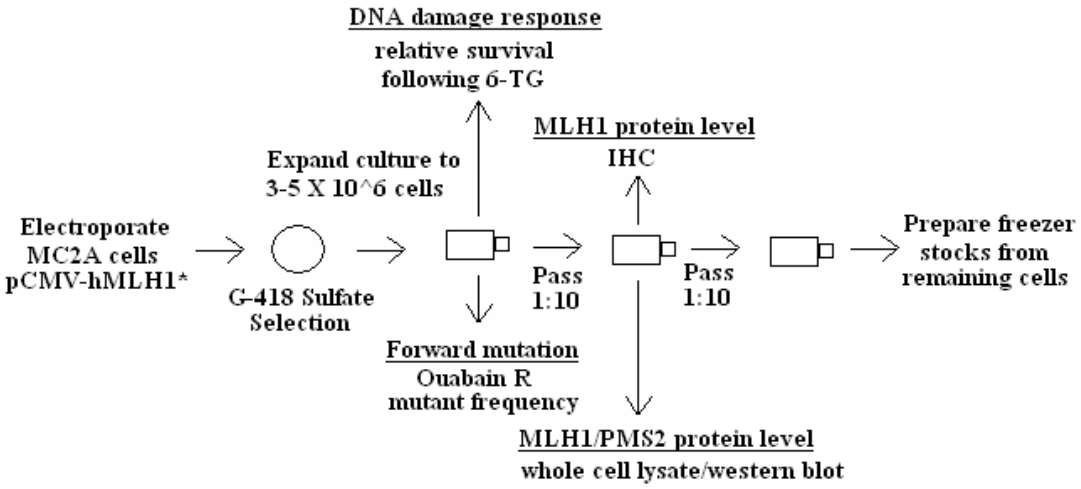


Figure 4.1: Overview of the pooled approach.

MLH1-deficient MEFs (MC2A cells) were electroporated with expression vectors for wild-type or variant hMLH1, selected for G-418 resistance, pooled, expanded and assayed for: a cytotoxic response to 6-TG, accumulation of ouabain^R mutants, hMLH1 and mPMS2 protein distribution and steady-state level.

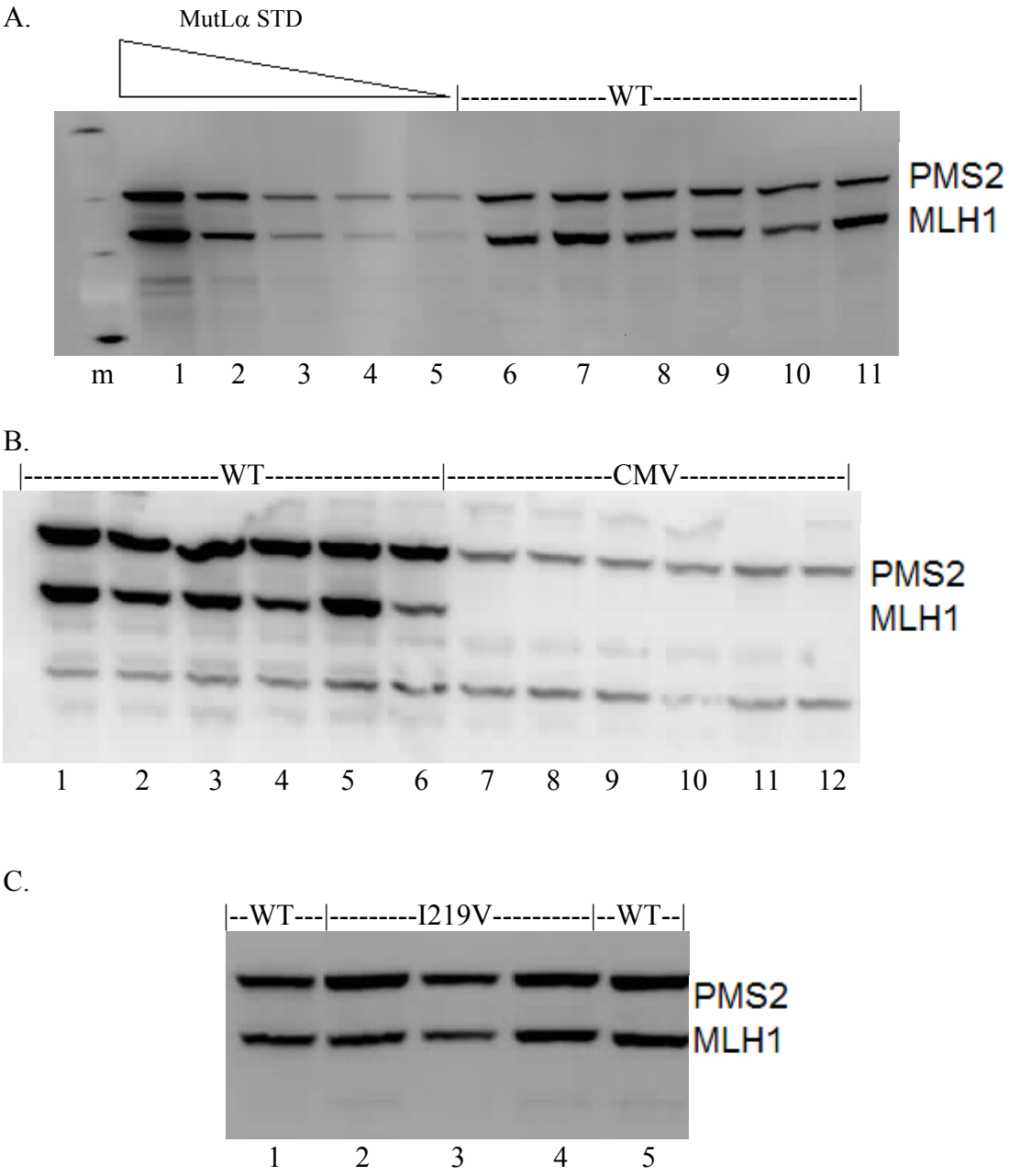


Figure 4.2: hMLH1 and mPMS2 accumulation in pooled transfected cultures.

Representative immunoblot analyses of steady-state hMLH1 and mPMS2 levels in whole cell lysates prepared from MLH1-deficient MEF cultures transfected with either wild-type hMLH1, I219V or empty vector. (**A**) Lanes 1-5 contain MutLα standards (STD) and include 8.13, 2.71, 0.9, 0.452, 0.3 fmol hMLH1 respectively, and 2.81, 0.938, 0.312, 0.156, 0.104 fmol hPMS2 respectively. Lanes 6-11 show representative wild-type (WT) transfected cultures, (**B**) wild-type (WT) and empty vector (CMV) transfected cultures and **(C)** wild-type (WT) and I219V.

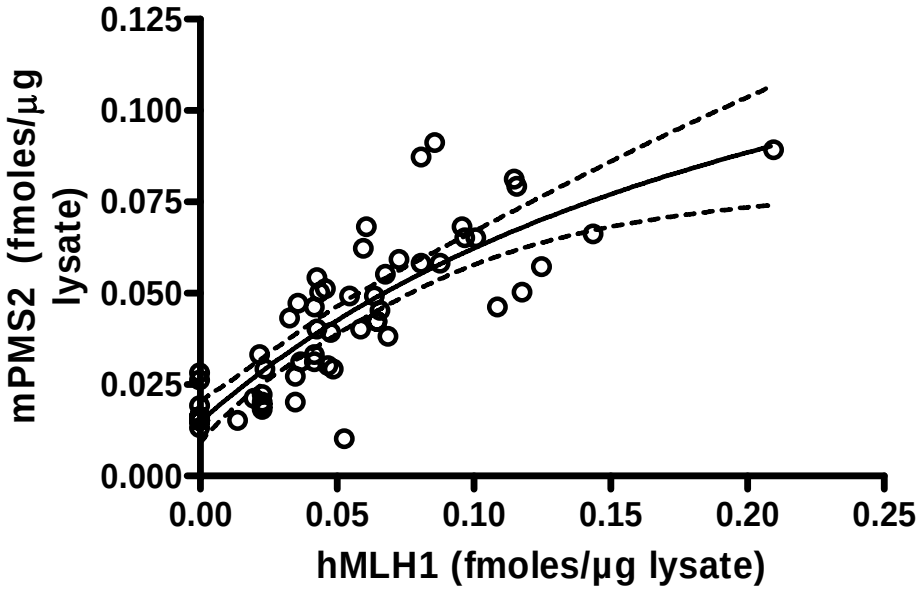


Figure 4.3: Quantitative analysis of hMLH1 and mPMS2 accumulation in pooled cultures.

Compiled results of hMLH1 and mPMS2 concentrations in whole cell lysates prepared from stably tranfected MC2A cultures expressing wild-type hMLH1. Open circles represent individual cultures generated in 6 independent transfections, the solid line the fitted modified hyperbolic regression curve (r^2=0.73) and the dashed line the 95% CI.

