Novel Roles for MLH3 Deficiency and TLE6-Like Amplification in DNA Mismatch Repair-Deficient Gastrointestinal Tumorigenesis and Progression

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Abstract
DNA mismatch repair suppresses gastrointestinal tumorigenesis. Four mammalian E. coli MutL homologues heterodimerize to form three distinct complexes: MLH1/PMS2, MLH1/MLH3, and MLH1/PMS1. To understand the mechanistic contributions of MLH3 and PMS2 in gastrointestinal tumor suppression, we generated Mlh3+/−:Apc638N and Mlh3+/−;Pms2+/−:Apc638N (MPA) mice. Mlh3 nullizygosity significantly increased Apc frameshift mutations and tumor multiplicity. Combined Mlh3/Pms2 nullizygosity further increased Apc base-substitution mutations. The spectrum of MPA tumor mutations was distinct from that observed in Mlh1+/−:Apc638N mice, implicating the first potential role for MLH1/PMS1 in tumor suppression. Because Mlh3/Pms2 deficiency also increased gastrointestinal tumor progression, we used array-CGH to identify a recurrent tumor amplico. This amplicon contained a previously uncharacterized Transducin enhancer of Split (Tie) family gene, Tle6-like. Expression of Tie6-like, or the similar human TLE6D splice isoform in colon cancer cells increased cell proliferation, colony-formation, cell migration, and xenograft tumorigenicity. Tie6-like:TLE6D directly interact with the gastrointestinal tumor suppressor RUNX3 and antagonize RUNX3 target transactivation. TLE6D is recurrently overexpressed in human colorectal cancers and TLE6D expression correlates with RUNX3 expression. Collectively, these findings provide important insights into the molecular mechanisms of individual MutL homologue tumor suppression and demonstrate an association between TLE-mediated antagonism of RUNX3 and accelerated human colorectal cancer progression.

Introduction
Colorectal cancer (CRC) is one of the common malignancies in industrialized countries. Lynch syndrome, a highly penetrant disorder that confers predisposition to cancer of the colorectum, endometrium and other extra-colonic sites [1], is caused by germline mutations in DNA Mismatch Repair genes (MMR). Including sporadic forms, defective MMR underlies ~12–15% of CRC [2]. MMR plays critical roles in the maintenance of genomic stability in both prokaryotes and eukaryotes [3]. The study of model organisms has yielded great insights into the mechanisms through which MMR prevents cancer [1,3,4,5,6,7,8]. Briefly, there are nine mammalian MMR genes (MLH1, MLH3, PMS1-2, MSH2-6). The mammalian E. coli MutS homologues (Msh) directly contact DNA, scanning along the genomic DNA for mismatches analogous to a “sliding clamp” until they encounter a base-pair containing a mismatch [9,10]. MSH2-MSH6 primarily recognizes single-base substitutions and 1 base-pair insertion-deletion loop (IDL) mutations, while MSH2-MSH3 recognizes 1–4 base-pair insertion-deletion mutations [1,3]. The IDL repair deficiency is commonly referred to as Microsatellite Instability (MSI). The MSH proteins interact with multiple proteins including the mammalian E. coli MutL homologues (MLH) and yeast post-meiotic segregation (PMS) homologue proteins (which have significant amino acid identity and structural similarity to the MLH proteins), as well as RPA, EXO1, RFC, HMG1, POLD1 and other proteins [1,8,11,12]. MLH1-PMS2 is the primary MutL complex that interacts with both MSH2/6 and MSH3 complexes. MLH1–MLH3 is less well characterized, but is believed to participate in IDL repair [13,14], DNA damage response [13], and possibly single-base point mutation repair (SBR) [15]. MLH1-PMS1 exists in mammalian cells but currently has no clearly defined roles in processes related to cancer prevention [16,17].
**Author Summary**

