

LEGENDS FOR THE SUPPLEMENTARY FIGURES AND TABLES

SUPPLEMENTARY FIGURE 1.

A. The kinetics of expression of the *MLH1* transgene in the HCT116 cells allowed control of the rate transfected cells remained stable for up to 48 hours and 72 hours post-transfection, *i.e.* during the time cells were exposed to MNNG treatments. Data represent means \pm SD from two independent experiments.

B. Results of the clonogenic assay showing a decrease in surviving colonies following MNNG treatment of HCT116 cells transfected with the *MLH1*-containing construct, contrary to HCT116 cells transfected with the random sequence-containing construct. Each column of three wells constitutes a triplicate. Colonies were stained with crystal violet.

SUPPLEMENTARY FIGURE 2. Dose response curves and LD50

A. Dose response curves of HCT116 cells transfected with the *MLH1* (in green) or the random (in red) sequence-containing construct and of HCT116-ch3 cells transfected with the random (in pink) sequence-containing construct after 1, 2 or 3 pulses of MNNG treatment. Response of the non-transfected HCT116 (in blue) and HCT116-ch3 (in black) cells is shown for comparison. The corresponding LD50 values are also represented (graph on the right).

B. Dose response curves of LoVo cells transfected with the *MSH2* (in green) or the random (in red) sequence-containing construct after 1, 2 or 3 pulses of MNNG treatment. Response of the non-transfected LoVo (in blue) is shown for comparison. The corresponding LD50 values are also represented (graph on the right).

SUPPLEMENTARY FIGURE 3. MT according to cell fluorescence intensity.

A. Representative mCherry fluorescence histogram of control non-transfected HCT116 cells (auto-fluorescence) (top) and HCT116 cells transfected with the *MLH1*-mCherry plasmid (bottom). Cells were sorted separately according to their fluorescence intensity (*i.e.* mCherry low or mCherry high).

B. Dose response curves of HCT116 cells transfected with either the *MLH1*-mCherry or the random-mCherry construct according to their fluorescence intensity. One, 2 or 3 pulses of MNNG treatment were performed and experiments were achieved in 2 or 3 independent clonogenic experiments, each performed in triplicate.

C. Graph showing the LD50 in the different experimental conditions.

SUPPLEMENTARY FIGURE 4. Location of all *MLH1* (A) or *MSH2* (B) variants screened in this study within the gene structure and functional domains of the protein.

VUS in italics are classified as potentially damaging or potentially neutral with the MT assay.

SUPPLEMENTARY FIGURE 5. ROC curves for the MT assay and 3 *in silico* prediction tools in the validation set of *MLH1* (left) or *MSH2* (right) variant controls.

While all *in silico* methods performed reasonably well, the MT assay was substantially better at distinguishing known pathogenic from known neutral variants. Cut-off was set to 20 for CADD and C15 for Align GVGD.

SUPPLEMENTARY TABLE 1. List of proven pathogenic and proven neutral *MLH1* and *MSH2* variants in the validation set.

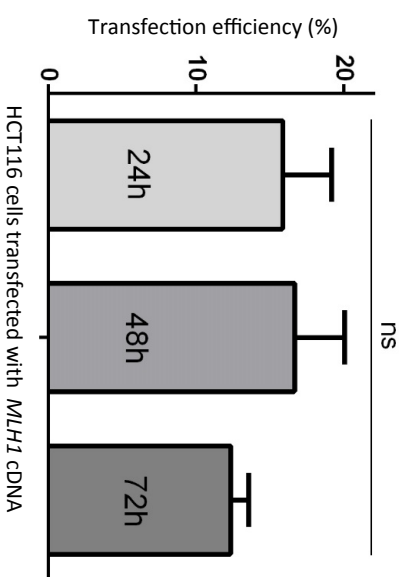
The current classification of the variants in the InSiGHT and French databases and the result of the methylation tolerance assay (expressed as mean survival scores) are indicated.

SUPPLEMENTARY TABLE 2. Clinical, population and functional data available in InSiGHT or French databases, together with *in silico* CADD prediction score and MT assay result, for the 25 *MLH1* VUS of the prospective set.

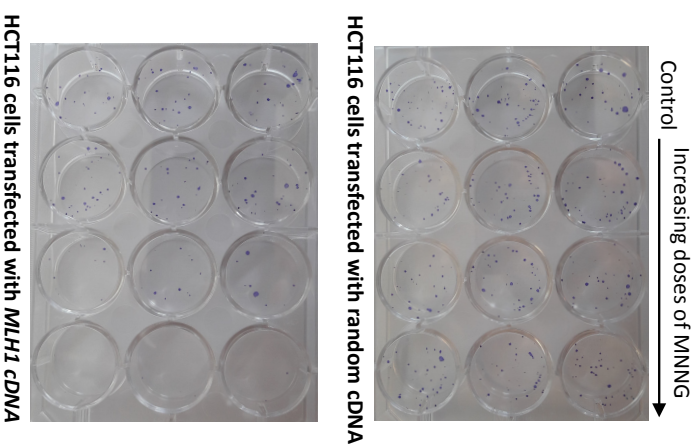
SUPPLEMENTARY TABLE 3. Clinical, population and functional data available in InSiGHT or French databases, together with *in silico* CADD prediction score and MT assay result, for the 23 *MSH2* VUS of the prospective set.