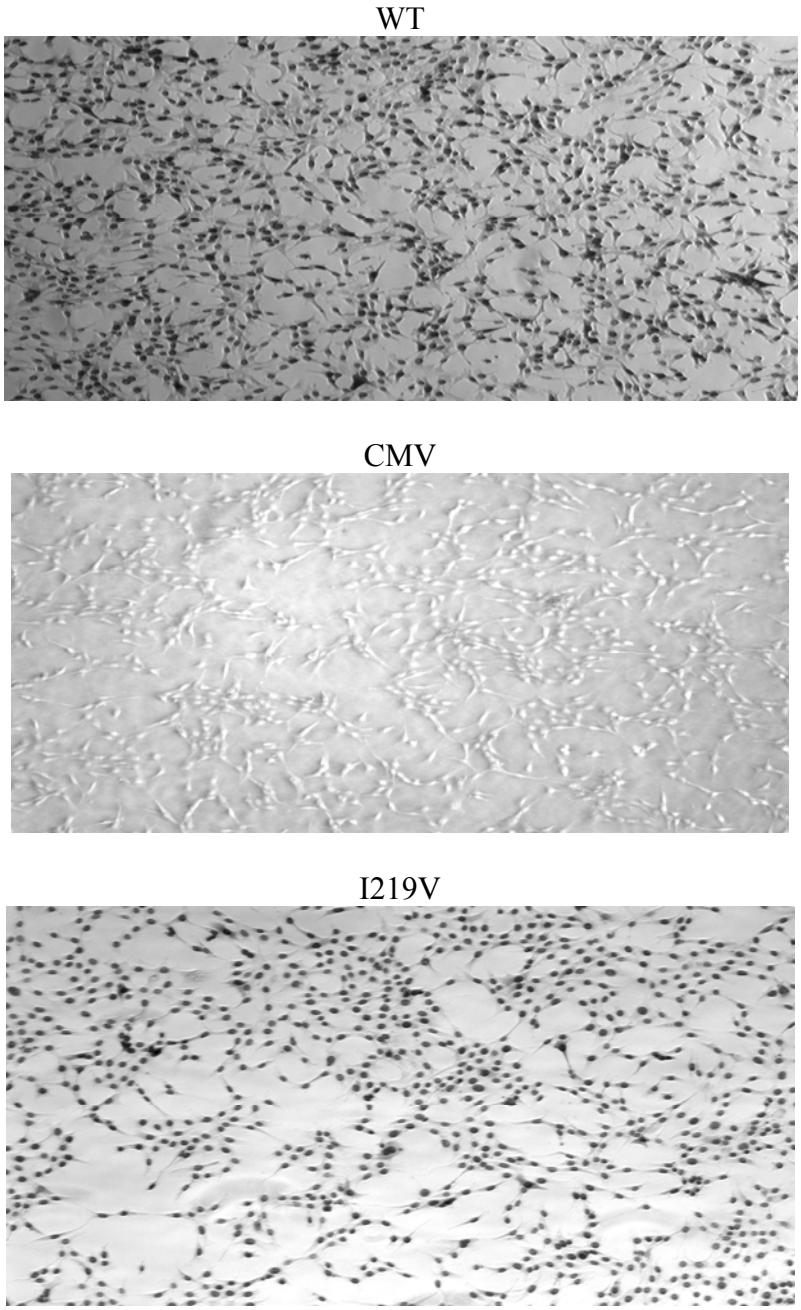
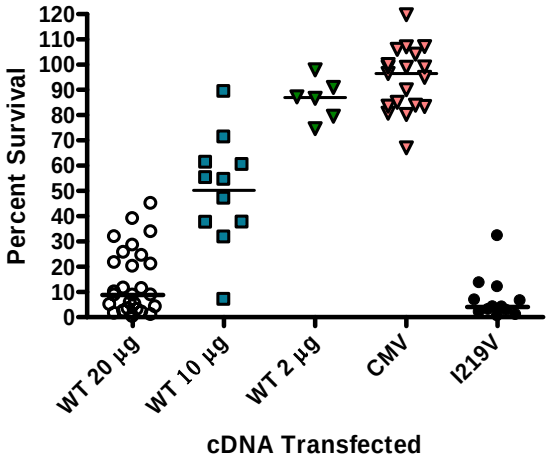


Figure 4.4: Sub-cellular localization and accumulation of hMLH1 in pooled transfected cultures.

hMLH1 accumulation and sub-cellular localization in pooled, transfected cultures of mMlh1-deficient MEFs was determined in fixed cells by immunohistochemistry, and visualized using colormetric staining and light-microscopy. Shown are representative cultures transfected with either an expression vector for wild-type hMLH1 (WT), the polymorphic variant I219V, or "empty" vector lacking the hMLH1 cDNA (CMV)

A.



B.

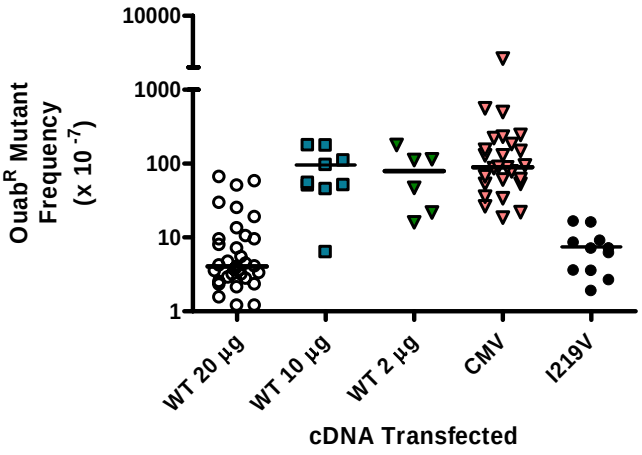
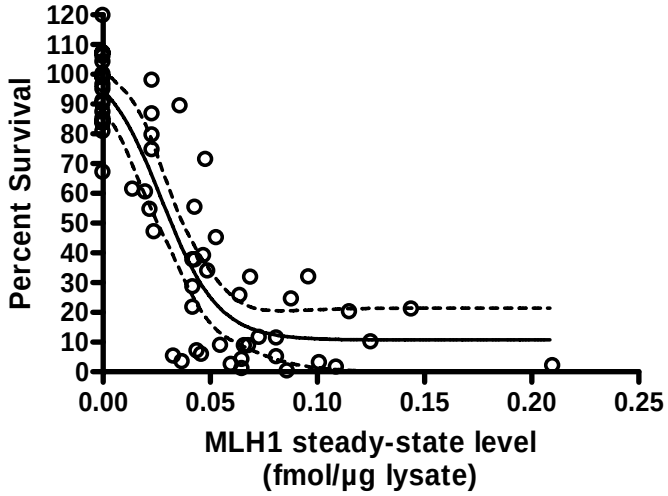


Figure 4.5: Functional responses of wild-type hMLH1 expressing cultures.

Functional responses of MLH1-deficient MEF cultures transfected with either 20 µg wild-type hMLH1 vector (WT 20 µg), 10 µg wild-type hMLH1vector (WT 10 µg), 2 µg wild-type hMLH1 (WT 2 µg), 20 µg empty vector (CMV) or 20 µg polymorphic variant (I219V). Results were compiled from 6 independent transfections. **(A)** 6-TG induced cytotoxicity. Cells were plated at cloning densities and exposed to 1.0 µM 6-TG for 24 hours. Surviving colonies were counted and reported relative to the plating efficiencies of mock-treated cells (set at 100%). Plotted is the measured relative survival for each pooled culture. (**B**) Ouabain^R mutant frequencies (MF). Cells were plated and cultured in the presence of 1mM ouabain. Plotted is the frequency of ouabain resistant mutants calculated based on the number of surviving colonies adjusted for plating efficiency.

A.



B.

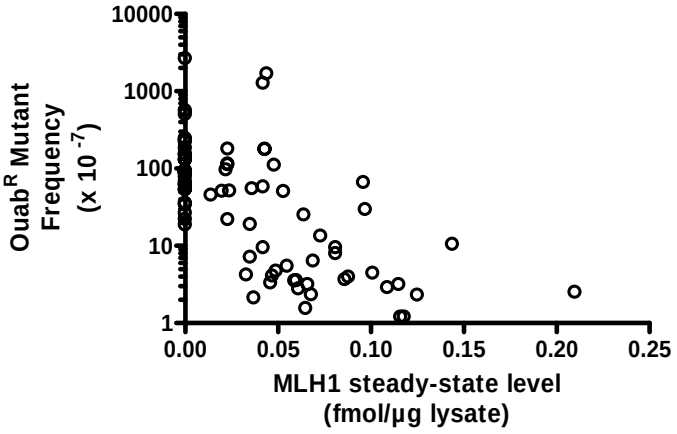
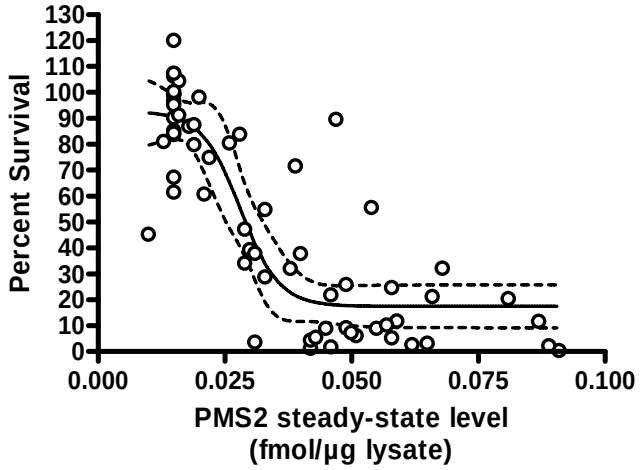


Figure 4.6: Functional responses and average hMLH1 accumulation.