Approximately one million people every year are diagnosed with colorectal cancer worldwide, and about five hundred thousand of these people subsequently perish from the disease. Colorectal cancer is thought to develop through a series of early and later stages (called cancer initiation and progression, respectively). Deaths from colorectal cancer are particularly tragic because the disease can usually be cured if discovered before full-blown progression. However, our knowledge of how these tumors progress remains very limited. DNA mismatch repair is known to be an important process in preventing ~15% of colorectal cancer initiation. In this study we describe how two of these genes (Mlh3 and Pms2) that have partial functional redundancy and therefore individually are rarely mutated are also important in preventing colorectal cancer progression. Additionally, we describe a new gene (Tle6-like) that, when overactive, makes these cancers progress more rapidly. The overall goal of this study is to understand colorectal cancer progression better so that we can come up with new ways to block it at the later stage.

To study the precise mechanisms through which MMR suppresses carcinogenesis in vivo, we and others [16,18,19,20,21,22,23,24] previously developed several mouse models carrying mutations in different MMR genes. Mlh1<sup>-/-</sup> and Msh2<sup>-/-</sup> mice develop early onset GI epithelial cancers, lymphomas and other types of cancer. Pms2<sup>-/-</sup> mice develop lymphomas, but not GI epithelial cancers. Mlh3<sup>-/-</sup> mice develop GI and extra-GI tumors, have decreased survival when compared with Wt mice, but with later onset than Mlh1<sup>-/-</sup> [13]. Mlh3<sup>-/-</sup>;Pms2<sup>-/-</sup> mice have increased cancer incidence, resistance to apoptosis and MSI [13]. However, the precise mechanisms in which Mlh3 and Pms2 participate to suppress GI epithelial tumorigenesis and progression remain poorly characterized. Germ-line mutations in tumor suppressor gene Apc lead to familial adenomatous polyposis (FAP) [25,26]. Mutations in APC are found in the majority of sporadic CRC and many Lynch syndrome tumors [27,28]. APC complexes with AXIN and CK1/2 and destabilizes β-Catenin by enhancing proteasomal destruction. Mutated APC proteins are unable to down-regulate β-Catenin, and the stabilized β-Catenin translocates into the nucleus where it acts as a transcriptional coactivator of the DNA binding protein TCF-4 [29,30]. More than 95% of APC germ-line mutations are truncating or nonsense mutations and most of the pathogenic mutations are located within the first 1500 codons. Apc mutations cooperate with MMR deficiency in both tumorigenesis and tumor progression. Apc<sup>1638N</sup> mice are a well characterized model that develops GI cancer [31]. Mlh1<sup>-/-</sup>;Apc<sup>1638N</sup> mice showed significantly increased GI tumor multiplicity and accelerated progression to adenocarcinoma compared to either mutation separately. Analyses of GI tumors from Mlh1<sup>-/-</sup>;Apc<sup>1638N</sup> and Msh3<sup>-/-</sup>;Msh6<sup>-/-</sup>;Apc<sup>1638N</sup> mice revealed that both single-base substitutions and MSI induced frameshift mutations in repetitive sequences were responsible for most mutations found in the remaining wild-type (Wt) Apc allele [32,33]. In contrast, tumor-associated Apc mutations found in the Wt Apc allele in Msh6<sup>-/-</sup>;Apc<sup>1638N</sup> tumors were predominantly single-base point mutations.