SUPPLEMENTARY FIGURE 1

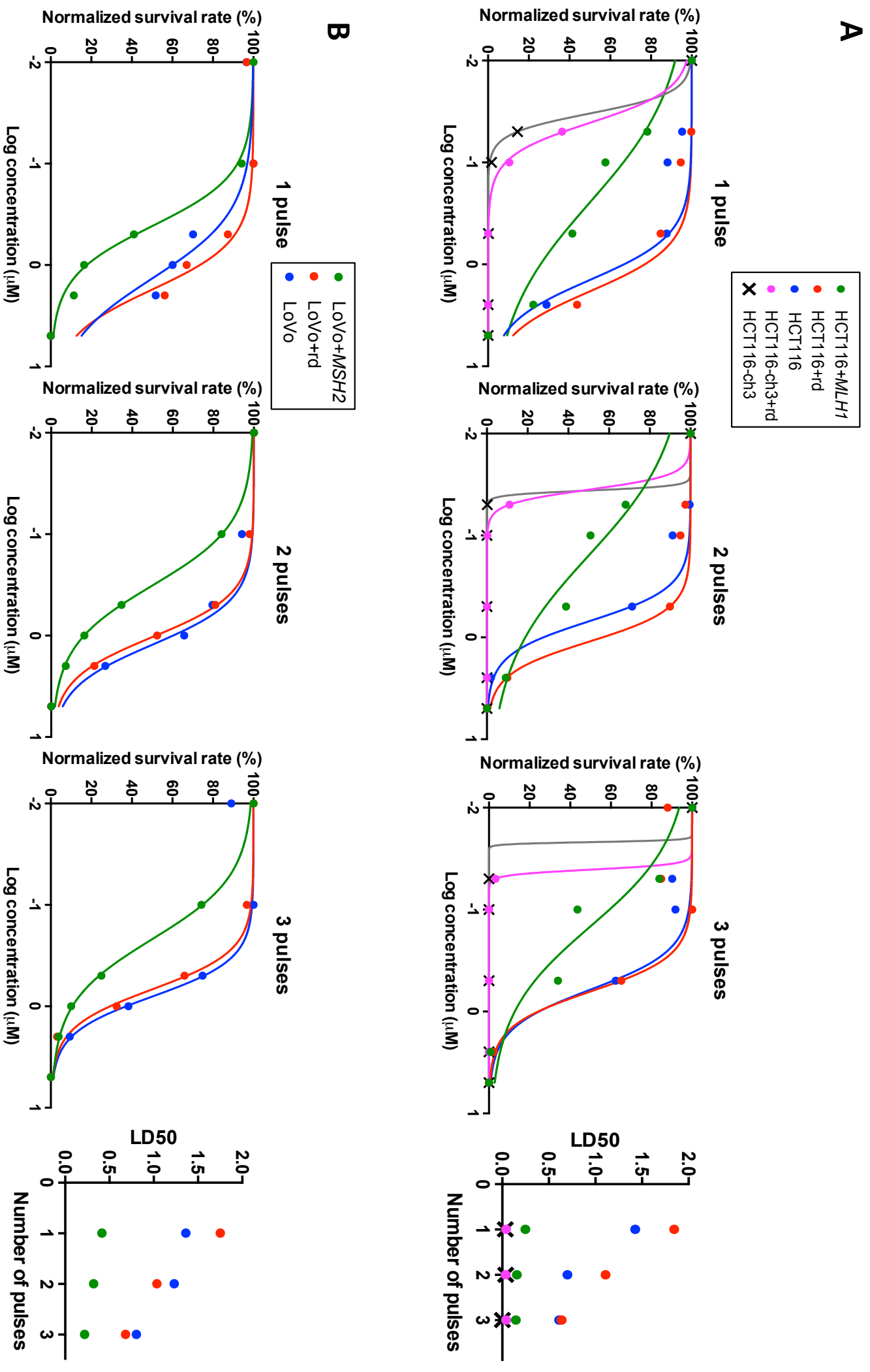
A



B



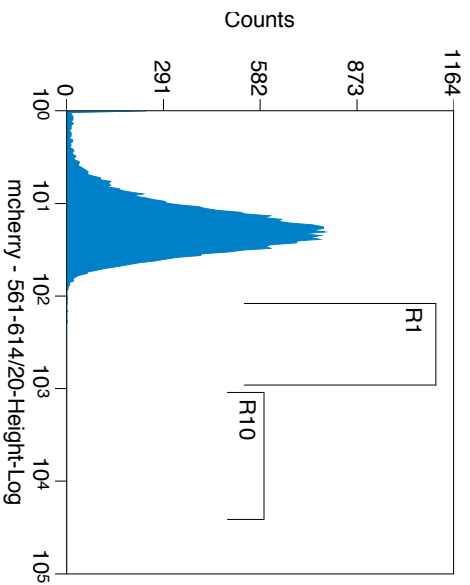
SUPPLEMENTARY FIGURE 2



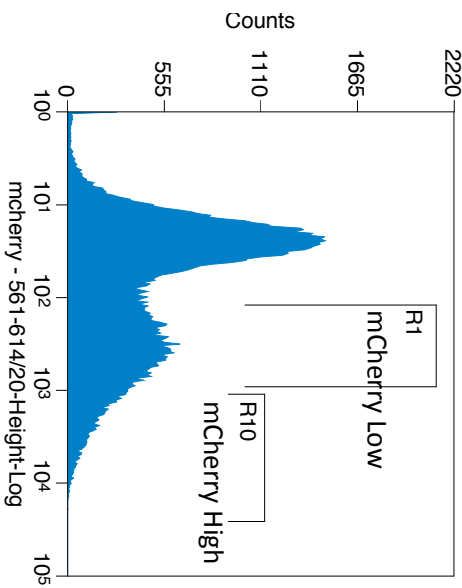
SUPPLEMENTARY FIGURE 3

A

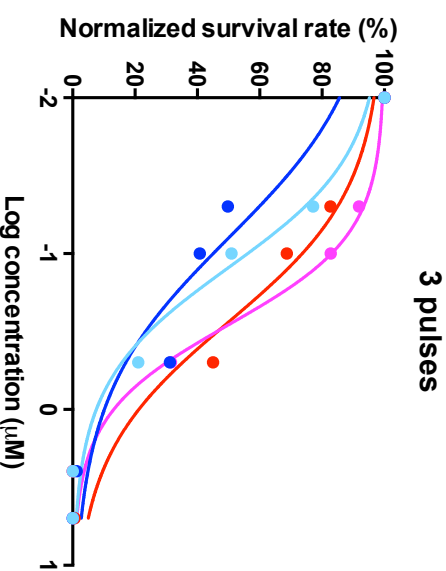