(A) The survival of wild-type transfected cultures following exposure to 6-TG was plotted as a function of the average steady-state hMLH1 concentration observed in lysates of each culture. Open circles represent individual cultures, the solid line the sigmoidal dose response (with variable slope) ($r^2 = 0.80$) and the dashed line the 95% CI. (**B**) Calculated ouabain MF plotted as a function of the average steady-state hMLH1 levels observed in wild-type transfected cultures. Open circles represent individual cultures. Data points did not fit a simple equation but there appeared to be a trend of very low hMLH1 levels being associated with a higher MF than cultures expressing high levels of hMLH1.

A.



B.

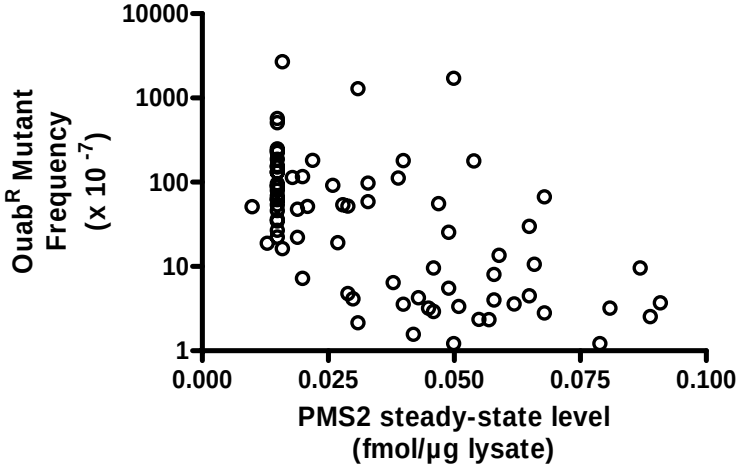
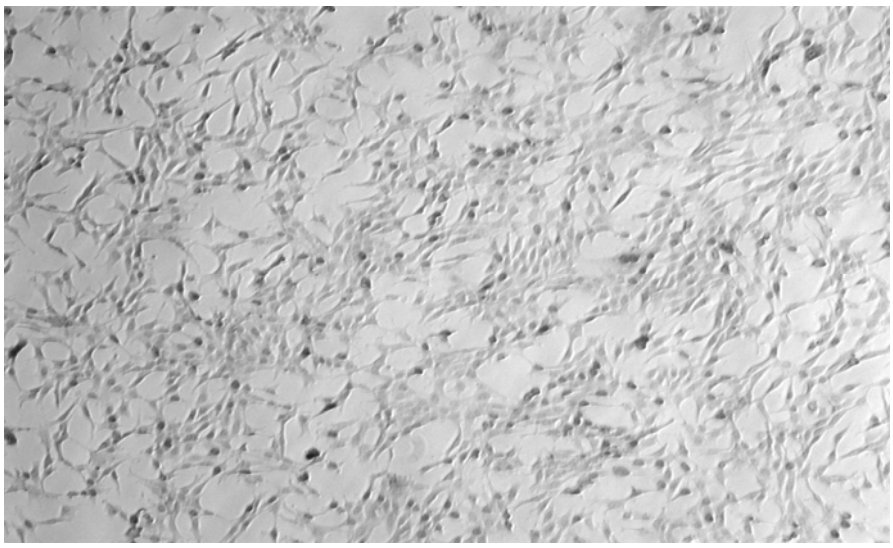


Figure 4.7: Functional responses and average mPMS2 accumulation.

(A) The survival of wild-type transfected cultures following exposure to 6-TG was plotted as a function of the average steady-state mPMS2 concentration observed in lysates of each culture. Open circles represent individual cultures, the solid line the sigmoidal dose response (with variable slope) ($r^2 = 0.76$) and the dashed line the 95% CI. (**B**) Calculated ouabain MF plotted as a function of the average steady-state mPMS2 levels observed in wild-type transfected cultures. Open circles represent individual cultures. Data points did not fit a simple equation but there appeared to be a trend of very low mPMS2 levels being associated with a higher MF than cultures expressing high levels of mPMS2.

A. K618A



B. K616Δ

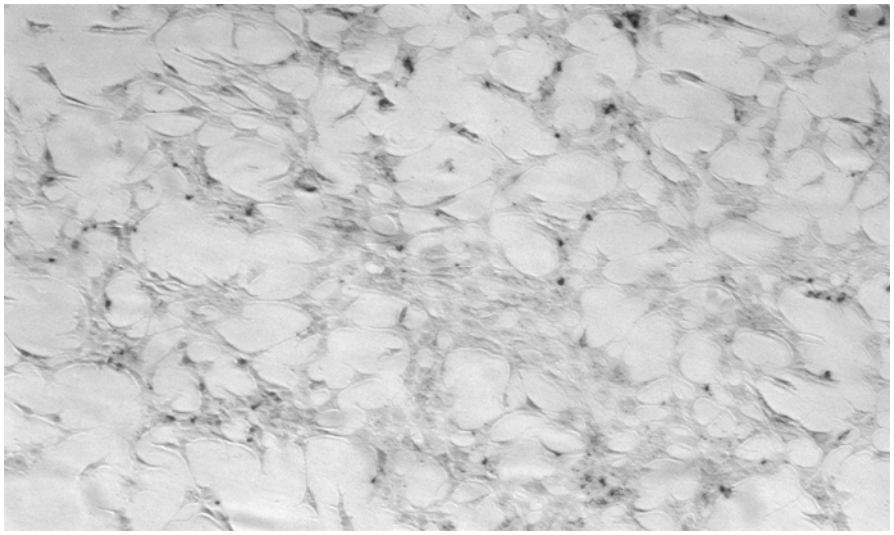


Figure 4.8: Sub-cellular localization and accumulation of representative hMLH1 variants with reduced stability.

Accumulation and localization of hMLH1 variants K618A (**A**) or K616Δ (**B**) expressed in MLH1-deficient MEFs was determined as described in the legend for Figure 4.4.

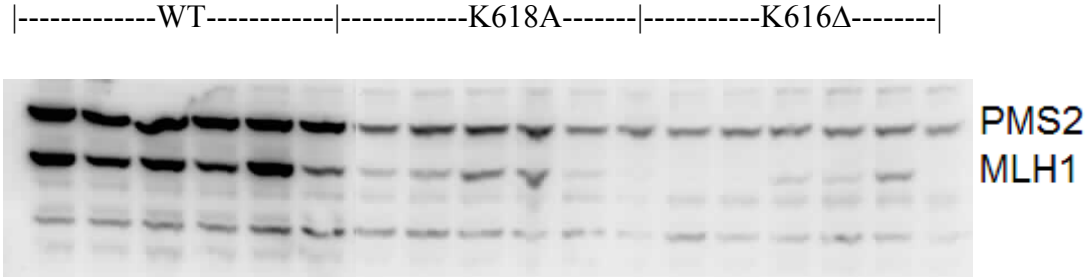
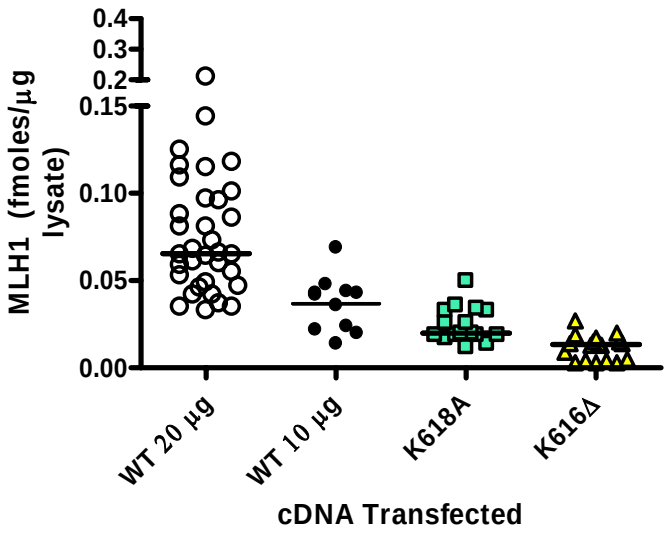


Figure 4.9: hMLH1 and mPMS2 levels of representative reduced stability variants.