To understand more precisely the mechanistic roles that Mlh3 and Pms2 play in GI tumor suppression, we generated Mlh3<sup>-/-</sup>;Apc<sup>1638N</sup> (M4) and Mlh3<sup>-/-</sup>;Pms2<sup>-/-</sup>;Apc<sup>1638N</sup> (MP4) mice. We show that in vivo Mlh3 mutations significantly increase frameshift mutation rates in Ap, and increase GI tumorigenesis. Unlike typical MSI-induced mutations, Mlh3 deficiency also results in frameshift mutations in non-repetitive sequences, a unique mutational signature among MMR deficient mice found only in Mlh3 deficient mice. Consistent with the role of Pms2 in SBR, combined Mlh3 and Pms2 mutations proportionally increase point mutations and show a sequence preference for a CpG mutation hotspot also previously seen in Mlh1<sup>-/-</sup> mice. Because MPA mutant mice also have significantly increased rates of GI adenocarcinomas vs. Ap<sup>1638N</sup> or MA mice, we investigated mechanisms of tumor progression. Using array-CGH, we identified a recurrent 5-Mb amplification on chromosome 12 in GI tumors from MPA mice. We defined the amplicon critical interval and demonstrated that it contains a previously uncharacterized member of the Transducin enhancer of Split (TLE)/Groucho family of transcriptional co-regulators, Tle6-like, that contributes to tumor progression. Tle6-like overexpression in colon cancer cell lines increases cell proliferation, colony-formation ability, cell migration and xenograft tumorigenicity. Human TLE6D, an alternatively spliced isoform of TLE6, with a domain structure similar to Tle6-like, has functional activity similar to Tle6-like. Both Tle6-like and TLE6D interact with GI tumor suppressor, RUNX3 [34], and antagonize RUNX3 gene target tranactivation. TLE6D is overexpressed in multiple human microsatellite stable (MSS) and microsatellite unstable (MSI-H) CRCs, and TLE6D expression levels correlate with RUNX3 expression levels. Collectively, these findings provide important insights into the molecular mechanisms through which MMR-deficiency contributes to GI tumorigenesis and implicate a novel association between TLE6 isoforms and antagonism of RUNX target gene expression in CRC tumor progression.

**Results**

*Mlh3, Pms2 and Apc Mutations Cooperate to Increase Tumor Incidence, Accelerate Progression and Decrease Overall Survival*

By 9.5 months of age, MA mice develop >50% more tumors than Ap<sup>1638N</sup> mice (P<0.001; Mann-Whitney) [Figure 1A and C]. However, the relative ratios of GI adenomas to carcinomas in Apc<sup>1638N</sup> mice (65% and 35% respectively) were very similar to that seen in MA mice (70% and 30% respectively) and overall survival is not significantly affected (9.5 vs. 10.5 months). No significant effect was seen on extra-GI cancer incidence or overall survival. These data suggest the primary role of Mlh3 is in suppression of GI tumor initiation and not tumor progression.

To study the effects of combined Mlh3 and Pms2 mutations in *vivo*, we generated MPA mice. MPA mice had significantly shorter survival vs. Apc<sup>1638N</sup> or MA mice (P<0.01, Mann-Whitney test; Figure 1A, C) and developed significantly more adenocarcinomas than MA or Apc<sup>1638N</sup> mice (Figure 1B, C) (P=0.0022 MP vs. MA and p=0.0003 MP vs. Apc<sup>1638N</sup>). These are consistent with a role for Mlh3;Pms2 combined loss both to increase GI tumor initiation and accelerates progression. However, mean overall survival of MPA mice is longer than that previously seen in Mlh1<sup>-/-</sup>;Apc<sup>1638N</sup> mice [35].

**Spectrum of Apc Mutations in MA and MPA Mouse Tumors**

In *vivo* studies have alternatively suggested that Mlh3 participates in either IDL repair [13] or SBR [15]. To understand the role of Mlh3 in these processes, we used the wild type Apc allele as a tumor-associated in *vivo* reporter gene to analyze the mutation spectrum from MA GI tumors. A total of 49 tumors from MA mice and 28 tumors from Apc<sup>1638N</sup> littermates were analyzed for Apc
truncation mutations by IVTT analysis. Truncated Apc products were detected in 27 of 49 (55%) MA tumors while only 9 of 28 (32%) were found in Apc1638N tumors. The current observed incidence of Apc somatic mutations of Apc1638N tumors is in agreement with the previous results (7 of 22, 32%) [36], hence for better understanding of mutational differences between the two strains, this and the previous data for Apc1638N tumors were combined and used for further comparisons. This 23% increase in somatic Apc mutations in MA mice was significant (P<0.0048; Fisher exact test) and was attributable to increased small insertion/deletion frameshift mutations (62.5%) vs. Apc1638N (33.3%) mice (P<0.001; Fisher exact test; Figure 2B and Tables 1 and 2). MA
mice had one recurrent insertion/deletion mutation “hotspot” also observed in Mlh1−/−Apc1638N mice (amino acid 1464) (Figure 2A). Furthermore, examination of the sequences surrounding each Apc mutation site in MA tumors showed that, unlike in other mismatch repair deficient tumors such as Mlh1−/−;Apc1638N or Msh6−/−; Mlh3−/−;Apc1638N [32,33], about 40% of frameshift mutations occurred at non-repetitive sequences within the Apc coding region. These data are consistent with a primary in vivo role for Mlh3 in DNA repair of small insertion/deletion mutations in GI epithelial cells.