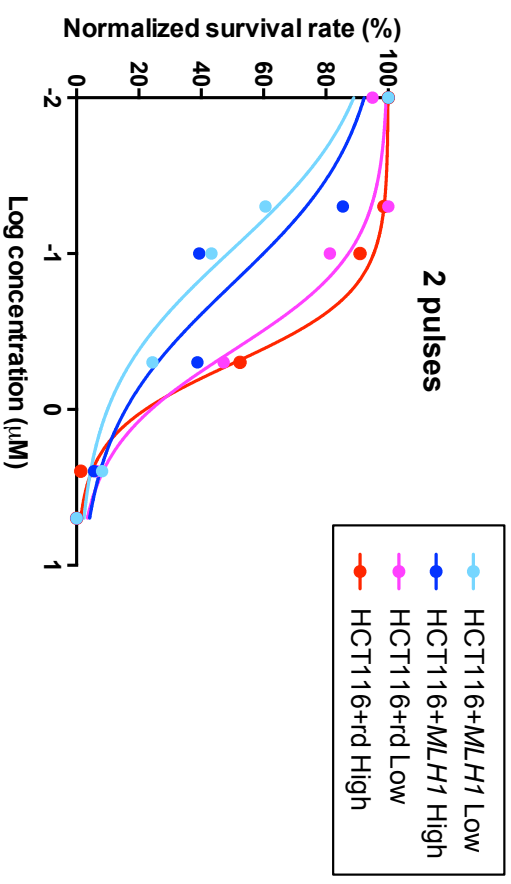
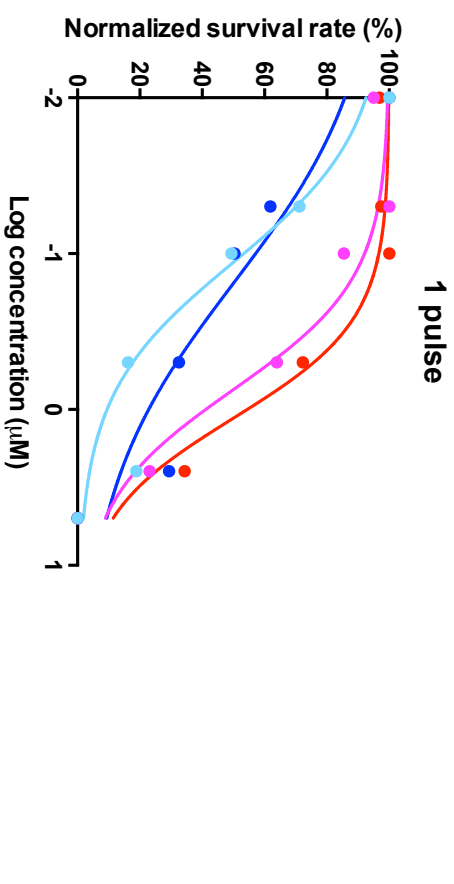
Control HCT116 cells (non-transfected)



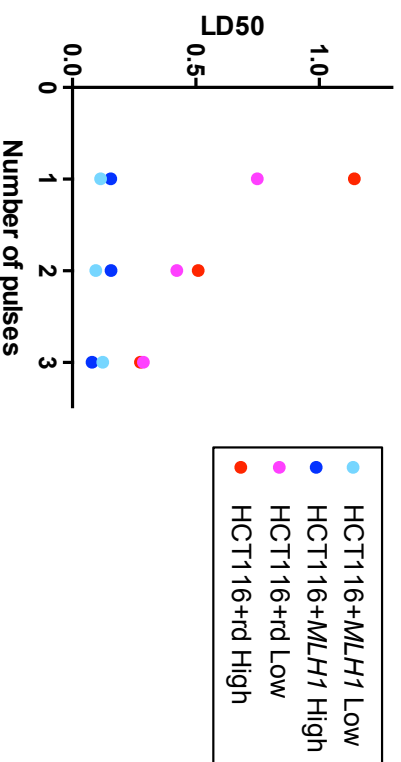
HCT116 cells transfected with the *MLH1*-mCherry plasmid