Representative immunoblot analyses of steady-state hMLH1 and mPMS2 levels in whole cell lysates prepared from MLH1-deficient MEF cultures transfected with either wild-type (WT), K618A or K616Δ hMLH1 vectors as described in legend of Figure 4.2.

A.



B.

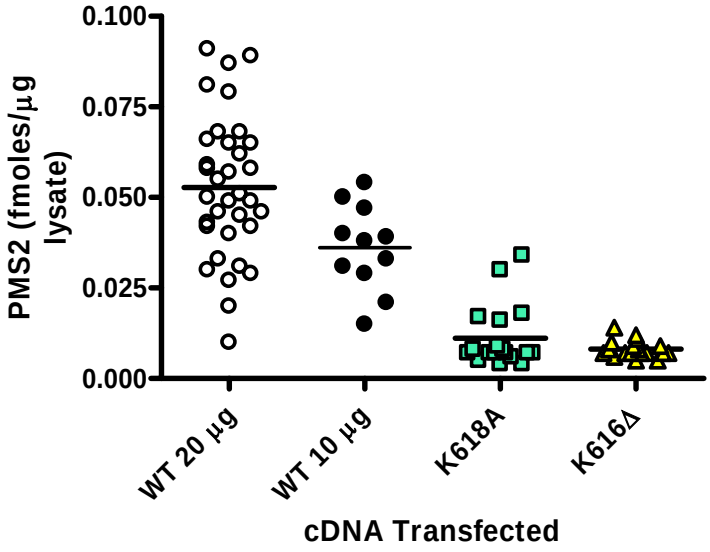
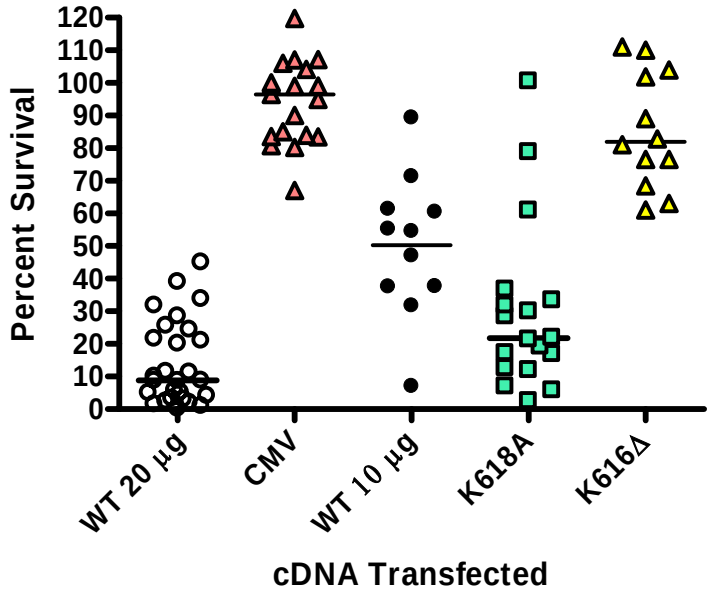


Figure 4.10: Quantitative analysis of hMLH1 and mPMS2 accumulation in cells expressing representative reduced stability variants.

Average (**A**) hMLH1 and (**B**) mPMS2 accumulation in cultures transfected with either 20 µg wild-type (open circles), 10 µg wild-type (solid circles), 20 µg K618A (squares) or 20 µg K616Δ (triangles) hMLH1 expression vectors as determined by quantitative immunoblot analyses. Plotted are the concentrations measured in individual cultures generated in 3-6 independent tranfections as described in legend to Figure 4.3.

A.



B.

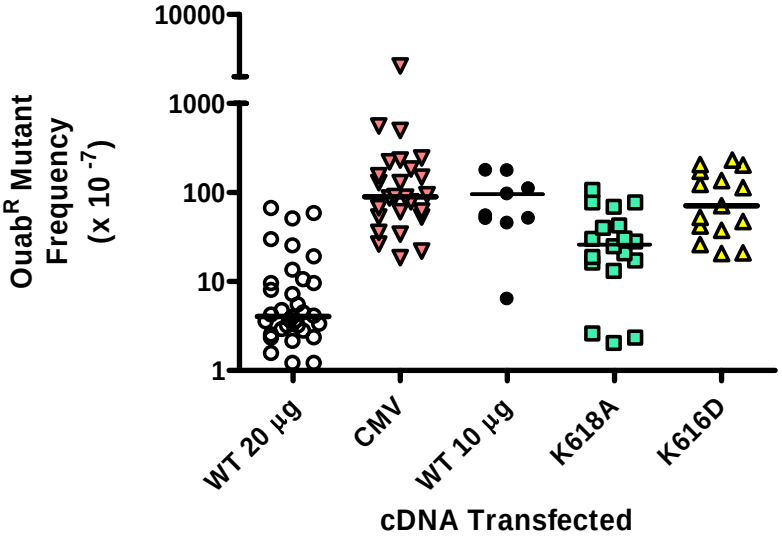
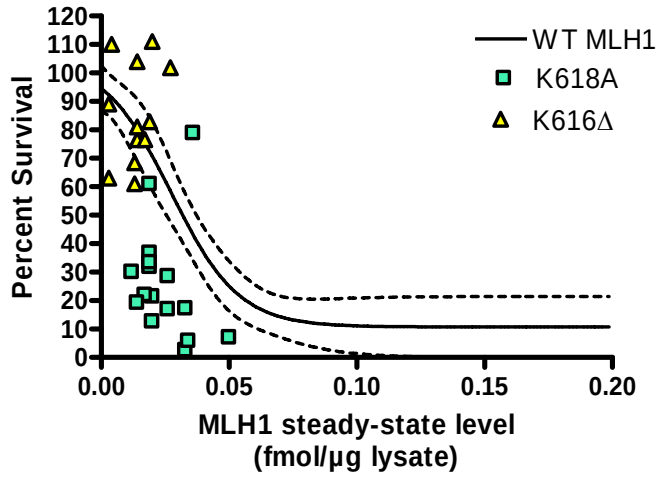


Figure 4.11: Functional responses of cells expressing representative reduced stability variants.

(**A**) Relative 6-TG survival and (**B**) ouabain^R MFs of MC2A cells transfected with either wild-type hMLH1 (WT 20 µg and WT 10 µg), empty vector (CMV), K616Δ or K618A variant hMLH1 expression vectors as determined in functional assays and described in the legend of Figure 4.5.

A.



B.

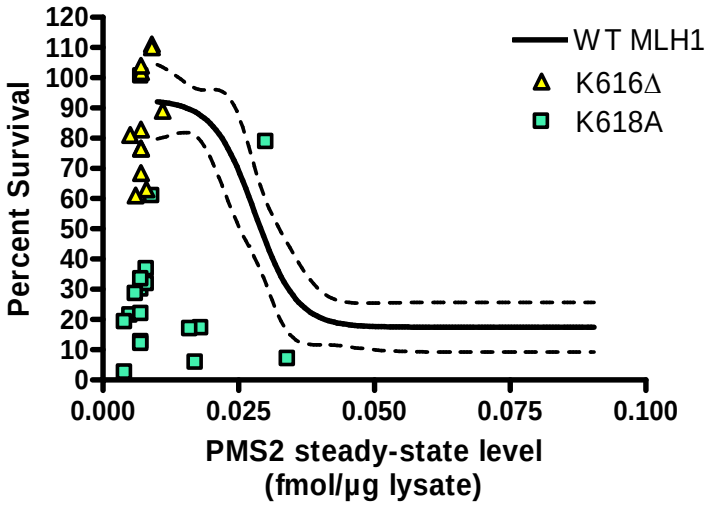
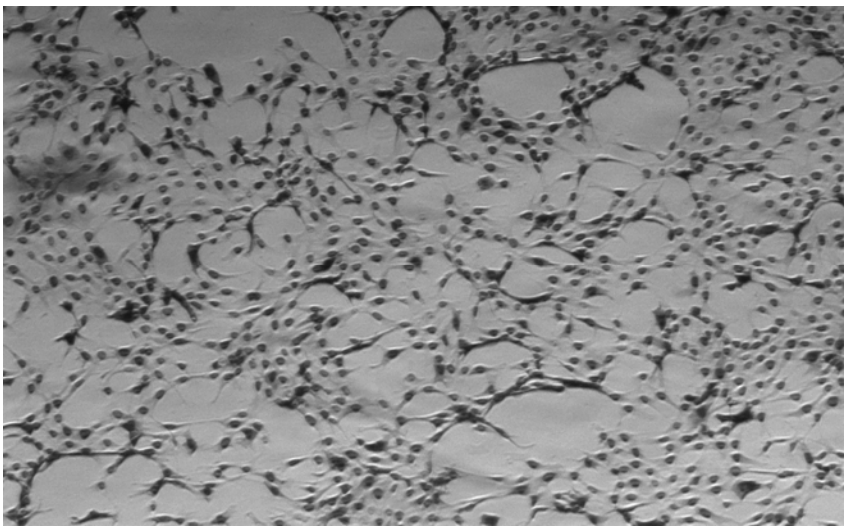


Figure 4.12: Functional responses and average hMLH1/mPMS2 accumulation in cells expressing reduced stability variants.