We also studied the tumor-associated Apc mutations in GI tumors from MPA mice. The overall incidence of Apc truncation mutations in MPA tumors were similar to that observed in MA tumors, yet the nature of mutations characterized was distinct. Compared with MA mice (37.5%), combined Mlh3;Pms2 deficiency caused a significant increase in the proportion of single-base point mutations (57.2%, P<0.01; Figure 2 and Table 2). Within the types of single-base point mutations, MPA tumors showed higher frequency of C:G→T:A transition mutations (12 of 16, 75%) compared to MA tumors (7 of 12, 58.3%). However, this
Table 1. Apc truncation mutations in intestinal tumors from MutL homolog deficient Apc\textsuperscript{1638N} mice.

| Tumor DNA samples analyzed                      | +/+ Apc\textsuperscript{1638N} | Mlh3\textsuperscript{-/-} Apc\textsuperscript{1638N} | Mlh3\textsuperscript{-/-} Pms2\textsuperscript{-/-} Apc\textsuperscript{1638N} | Mlh1\textsuperscript{-/-} Apc\textsuperscript{1638N} |
|------------------------------------------------|-------------------------------|-----------------------------------------------|-------------------------------------------------|-----------------------------------------------|
| Tumors samples with Apc truncations detected by I/TT | 50 (100%)                    | 49 (100%)                                     | 49 (100%)                                        | 44 (100%)                                     |
| Tumors samples showing \(>1\) mutant allele     | 16 (32%)                      | 27 (55%)                                      | 25 (51%)                                         | 37 (84%)                                      |
| Total Apc mutations characterized by sequencing | 18 (100%)                     | 32 (100%)                                     | 28 (93%)                                         | 91 (100%)                                     |

\*Combined with previous data from Wong et al 2002 [36].
\*Data from Kuraguchi et al 2000 [32] for comparison.

Mechanisms of MMR Suppress GI Tumorigenesis

Apc mutation is thought to be an early event in CRC carcinogenesis. The significantly increased number of adenocarcinomas vs. adenomas seen in MPA vs. MA or Apc\textsuperscript{1638N} mice suggested that MPA tumors have accelerated tumor progression. While there is extensive evidence that increased mutation rates and decreased apoptosis contribute to MMR defective CRC, it is likely that additional mechanisms participate in tumor progression as well. Because chromosomal and segmental aneuploidy has been described in a subset of MMR deficient adenocarcinomas [37,38,39], we performed array comparative genomic hybridization (aCGH) analyses of GI tumor vs. E18.5 C57BL/6 embryonic control DNA from Apc, MA, and MPA mice to identify specific genetic changes that accelerate MPA GI tumor progression. Comparison of aCGH profiles revealed a recurrent 5-Mb base pairs amplification on chromosome 12F2 (66.7%–83.3%; see Table 3 for detail; Figure 3A and B) in MPA GI tumors not seen in Apc\textsuperscript{1638N} or MA tumors (Figure S1). To define the critical interval for this amplification on chromosome 12F2 we bred a new cohort of MPA mice and quantified copy number variation in the tumor region and two flanking genes, we identified one gene that showed recurrent increased level of genomic DNA in tumor tissue (Figure 3C). Transduction-like enhancer protein 6-like, (Tle6-like), TLE family members act as transcriptional corepressors [40,41] without any intrinsic DNA-binding activity. They are recruited to specific gene regulatory sequences in a context-dependent manner by forming complexes with different DNA-binding transcription factors. Two evolutionarily conserved domains define the TLE gene family: an N-terminal glutamine-rich (Q) domain that mediates TLE family member heterodimerization, and a C-terminal domain of WD motif repeats that mediates direct interactions with sequence specific DNA binding transcription factors [41,41]. Previously TLE family members have been described containing only the Q domain, such as Grg6/Tle6 [42], or only the WD repeat motif, such as Grg6/Tle6 [43]. Tle6-like similarly contains only the C-terminal WD repeat domain and had highest amino acid identity (84.4%) with TLE6 (Figure 4A and Figure S2).