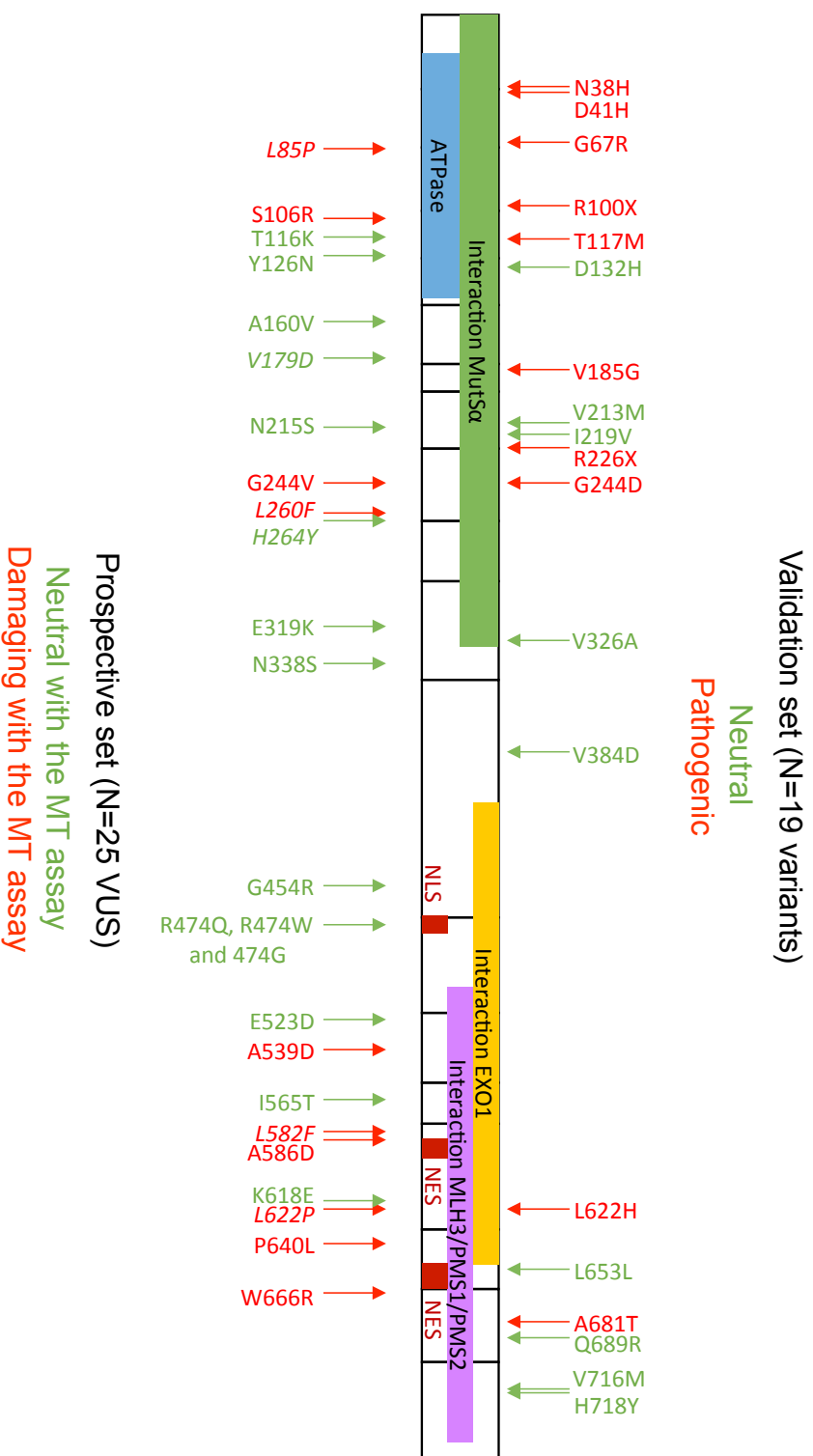
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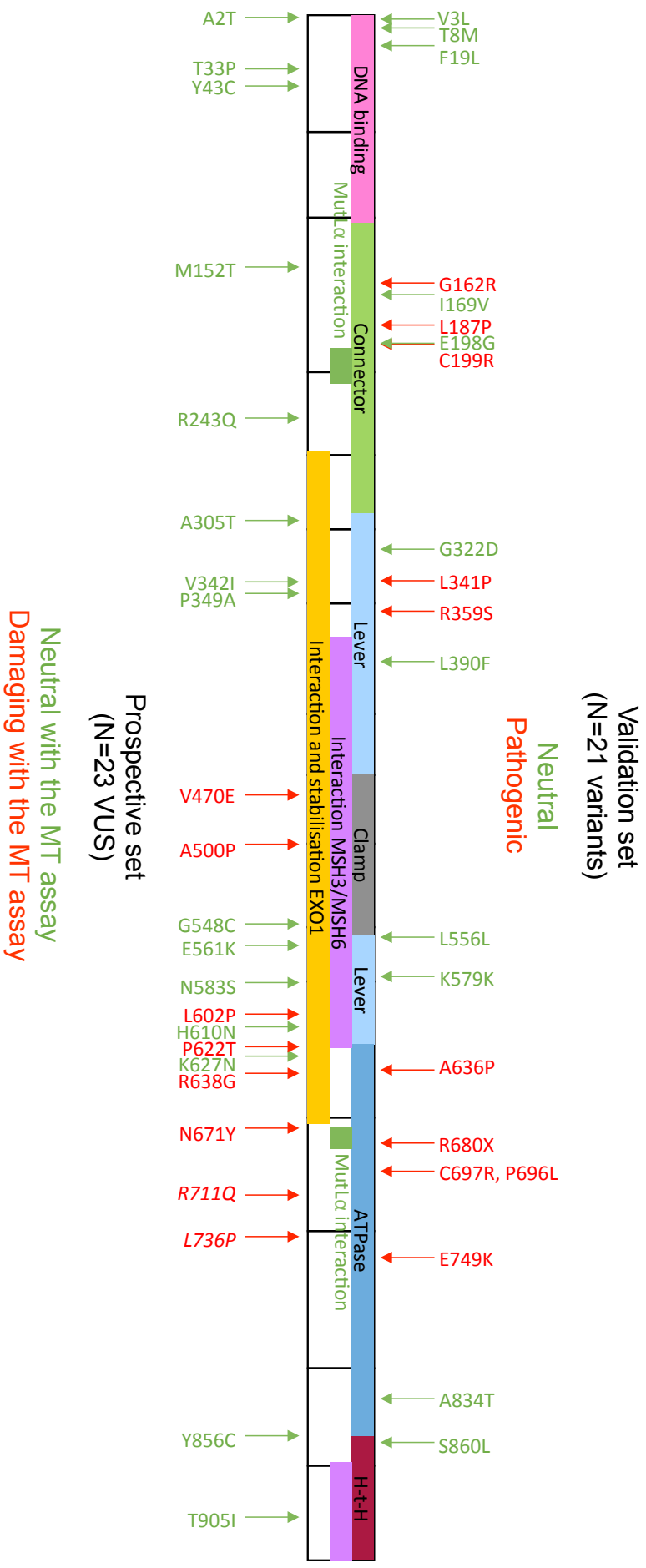
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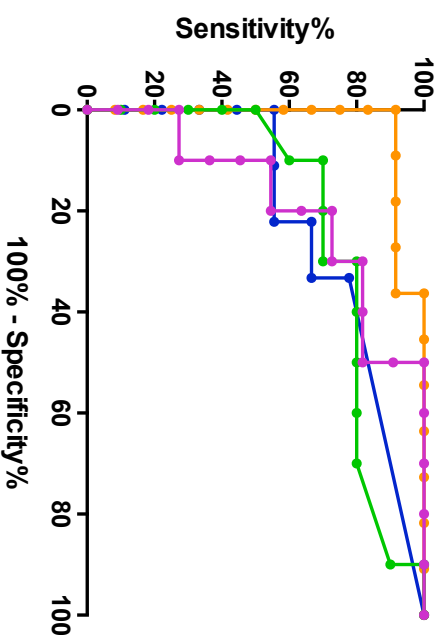
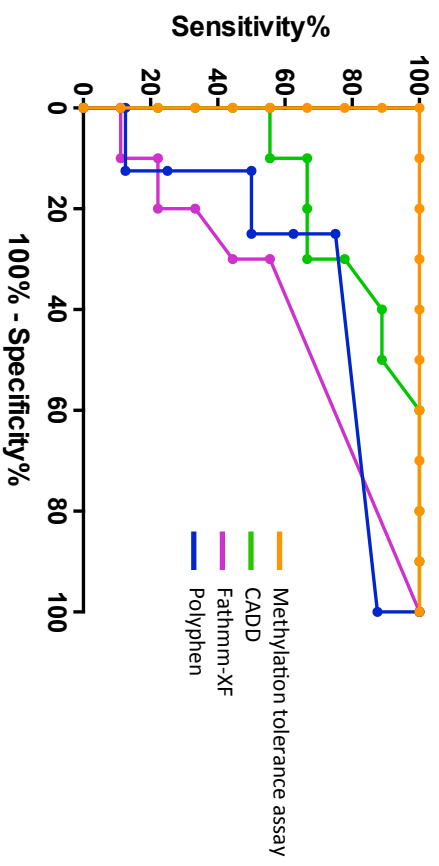
SUPPLEMENTARY FIGURE 4A