The survival of pooled cultures expressing K616Δ (triangles) or K618A (squares) hMLH1 following exposure to 6-TG was plotted as a function of the average steady-state **(A)** hMLH1 and **(B)** mPMS2 concentrations observed in lysates. The solid line (reproduced from Figures 4.6A and 4.7A) represents the sigmoidal dose responses (with 95% CI, dashed line) of wild-type expressing cultures.

A. R755W



B. K751R

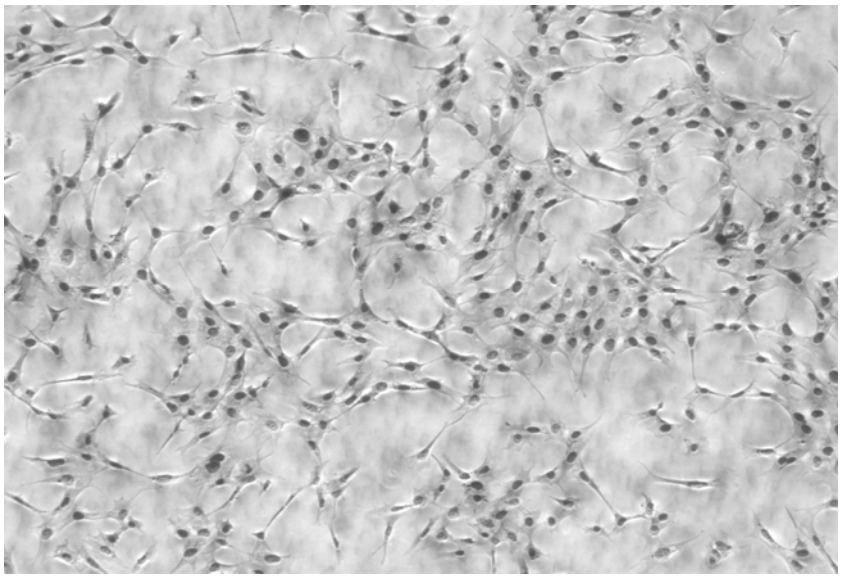


Figure 4.13: Sub-cellular localization and accumulation of representative stable hMLH1 variants.

Accumulation and localization of hMLH1 variants R755W (**A**) or K751R (**B**) expressed in mMLH1-deficient MEFs was determined as described in the legend for Figure 4.4.

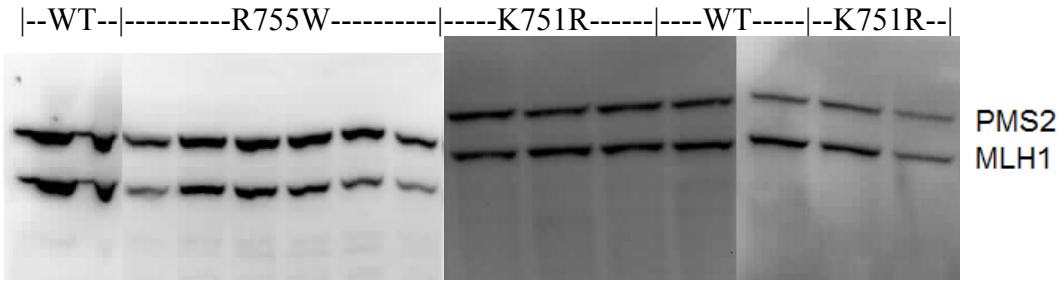


Figure 4.14: hMLH1 and mPMS2 accumulation in cells expressing representative stable variants.

Quantitative immunoblot analyses of hMLH1 and mPMS2 levels in whole cell lysates prepared from MC2A cultures transfected with either wild-type (WT), R755W, or K751R hMLH1 expression vectors.

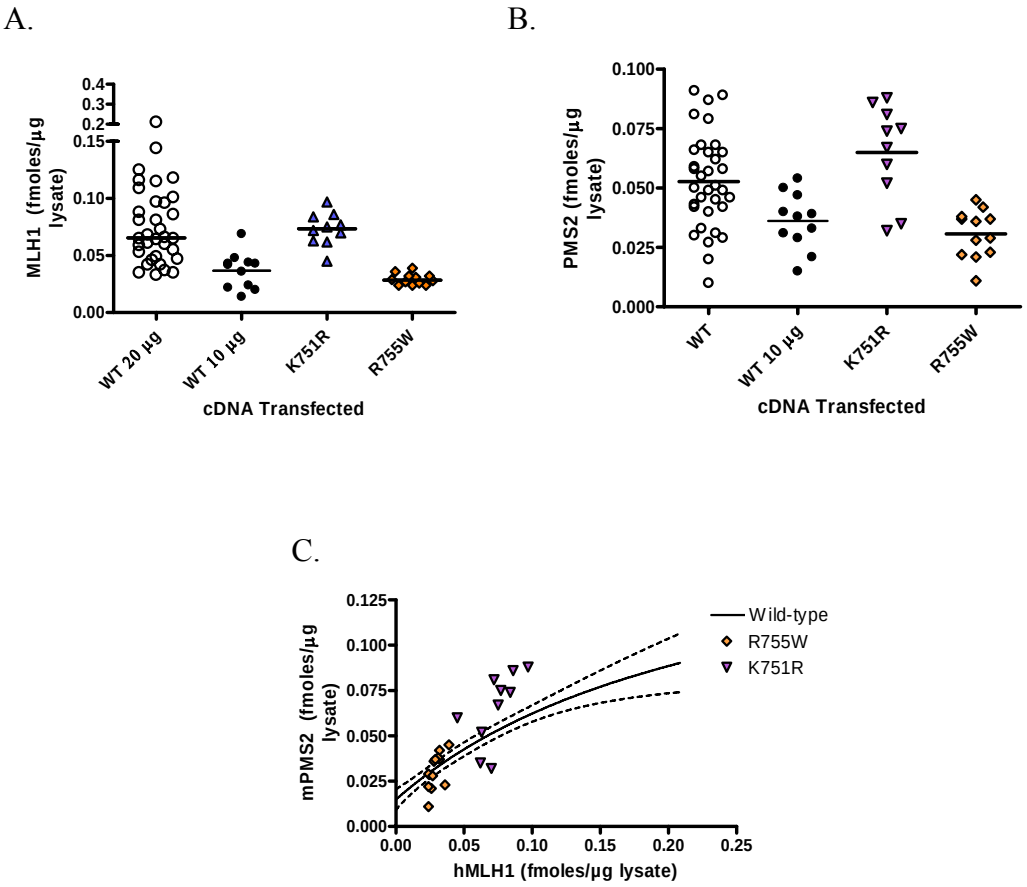
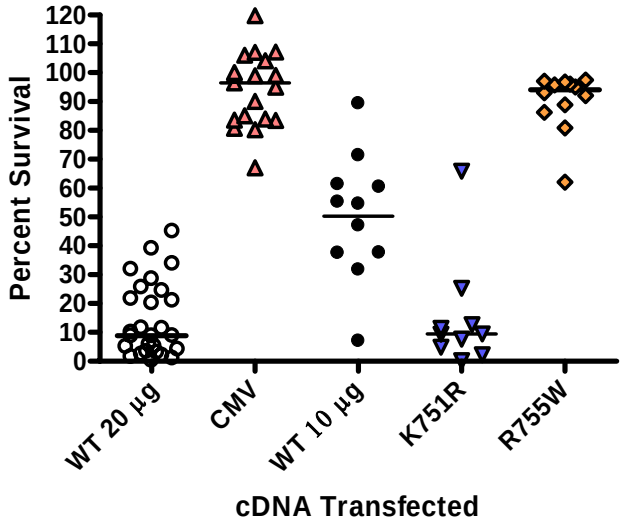


Figure 4.15: Quantitative analysis of hMLH1 and mPMS2 levels in cells expressing representative stable hMLH1 variants.

Average (**A**) hMLH1 and (**B**) mPMS2 accumulation in cultures transfected with either wild-type (WT 20 µg, WT 10 µg), K751R or R755W hMLH1 expression vectors as determined by quantitative immunoblot analysis. **(C)** Stabilization of mPMS2 by R755W (diamonds) and K751R (triangles). The solid line represents the fitted modified hyperbolic regression curve of wild-type hMLH1 expressing cultures and the dashed line the 95% CI (reproduced from Figure 4.3). Points represent individual pooled cultures.