RNA and Protein Expression Levels of Tle6-like Are Increased in MPA Tumors

To understand the impact of gene amplification on Tle6-like expression, we isolated total RNA from tumor and normal tissues from MPA mice and used qPCR to quantify relative Tle6-like mRNA expression. As a result of copy number amplification, Tle6-like mRNA levels were significantly increased in tumors compared with adjacent normal GI tissue (Figure 3D). To understand whether Tle6-like protein levels are subsequently increased, we generated anti-Tle6-like specific antisera. Western blot analysis with this antisera demonstrated that Tle6-like protein levels are significantly increased in GI tumors compared to surrounding normal GI epithelial tissue from MPA mice (Figure 3E). Overall, these data suggest increased genomic DNA copy number of Tle6-like causes increased mRNA and protein expression of Tle6-like in MPA tumors.

Expression Level of TLE6 Alternative Spliced Isoform D (TLE6D) Is Increased in Human Colorectal Tumors

Gene diversity can be generated by several mechanisms, including gene duplication and paradigm evolutionary divergence, and the generation of alternative mRNA splice isoforms that modify coding sequence. The mouse Tle6-like-containing amplicon is syntenic to human chromosome 14q33, but amplification of this chromosomal region is not associated with CRC. Upon further analysis, we discovered that 14q33 contains no human ortholog of mouse Tle6-like, or any other TLE family member. However, when we analyzed TLE6 mRNAs bioinformatically, we identified a previously identified alternative spliced isoform of TLE6 (TLE6D) (Genbank Accession #BX375733) that contains only the C-terminal WD repeat domain of TLE6, and therefore has the same domain structure as mouse Tle6-like (Figure 4B) To understand expression of TLE6D (full-length isoform) and TLE6D in human CRC, we generated three sets of RT-PCR primers: one for the TLE6D N-terminus, one crossing the splice junction that is specific for TLE6D and one that detects TLE6A but not TLE6D (Figure 4B). We then calculated expression of these transcripts in 40 human CRC samples and normal tissue. Compared to adjacent normal tissue, the TLE6D-specific and TLE6 C-terminus qPCR showed significantly increased expression in a subset of human CRCs (Figure 5A), but not for the TLE6 N-terminal or TLE6A qPCR (data not shown). These data suggest that the TLE6D isoform specifically is overexpressed in a subset of human CRCs.
Table 2. Sequences of Apc mutations in MutL homolog deficient Apc1638N tumors.