SUPPLEMENTARY FIGURE 4B



SUPPLEMENTARY FIGURE 5



Supplementary Table 1. Proven pathogenic and non-pathogenic *MLH1* and *MSH2* variants used in the validation study

Variant		Classification		MT assay result (%)
Protein	DNA	InSiGHT	French database	
<i>MLH1</i> variants				
N38H	112A>C	5	-	98.03
D41H	121G>C	5	4	87.27
G67R	199G>A	5	5	94.36
R100X	298C>T	5	5	91.49
T117M	350C>T	5	5	91.61
V185G	554T>G	5	-	87.96
R226X	676C>T	5	5	97.63
G244D	731G>A	5	5	87.86
L622H	1865T>A	5	-	82.18
A681T	2041G>A	5	5	84.50
D132H	394G>C	1	1	37.86
V213M	637G>A	1	1	28.06
I219V	655A>G	1	1	44.66
V326A	977T>C	1	1	55.23
V384D	1151T>A	1	1	36.64
L653L	1959G>T	1	1	35.32
Q689R	2066A>G	1	1	54.42
V716M	2146G>A	1	1	53.71
H718Y	2152C>T	1	1	31.15
<i>MSH2</i> variants				
G162R	484G>A	5	4	68.27
L187P	560T>C	5	-	77.48
C199R	595T>C	5	5	70.76
L341P	1022T>C	4	5	58.90
R359S	1077A>T	5	5	83.14
A636P	1906G>C	5	5	66.58
R680X	2038C>T	5	5	68.03
P696L	2087C>T	5	4	65.48
C697R	2089T>C	5	5	67.66
E749K	2245G>A	4	5	70.65
V3L	7G>T	-	2	20.15
T8M	23C>T	1	2	25.90
F19L	55T>C	-	2	31.49
I169V	505A>G	2	-	20.96
E198G	593A>G	1	-	67.46
G322D	965G>A	1	1	32.17
L390F	1168C>T	1	1	29.03
L556L	1666T>C	1	1	25.40
K579K	1737A>G	1	-	20.05
A834T	2500G>A	2	-	25.10
S860L	2579C>T	2	-	10.53