A.



B.

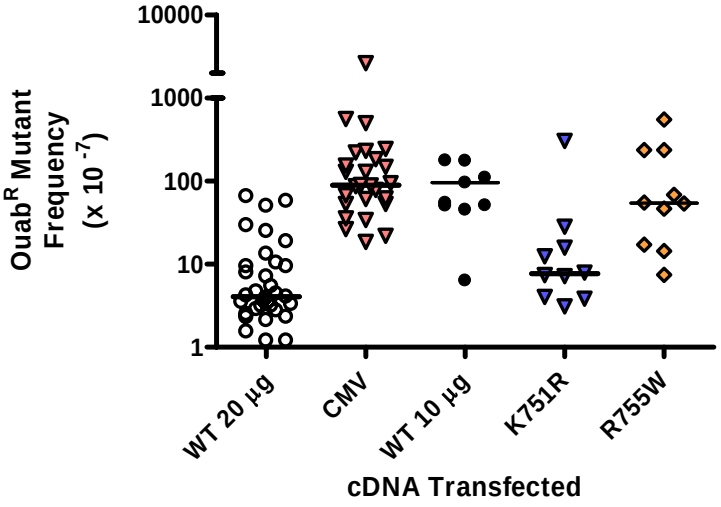
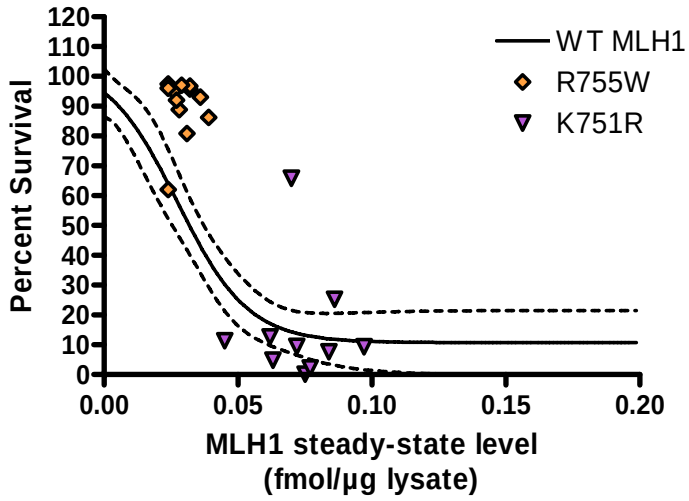


Figure 4.16: Functional responses of cells expressing representative stable MLH1 variants.

(**A**) Relative 6-TG survival and (**B**) ouabain^R MF of MC2A cells transfected with either empty vector, wild-type, K751R or R755W hMLH1 expression vectors as determined in functional assays, as described in Figure 4.5.

A.



B.

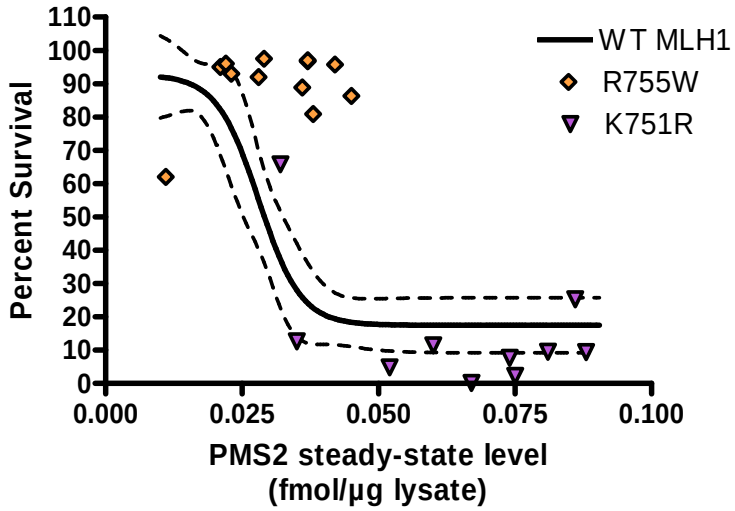


Figure 4.17: Functional responses and hMLH1/mPMS2 levels in cells expressing representative stable hMLH1 variants.

The survival of pooled cultures expressing R755W (diamond) or K751R (triangle) hMLH1 following exposure to 6-TG was plotted as a function of the average steady-state **(A)** hMLH1 and **(B)** mPMS2 concentrations observed in lysates. The solid line (reproduced from Figures 4.6A and 4.7A) represents the sigmoidal dose responses (with 95% CI, dashed line) of wild-type expressing cultures.

A. E578G

B. D132H

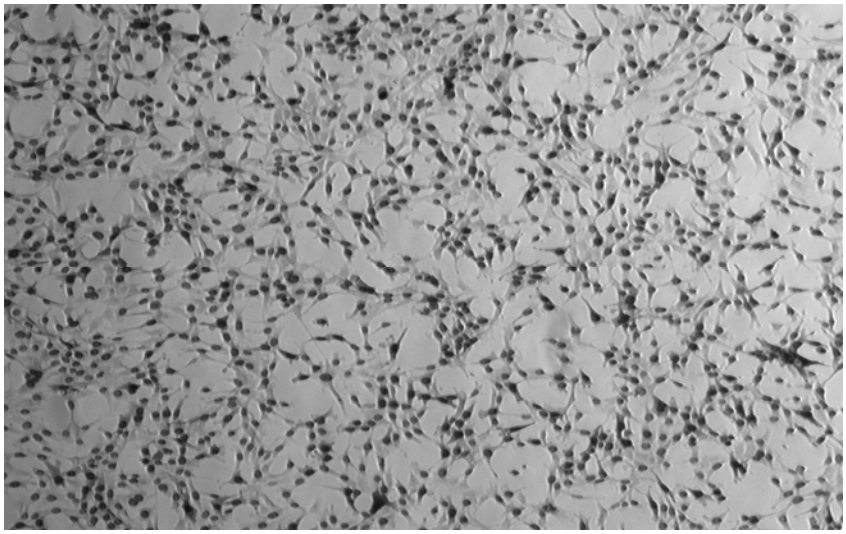


Figure 4.18: Sub-cellular localization and accumulation of representative controversial and weakly penetrant hMLH1 variants.

Accumulation and localization of hMLH1 variants E578G (**A**) or D132H (**B**) in mMLH1-deficient MEFs was determined as described in the legend for Figure 4.4.

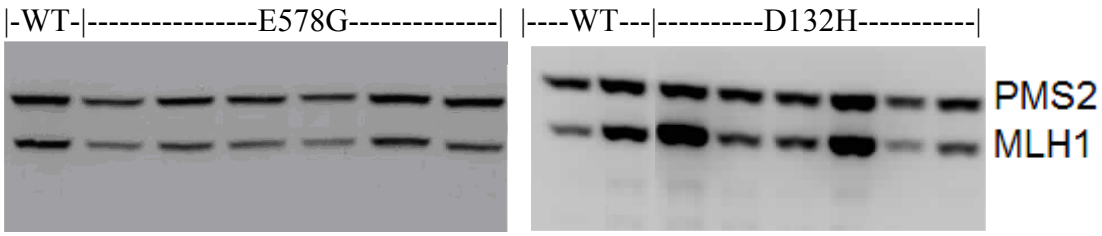


Figure 4.19: hMLH1 and mPMS2 accumulation in cells expressing representative controversial and weakly penetrant hMLH1 variants.

Quantitative immunoblot analysis of hMLH1 and mPMS2 levels in whole cell lysates prepared from MC2A cultures transfected with either wild-type (WT), E578G, or D132H hMLH1 expression vectors.