| Codon | Mutation | Consequence | Wild-type Sequence | Apc1638N | Mlh3 /−/ Apc1638N | Mlh3 Pms2 /−/ Apc1638N |
|-------|----------|-------------|-------------------|----------|-------------------|--------------------------|
| 769   | G→T     | Glu→Stop   | TTA TCA GAA ACC TTC | -        | 1                 | -                        |
| 778   | ΔT       | frameshift  | AAC CTA AGT CCC AGG | -        | -                 | -                        |
| 803   | C→T     | Arg→Stop   | GCC AAT CCA CAT GAT | -        | 2                 | -                        |
| 808   | ΔG       | frameshift  | GAT AGT AGG TCA GAC | 1        | -                 | -                        |
| 819   | ΔTG      | frameshift  | ATG ACT TTG TTC TCA | -        | 1                 | -                        |
| 847   | ΔAC      | frameshift  | GAG AAA GAC AGA AGT | -        | 1                 | -                        |
| 848   | ΔAG      | frameshift  | AAA GAC AGA AGT TTG | -        | 1                 | -                        |
| 853   | ΔAG      | frameshift  | GAG AGA AGA CGA GGT | -        | 1                 | -                        |
| 854   | C→T     | Arg→Stop   | GAG AGA AGA CGA GGT | -        | 1                 | 4                        |
| 866   | G→T     | Glu→Stop   | ACA ACA GAA AAT GCA | -        | -                 | 1                        |
| 871   | ΔTC      | frameshift  | GGA ACC TCA TCA AAA | -        | 1                 | -                        |
| 872   | C→A     | Ser→Stop   | ACC TCA TCA AAA CGA | -        | -                 | -                        |
| 874   | C→T     | Arg→Stop   | TCA AAA CGA GGG CTTG | 1        | -                 | 4                        |
| 884   | C→T     | Gln→Stop   | GCA GCC CAG ATG GCC | -        | -                 | 1                        |
| 902   | ΔAG      | frameshift  | GAC GAC AGA AGT TCT | -        | 1                 | -                        |
| 909   | G→T     | Glu→Stop   | ACC ACC GAG TTC CAT | -        | 1                 | -                        |
| 913   | ΔTG      | frameshift  | CAT TGT GCA GAC | -        | 1                 | -                        |
| 921   | C→T     | Arg→Stop   | GGC GCA AGA AGC | 3        | 2                 | -                        |
| 933   | C→A     | Tyr→Stop   | AAC ACA TAC AAT TCA | 1        | -                 | -                        |
| 934   | +TACA    | frameshift  | AAC ACA TAC AAT TCA | 1        | -                 | -                        |
| 939   | G→T     | Glu→Stop   | AAG TCG GAA AAT TCA | 2        | -                 | 1                        |
| 944   | Δ8bp     | frameshift  | TCAATTTAGCGATAGCTTTGCTTCATGCGCAAATGTTCA TCT TCA AAT | 1        | -                 | -                        |
| 956   | C→T     | Arg→Stop   | TAT AAA CGA TCT TCA | 1        | 1                 | 1                        |
| 959   | ΔA       | frameshift  | TCT TCA AAT GAC AGT | -        | -                 | 1                        |
| 974   | ΔAA      | frameshift  | GGT AAA AGA GCC CAA | 1        | -                 | -                        |
| 974   | ΔGA      | frameshift  | GGT AAA AGA GCC CAA | 1        | -                 | -                        |
| 984   | T→A     | Tyr→Stop   | GAA TCC TAT TCT GAA | -        | 1                 | -                        |
| 992   | +T       | frameshift  | AAA TTT TGC AGT TAT | -        | 2                 | -                        |
| 992   | Δ8bp+A   | frameshift  | AAA TTT TGC AGT TAT | 1        | -                 | -                        |
| 1004  | ΔC       | frameshift  | GAC CTA GCC CAT AAG | -        | 1                 | -                        |
| 1018  | G→T     | Glu→Stop   | GAT GGA GAA CTTG GAT | 1        | -                 | -                        |
| 1025  | C→A     | Tyr→Stop   | ATA AAT TAC AGT CTT | -        | 1                 | -                        |
| 1025  | ΔAC      | frameshift  | ATA AAT TAC AGT CTT | 1        | -                 | 2                        |
| 1047  | G→A     | Tyr→Stop   | GAA AGG TGG GCA AGA | 1        | 1                 | 1                        |
| 1127  | +T       | frameshift  | CAG TCT TCT GCT CAG | 1        | -                 | -                        |
| 1127  | ΔCT      | frameshift  | CAG TCT TCT GCT CAG | 1        | -                 | -                        |
| 1128  | ΔGT      | frameshift  | TCT TCG TGG TGC GAA | -        | 1                 | -                        |
| 1141  | C→A     | Tyr→Stop   | ACC AAT TAC AGT GAA | 1        | -                 | -                        |
| 1143  | G→T     | Glu→Stop   | TAC AGT GAA CTT TAT | 1        | -                 | -                        |
| 1154  | G→T     | Glu→Stop   | GAA GAA GAA GAA GAG | 1        | -                 | -                        |
| 1157  | ΔGA      | frameshift  | GAA GAG AGA CCG ACA | 1        | -                 | -                        |
| 1211  | ΔTC      | frameshift  | CAT CTC TCT CCA AGC | -        | 1                 | -                        |
| 1219  | ΔG       | frameshift  | ACA GCT GTA CCA CCA | -        | -                 | 1                        |
| 1227  | C→T     | Gln→Stop   | AAA AGG CAG AAT CAG | -        | -                 | 1                        |
| 1227  | C→A     | Cys→Stop   | TCA AGG TGC AGT TCA | -        | -                 | 1                        |
| 1234  | C→A     | Ser→Stop   | CCA AGT TCA GCA CAA | -        | 1                 | -                        |
| 1244  | ΔG       | frameshift  | CAA AAA GAC ACT AGT | -        | -                 | 1                        |
| 1370  | ΔA       | frameshift  | ACA CCC AAA AGT CCC | 1        | -                 | 1                        |
| 1464  | ΔAG      | frameshift  | GAG AGA AGA GAG AGT | -        | 5                 | 4                        |