Supplementary Table 2. Clinical, population and functional data available in InSiGHT or French databases, together with *in silico* CADD prediction score and MT assay result, for the 25 MLH1 VUS of the prospective set

Variant	DNA	Classification		Tumor and age (years) ^b	MSI	IHC and/or other somatic data ^c	Amsterdam or Bethesda criteria	Cancer and age (years) in relatives ^b	Allele frequency (%)	CADD score ^d	Previous functional tests	MT assay result ^e
		InSiGHT	French ^a									
L85P	254T>C	/	3*	CRC 49	YES	MLH1+ PMS2-	/	/	/	28.3	/	77.12% Potentially damaging
S106R	318C>A	3	3	/	YES	MLH1+ MSH2+ MSH6+ PMS2+	/	/	/	33	/	84.96% Damaging
T116K	347C>A	3	3	CRC 28	/	/	/	mat uncle: CRC 58	/	34	/	44.83% Neutral
Y126N	376T>A	3	3	LGD Ad 30 BC 73	/	/	/	non carrier sister: CRC 74; several BC in family	0.0074	31	/	51.99% Neutral
A160V	479C>T	3	/	CRC 68	YES	MLH1-	Amsterdam I	/	0.014	23	102% repair activity in vitro; dominant mutator effect; expression >75% in transfected HCT116 cells ¹	27.69% Neutral
V179D	536T>A	/	3*	CRC 59	/	/	/	Mother: CRC 65; mat aunt: GC 61; father: PrC PC 79; pat uncle: HCC; pat uncle's son: MSTO (asbestos)	/	33	/	61.88% Potentially neutral
N215S	644A>G	3	3	Spo CRC <50	YES	/	/	/	0.0016	26.4	/	46.73% Neutral
G244V	731G>T	3	3	/	/	MLH1-	Bethesda	/	/	27.4	24% repair activity in vitro; No dominant mutator effect; expression >75% in transfected HCT116 cells ¹	101.24% Damaging
							/	/	/	/	/	
							/	/	/	/	/	
							/	/	/	/	/	
L260F	778C>T	3	3	CRC 41	/	MLH1+ PMS2+	Amsterdam II	Tested individuals: CCR 36; PrC 68; Untested individuals: CCR 34; CCR 45; CCR 55; CCR 45; EC 49; GC 58	/	32	/	76.37% Potentially damaging
							/	/	/	/	/	

				Spo BC	YES	MLH1+ MSH2+ No MLH1 methylation	/	/											
H264Y	790C>T	3	3	CRC 30	YES	/	/				0.00087	32	/	63.65% Potentially neutral					
E319K	955G>A	3	3	BC 45	/	MLH1+ MSH2+ MSH6+ PMS2+	/	Mother: BC 40; mat aunt: BC 40; mat aunt's daughter: BC 40			0.0082	35	/	32.28% Neutral					
N338S	1013A>G	3	3	CRC 38	/	/	Bethesda	Father: lymphoma ; uncle: CRC			0.0058	15.9	/	44.57% Neutral					
				CRC 33	YES	MLH1- PMS2-	Bethesda	Untested individuals: skin: CCR 80; PTC 80; skin: CCR 50											
G454R	1360G>C	3	1	/	NO	/	/	/			0.015	0.5	/	38.42% Neutral					
				/	/	MLH1+ MSH2-	/	/											
				Ad	/	/	Bethesda	One unaffected carrier; Untested individuals: CRC 39; EC 42											
R474Q	1421G>A	3	1	/	/	/	/			0.00082	23.9	102% repair activity in vitro ; dominant mutator effect; expression >75% in transfected HCT116 cells ¹	35.77% Neutral						
R474W	1420C>T	3	/	/	/	/	/			0.0016	27.8	/	29.17% Neutral						
R474G	1420C>G	/	3*	/	/	/	/			0.0082	23.5	/	28.28% Neutral						
E523D	1569G>T	3	/	/	/	/	/	/			/	13.2	96% repair activity in vitro; dominant mutator effect; expression >75% in transfected HCT116 cells ¹	49.04% Neutral					
A539D	1616C>A	3	3	CRC <50	/	/	/	2 affected carriers in the family			/	25.7	/	98.51% Damaging					
I565T	1694T>C	/	3*	/	/	/	/	/			/	26.9	/	45.13% Neutral					
L582F	1744C>T	3	3	CRC 37	YES	MLH1- MSH2+ MSH6+ PMS2- No MLH1 methylation	Bethesda	/			/	28	30% repair activity in vitro ² ; nuclear localization in murine NIH-3T3; defective PMS2 interaction ³	75.67% Potentially damaging					
A586D	1757C>A	3	3	/	/	/	Amsterdam II	/			/	32	/	87.77% Damaging					
K618E	1852A>G	3	3	/	/	/	Amsterdam II	/			0.34	27.8	/	50%					