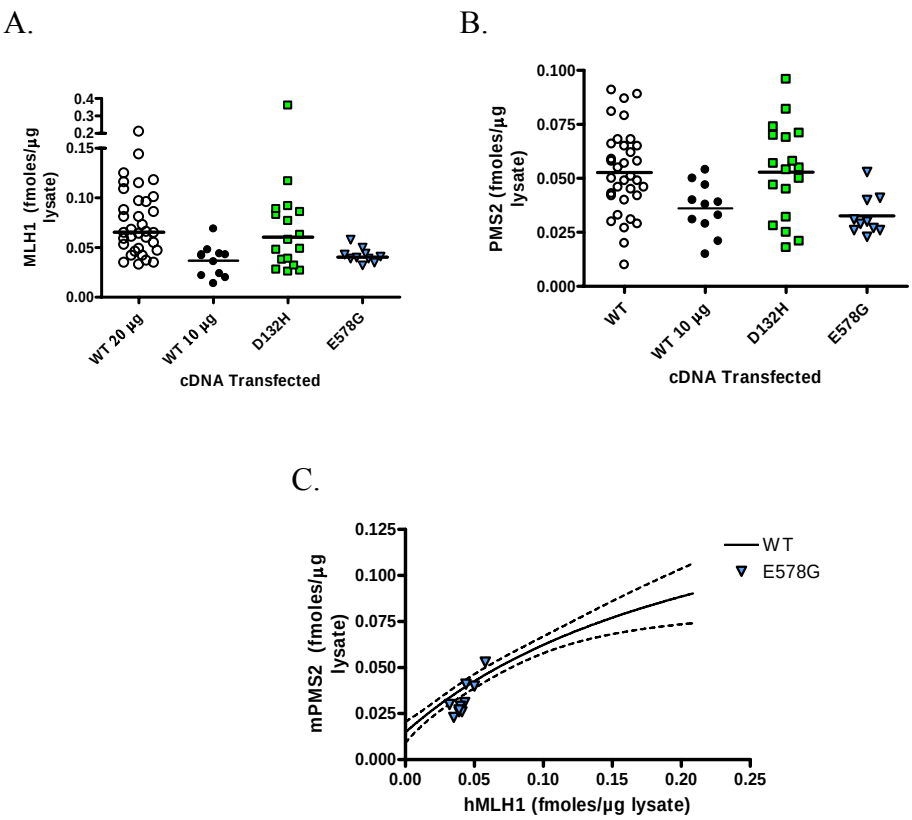
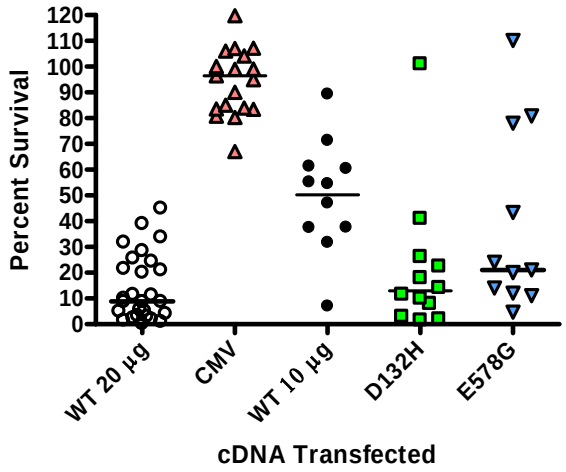


Figure 4.20: Quantitative analysis of hMLH1 and mPMS2 levels in cells expressing representative controversial and weakly penetrant hMLH1 variants.

Average (**A**) hMLH1 and (**B**) mPMS2 levels in cultures transfected with either wild-type, D132H or E578G hMLH1 expression vectors as determined by quantitative immunoblot analysis. **(C)** Stabilization of mPMS2 by E578G (triangles). The solid line represents the fitted wild-type modified hyperbolic regression curve and the dashed line the 95% CI (reproduced from Figure 4.3). Points represent individual pooled cultures.

A.



B.

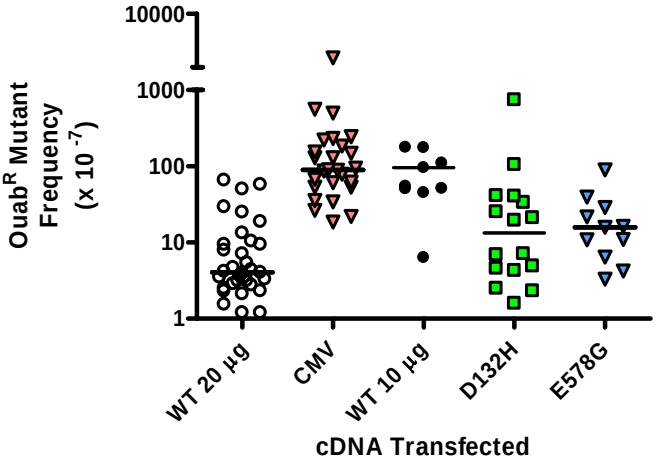
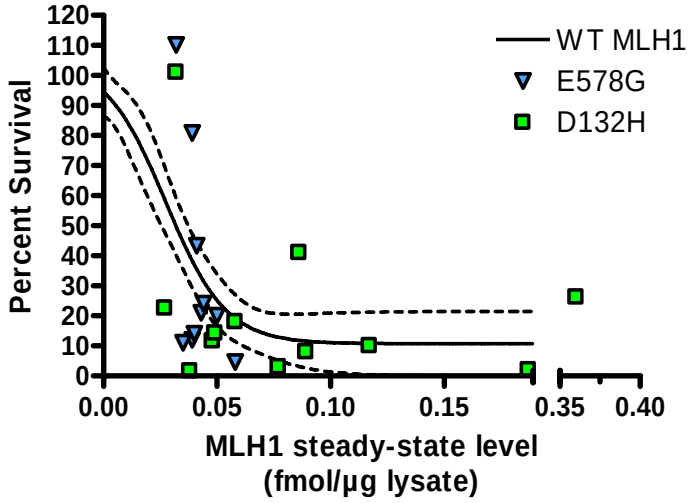


Figure 4.21: Functional responses of cells expressing representative controversial and weakly penetrant hMLH1 variants.

(**A**) Relative 6-TG survival and (**B**) ouabain MF of MC2A cells transfected with either empty vector, wild-type, D132H or E578G hMLH1 expression vectors as determined in functional assays.

A.



B.

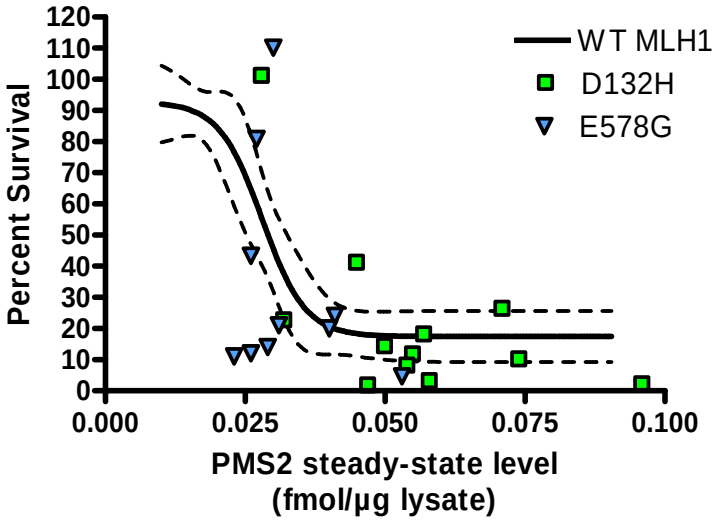


Figure 4.22: Functional responses and hMLH1/mPMS2 levels in cells expressing controversial and weakly penetrant hMLh1 variants.

The survival of pooled cultures expressing D132H (squares) or E578G (triangles) hMLH1 following exposure to 6-TG was plotted as a function of the average steady-state **(A)** hMLH1 and **(B)** mPMS2 concentrations observed in lysates. The solid line (reproduced from Figures 4.6A and 4.7A) represents the sigmoidal dose responses (with 95% CI, dashed line) of wild-type expressing cultures.

CHAPTER 5: GENERAL DISCUSSION

5.1 Introduction

There have been many approaches with the goal of identifying the effects of mutations in hMLH1 on DNA mismatch repair function. Generally, these approaches have evaluated either protein-protein interactions or variant protein repair activities. Common methods include yeast two-hybrid binding assays, GST pulldown assays, analysis of protein sub-cellular localization or functional studies in yeast systems, human systems, or cell free extracts. This chapter discusses the strengths and weakness of our half-life and pooled approaches compared to three other methods that were also developed to classify the pathogenicity of hMLH1 variants.

5.2 Comparison of approach to other methods

5.2.1 Half-life and pooled approach:

The half-life and pooled approaches described in this thesis together assess the hMLH1 variants for protein stability, interaction with and stabilization of PMS2 and MMR function in transfected mouse cells deficient for MLH1. By investigating both variant protein stability and its ability to function in mutation avoidance and ability to induce a cytotoxic response, we can assess potential pathogenic variants and determine a likely cause for any failure to function like wild-type.

Using these approaches we determined the half-lives and degradation rates of 9 variants, then demonstrated a simple quantitative relationship between degradation rate and yield. We found that a measurement of the relative yield alone is a sufficient and sensitive measure of decreased hMLH1 stability. In both assays polymorphic variants I219V and S406N behaved similar to wild-type. Results from the pooled approach demonstrated that variants K618A and K616Δ with reduced stability also show partial to complete MMR deficiencies. Therefore, it is likely that other variants with similarly reduced stability would also show MMR defects. Both controversial variants, D132H and E578G, examined in the transient and pooled assays behaved similarly to wild-type. This suggests that these and other previously classified weakly penetrant variants may not be deficient for MMR and may have been identified in cancers due to a linkage to another more pathogenic mutation. To confirm this conclusion, more replicates should be analyzed. Stable hMLH1 variants that are capable of stabilizing hPMS2 in the transient transfection assay may or may not function like wild-type hMLH1 in functional assays. While both K751R and R755W appeared similar to wild-type in the transient assay, only K751R-expressing cultures were able to restore a wild-type MMR phenotype. This suggests that biochemically inactive variants are not necessarily identified and eliminated from cells.

There are both advantages and disadvantages to using the half-life and pooled approaches to evaluate the effect of mutations in hMLH1. Assessing the behavior of pooled cultures, as opposed to individual clones, requires less

time and measures the activity of multiple populations consisting of 35-50 individual clones. The analysis of multiple clones greatly reduces the influence of second site mutations that arise in any individual clone and that can influence phenotypic behavior. By comparing functional responses to protein levels and variant protein stability, an explanation for variant-expressing cells mutant phenotype may be inferred. Disadvantages of this approach include the use of human MLH1 proteins expressed in mouse cells, which introduces the possibility that species differences could influence behavior in the assay. Also, the ouabain^{R} mutant frequency assay is relatively insensitive to subtle changes in mutation rate and did not display an easily definable hMLH1 concentration-dependent dose response.

5.2.2 Yeast dominant negative assay:

Shimodaira et al (1998) examined the expression of variant human MMR genes in yeast to evaluate the effect of hMLH1 mutations. When expressed at high levels in MMR-proficient *Saccharomyces cerevisiae*, wild-type hMLH1 cDNA produced a dominant mutator effect by interfering with yeast MMR and causing an increased mutation rate that could be measured using a frameshift mutation reporter plasmid. Introduction of a mutation in the reporter generates a functional LacZ gene which can be ascertained as blue colonies on appropriate indicator plates. The ability of hMLH1 to interfere with yeast MMR presumably involves protein-protein interactions with yeast MMR proteins. However the identity of the interacting proteins and the nature

of the interaction remain unclear. hMLH1 variants were assessed for their ability to produce blue colonies similar to wild-type hMLH1. Variants were then classified as either potentially harmful mutations (if they failed to produce a dominant negative effect) or polymorphism (if they behaved similar to wild-type).

Shimodaira et al (1998) examined 27 sequence variants in hMLH1, 25 of which had been reported as cancer-causing mutations. They determined that 4 of the 27 variants, two reported polymorphisms (I219V and R265H) and two reported mutations (V326A and Q542L) behaved similarly to wild-type whereas the other 23 inactivated the dominant negative effect. Contrary to our transient assay and pooled approach, which identify E578G as similar to wild-type in functional assays, Shimodaira et al (1998) concluded E578G to have a reduced mutator effect. Shimodaira et al (1998) conclude that most hMLH1 missense mutations alter normal hMLH1 function.

There are advantages and disadvantages to using the approaches described by Shimodaira et al (1998) to identify pathogenic hMLH1 variants. This assay provides a rapid screening method useful for classifying a wide range of hMLH1 variants over a short period of time. The primary disadvantage is that the mechanism of the dominant negative effect is not known. Thus the mechanism by which mutations in hMLH1 interfere with the dominant negative effect is also not known. The assay presumably will identify mutants that destabilize hMLH1 in yeast, or that interfere with the critical protein-protein interactions necessary for the dominant effect. This approach

may also be ineffective for identifying mutations that alter protein function unrelated to the dominant negative effect. The dominant mutator effect of wild-type hMLH1 protein in yeast cells is useful for identifying variants that behave differently from wild-type protein in a foreign system but because of its limitations, it cannot be used as the sole method to determining variant behavior.

5.2.3 Protein-protein interactions approach:

Kondo et al (2003) used two approaches to investigate protein-protein interactions of wild-type or variant hMLH1 with either wild-type hPMS2 or wild-type hEXO1. Using a yeast two-hybrid assay, researchers determined relative protein binding affinities of wild-type and variant hMLH1 by measuring β-galactosidase (β-gal) activity. If the proteins bound, the activation domain fused to the PMS2 or EXO1 protein was brought into close proximity with the DNA binding domain fused to full-length MLH1 protein and transcription of the gene encoding β-gal was activated. If the proteins did not interact, the enzyme was not made. Kondo et al (2003) also investigated protein binding using a Glutathione *S*-transferase (GST) binding assay. Variant or wild-type hMLH1 proteins produced in vitro were added to GST-fused hPMS2 absorbed onto glutathione beads, incubated, washed to remove unbound hMLH1 protein, with the bound hMLH1 protein levels subsequently quantified by SDS-PAGE analysis. Through the use of protein binding assays,

Kondo was able to categorize variants as either similar to wild-type or not under these conditions.

Kondo et al (2003) examined 23 hMLH1 germline mutations and nine identified polymorphisms. Based on their analysis they concluded that 18 of the 32 variant were likely pathogenic and causative for colorectal cancer. All nine of the presumptive polymorphic variants behaved similar to wild-type. They concluded that the yeast two-hybrid system is effective for identifying protein interaction deficiencies in MLH1 variants. They proposed that even mutations outside the interaction domain affect binding due to conformational changes which ultimately effect the C-terminal interaction domain.

There are both advantages and disadvantages to using yeast binding assays to predict variant hMLH1 behavior in humans. Advantages of this approach include the ability to screen a wide range of variants in a short period of time. Disadvantages of this approach include the use of human proteins in a yeast system. Modifications that occur in human cells could be absent in yeast resulting in false negatives. This system also examines proteins that are over-expressed which could lead to non-specific interactions and false positives. In vitro studies also ignore protein function and other cellular influences that are present in human cells. While this approach is useful for categorizing hMLH1 variants, it is limited to being only one piece of the variant classification puzzle, which requires other assays to come to any firm conclusions on variant effects in humans.

5.2.4 Phenotypic complementation in human carcinoma cells:

Blasi et al (2006) used a human cell-based approach to identify the effects of variant hMLH1 proteins on MMR function. Human ovarian carcinoma cells lacking expression of hMLH1 were transfected to express either wild-type or variant hMLH1 cDNAs, individual clones were then cultured and assessed for forward mutation rate using the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus, cytotoxic response to methylating agents, microsatellite instability and steady-state levels of 8-oxoguanine. The same line was used in a similar analysis of individual clones expressing a truncated, MMR-deficient hMLH1 variant (L749X) in the Buermeyer laboratory (Mohd et al, 2006). Pathogenicity of the hMLH1 variants was then assessed based on independent conclusions from the various functional assays.

Blasi et al (2006) examined six hMLH1 variants and determined four (G67R, G224D, K618A and N635S) to be likely pathogenic due to deficiencies in suppression of spontaneous mutations and cytotoxic response to genotoxins. They conclude the other two previously identified polymorphisms (I219L, I219V) to function similarly to wild-type in their assay. Contrary to our findings, in a clonally derived line they concluded K618A to be expressed at a similar level to wild-type level and likely to be pathogenic due to biochemical deficiencies, not expression level. One explanation for K618A appearing deficient for methylation-induced cytotoxicity by Blasi at al (2006) and proficient for 6-TG induced cytotoxic response in our pooled experiment is the

origin of the cultures. Blasi et al examined cultures derived from a single clone which may have contained a second site mutation that influenced its functional phenotype, while we assayed pooled cultures where the effects of second site mutations would likely be minimized.

Like other methods, the approach described by Blasi et al (2006) has both advantages and disadvantages. The use of human cells to evaluate the function of variant hMLH1 protein is advantageous because it eliminates the likelihood of interspecies protein incompatibilities. This approach also assesses the activity of four different aspects of DNA MMR, which reduced the chance of falsely characterizing a variant as a polymorphism if it is deficient for some activities. The principal limitation of this approach is that it examines cultures arising from individual single clones. Characterization of many such clones typically is necessary (and labor intensive) to identify clones with an appropriate level of expression. The level of expression is chosen either to match the endogenous level of hMLH1 in other MMR-proficient lines, or to match the level achieved with transfection with wild-type cDNA. Neither approach takes into account the possibility that a hMLH1 variant might have reduced stability. A second limitation is the possibility that an individual clone might accumulate additional second site mutations which could influence MMR phenotype. In the case of I219V one culture showed high hMLH1 protein expression and behaved similarly to wild-type in functional assays while another showed a reduced hMLH1 protein level and had a decreased response compared to wild-type. Which protein level would be more

representative of the protein level expected in human cells expressing I219V from the endogenous hMLH1 promoter? This approach is useful because it examines a wide range of MMR functions but because it only looks at few examples of each variant, the results could be misleading.

5.3 Conclusions:

No single assay has been able to definitively determine the pathogenicity of missense mutations in hMLH1. While each approach contributes to the general understanding of hMLH1 variant behavior, the inclusion of results from various assays is required to culminate in a true understanding of these hMLH1 mutations. In order to determine the likely effect of hMLH1 variants on phenotype, it would be beneficial to examine in vitro protein-protein interactions as demonstrated by Kondo et al (2003), the steady-state MLH1 and PMS2 levels as described in Mohd et al (2006) and Chapter 3, and responses in functional assays as described in Chapter 4. By comparing the conclusions of these assays to results from clinical analyses, a general understanding of variant behavior may likely be identified.

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