				CRC, extracolonic LS and non LS tumor	/	/	No Amsterdam	Father: extracolonic HNPCC tumor; aunt and cousin: CCR						Neutral	
L622P	1865T>C	3	/	BC 38, CRC 48 CRC 77 CRC 43	YES YES YES	MLH1-MSH2+ MSH6+PMS2+ MLH1 methylation (region H not D)	No Bethesda no Amsterdam	no	Untested individuals: GC 70; bladder; unknown cancer	/	29.2	/	Potentially damaging	75.68%	
P640L	1919C>T	3	5	CRC 37	YES	MLH1-PMS2- No MLH1 methylation	/	Mother: CRC 45							
				/	YES	MLH1-PMS2-	Amsterdam I	/							
				EC 36, DC 48, CRC 56, UC 64	/	/	Amsterdam I	Father: CRC 38; pat uncle and grand-father: CRC							
				CRC 31	/	MLH1-PMS2-	Bethesda	Untested individuals: CRC 29; GC 55; CRC 81							
W666R	1996T>C	3	3	CRC 48, 55	YES	MLH1-	Bethesda	Untested relatives: EC; CRC; unknown							
				/	YES	/	Amsterdam I	1 affected carrier relative							
				/	/	/	/	/	/	/	26.2	/		88.21% Damaging	

^a An asterisk indicates a private variant not yet reported in the French database

^b Ad: adenoma; BC: breast cancer; CRC: colorectal cancer; EC: endometrial cancer; GC: gastric cancer; HCC: hepatocellular carcinoma; LGD : low grade dysplasia; mat: maternal; LS: Lynch syndrome; MSTO: mesothelioma; pat: paternal; PC: pancreatic cancer; PrC: prostate cancer; SpO: sporadic

^c IHC: immunohistochemistry; + indicates a staining of the corresponding protein; - indicates an absence of the corresponding protein

^d CADD algorithm prediction score

^e results of the methylation tolerance assay expressed as the mean survival score and proposed interpretation

1. Takahashi M, Shimodaira H, Andreutti-Zaugg C, et al. Functional analysis of human MLH1 variants using yeast and in vitro mismatch repair assays. *Cancer Res* 2007;67:4595-604.
2. Drost M, Zonneveld J, van Dijk L, et al. A cell-free assay for the functional analysis of variants of the mismatch repair protein MLH1. *Hum Mutat* 2010;31:247-53.
3. Andersen SD, Liberti SE, Lutzen A, et al. Functional characterization of MLH1 missense variants identified in Lynch syndrome patients. *Hum Mutat* 2012;33:1647-55.
4. Hardt K, Heick SB, Betz B, et al. Missense variants in hMLH1 identified in patients from the German HNPCC consortium and functional studies. *Fam Cancer* 2011;10:273-84.

Supplementary Table 3. Clinical, population and functional data available in InSiGHT or French databases, together with *in silico* CADD prediction score and MT assay result, for the 23 MSH2 VUS of the prospective set

Protein	Variant	DNA	Classification		Tumor and age (years) ^b	MSI	IHC and/or other somatic data ^c	Amsterdam or Bethesda criteria	Cancer and age (years) in relatives ^b	Allele frequency (%)	CADD score ^d	Previous functional tests ^e	MT assay result ^f
			InSiGHT	French ^a									
A2T	4G>A		3	1	LS spectrum cancer < 50	/	/	Amsterdam I	/	0.039	32	/	22.63% Neutral
					/	/	Bethesda	6 families ; 1 family: pathogenic MSH2 mutation c.1835C>G <i>In cis</i>	Brother: CRC 46, sister: EC 47; sister: CRC 68; father: liver cancer 52; uncle: CRC 30; niece: CRC 45; niece: EC 50; nephew: CRC 37; cousin: CRC 60; cousin: GC 31; great-cousin: CRC, brain tumor 18; cousin: CRC 42, 47; segregation with the disease in the family (at least over 10 meioses)				
					CRC 39	YES	MSH2-	Bethesda					
					Cancer 28	/	MSH2-, MSH2 methylation, germline EPCAM mutation, WT KRAS, WT BRAF	/		/			
					Cancer 45	/	MSH2-, MSH2 methylation, germline EPCAM mutation, WT KRAS, WT BRAF	/		/			
					CRC 50	YES	MSH2-	/		/			
					/	YES	MSH2-	/		/			
					/	YES	MSH2-,	/		3 families			

								EPCAM deletion (last exon in cis)								
								MSH2+	MSH1+ MSH2+ MSH6+							
T33P	97A>C	3	3	EC 45	NO	YES	MLH1+ MSH2+ MSH6+	Bethesda	2 families	0.0064	33	decreased repair activity in vitro ¹ , proficient mismatch binding and reduced ATP-dependent release ² , intact MSH6 interaction ¹ , MSH2 expression similar to WT ^{1,2}	21.11% Neutral			
Y43C	128A>G	3	3	Synchrononus CRC 50	NO	MLH1+ MSH2+ MSH6-	/	father: CRC 50; sister: CRC 50; sister: CCR 52; daughter: EC 39	0.0028	33	/	15.53% Neutral				
					CRC 49	/	mother: CRC 73									
					GC 65	/	sister: ovary 65; sister: EC, sarcoma 64; father and mother: brain tumor									
M152T	455T>C	/	3	Spo EC < 50	NO	MLH1+ MSH2+ MSH6+ PMS2+	/	/	16.7	/	10.26% Neutral					
R243Q	728G>A	3	3	Spo CRC <50	NO	MSH2-MSH6-	/	/	0.0017	33	/	23.56% Neutral				
					CRC < 50	NO	/	Amsterdam I	/	0.005	32	53% repair activity in vitro ³ , proficient mismatch binding & ATP-dependent release; 2.2-fold induction of mutation rate in bacteria; nuclear localization in MEF; intact EXO1 interaction; expression	30.54% Neutral			
A305T	913G>A	3	3	CRC 47	YES	MLH1- PMS2- MSH2+	/	brother: GC 41								

V342I	1024G>A	3	3	CRC < 40 /	YES /	MLH1+ MSH2- /	Amsterdam I /	/	3 recurrent CRC families	/	22.3	similar to WT ⁴ /	24.08% Neutral
P349A	1045C>G	3	3	Spo renal cell carcinoma 63	/	/	/	/	/	0.009	25	/	31.54% Neutral
				CRC 37	YES	/	Amsterdam II	daughter (not tested) : MSH2-MSH6-					
				/	/	/	/						
				BC 38, CRC	/	/	Amsterdam I	/					
				/	YES	MLH1+ MSH2- MSH6- PMS2+	/	/					
V470E	1409T>A	3	3	CRC 40	YES	MLH1+ MSH2+ MSH6+ PMS2+	/	pat grand-mother: CRC 50; pat grand-father: CRC 57; 2 2nd degree relatives LS tumors < 50	/	26.5	/	79.63% Damaging	
A500P	1498G>C	/	3	UC 68, CRC 74	YES	MLH1+ MSH2+ MSH6+ PMS2+	No Amsterdam	Variant carrier brother: CRC 40, 51, GC 51; sister: CRC 38, EC; sister: CRC 30, ovary 32; pat grand-father: CRC 55; half-sister (same father); BC 63, ad; half-sister's son: ad	/	32	/	66.01% Damaging	
				/	/	Amsterdam II	/						
G548C	1642G>T	3	3	/	/	MLH1+ MSH2+ No MLH1 methylation WT BRAF	/	no	/	34	/	29.82% Neutral	
E561K	1681G>A	3	3	/	/	/	/	/	0.0017	24.5	/	8.70% Neutral	
N583S	1748A>G	3	3	CRC 35	/	/	Amsterdam I	/	0.01	22.9	/	12.65% Neutral	
				CRC <50	/	/	Amsterdam I	/					
L602P	1805T>C	/	3	CRC 38	YES	/	Amsterdam	brother: CRC 40; father: UC 57, 58, 60, lymphoma	/	26.2	/	78.64% Damaging	
H610N	1828C>A	3	3	/	/	/	/	/	/	21.5	/	18.20%	

P622T	1864C>A	3	/	CRC 23	YES	/	Amsterdam I	/	/	Neutral		
				Ad 40, CRC 51, HGD Ad 52	YES	MSH2-MSH6-	No Amsterdam	father: CRC 55; mother: EC 45, CRC 55	/	Damaging		
K627N	1881A>C	3	3	/	/	/	/	/	/	20.71% Neutral		
R638G	1912A>G	3	3	CRC 44	YES	MSH2-	/	/	/	59.66% Damaging		
N671Y	2011A>T	3	3	CRC 42	/	/	Bethesda	/	/	67.36% Damaging		
R711Q	2132G>A	/	3	/	YES	MLH1+MSH2+	Amsterdam	/	/	53.08% Potentially damaging		
L736P	2207T>C	/	3*	Sebaceoma 66	YES	MSH6-	No Amsterdam	No family history	/	55.58% Potentially damaging		
Y856C	2567A>G	3	3	/	/	/	Amsterdam	/	/	21.46% Neutral		
T905I	2714C>T	3	3	/	/	/	/	multiple late-onset LS cancers in the family	0.0076	14.6	/	20.99% Neutral

^a An asterisk indicates a private variant not yet reported in the French database

^b Ad: adenoma; BC: breast cancer; CRC: colorectal cancer; GC: gastric cancer; EC: endometrial cancer; HGD: high grade dysplasia; Spo: sporadic

^c IHC: immunohistochemistry; WT: wild type; + indicates a staining of the corresponding protein; - indicates an absence of the corresponding protein

^d CADD algorithm prediction score

^e WT: wild type

^f results of the methylation tolerance assay expressed as the mean survival score and proposed interpretation

- Ollila S, Sarantaus L, Kariola R, et al. Pathogenicity of MSH2 missense mutations is typically associated with impaired repair capability of the mutated protein. *Gastroenterology* 2006;131:1408-17.
- Ollila S, Dermadi Bebek D, Jiricny J, et al. Mechanisms of pathogenicity in human MSH2 missense mutants. *Hum Mutat* 2008;29:1355-63.
- Drost M, Zonneveld JB, van Hees S, et al. A rapid and cell-free assay to test the activity of Lynch syndrome-associated MSH2 and MSH6 missense variants. *Hum Mutat* 2012;33:488-94.
- Lutzen A, de Wind N, Georgijevic D, et al. Functional analysis of HNPCC-related missense mutations in MSH2. *Mutat Res* 2008;645:44-55.