*Previous data from Wong et al 2002.

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**Tle6-like and TLE6D Enhance Cell Proliferation, Colony Formation, and Cell Migration**

Because GI tumors from **ApC** mice showed increased number of adenocarcinoma than **ApCA** or **MA** mice, we evaluated whether increased levels of **Tle6-like** can contribute to mechanisms that underlie tumor progression. We generated stable cell line 293 cells that express **Tle6-like** or **TLE6D**. For both **Tle6-like** and **TLE6D** overexpressing cell lines, proliferation rates were significantly increased compared with vector-transfected control cells (Figure 6A). Similar results were also seen in HCT116 and 3T3 cells (data not shown). We next tested the effect of **Tle6-like** or **TLE6D** expression on the ability to form colonies in vitro. Mouse embryonic fibroblasts transfected with **Tle6-like** or **TLE6D** significantly increased colony formation (four-fold and two-fold, respectively) compared with empty vector-transfected control cells (Figure 6B and C). We also tested the mobility of the cells transfected with **Tle6-like**/**TLE6D** by in vitro migration assay. Cell lines stably expressing **Tle6-like** or **TLE6D** were able to migrate a significantly longer distance when compared with control cell lines expressing only the vector (Figure 6D). In contrast, no effect of **Tle6-like** or **TLE6D** ectopic expression was seen on induction or resistance of apoptosis induced by serum-depletion in culture medium (data not shown). In summary, these results are consistent with a proliferation and migration advantage for tumor cells expressing **Tle6sh** or **TLE6D**.

**Tle6-like and TLE6D Expression Increases Xenograft Tumor Proliferation in vivo**

Because **Tle6-like** or **TLE6D** ectopic expression increased cell proliferation and migration in vitro, we evaluated their impact in vivo. We injected HCT116 cells stably expressing **Tle6-like**, **TLE6D** or vector s.c. into nude mice and quantified tumor growth. As expected, HCT116 cells transfected with vector formed xenograft tumors. In parallel, HCT116 cells expressing **Tle6-like** and **TLE6D** formed significantly larger tumors (Figure 7). These results suggest that **Tle6-like** and **TLE6D** expression increases CRC cell proliferation and growth, in vivo.

**Tle6-like and TLE6D Interact with the RUNX3 GI Tumor Suppressor and Antagonize RUNX3 Mediated Transactivation**

**RUNX** genes encode transcription factors that activate or repress transcription of key regulators of growth, survival and differentiation pathways [44,45]. This gene family is defined by the Runt domain, which mediates both protein-DNA and protein-protein interactions with transcriptional co-regulators. **TLE** proteins interact with, and regulate the function of, **RUNX** proteins through direct interactions between the **TLE** **WD** domain and the Runt domain and the interactions antagonize **RUNX3**-mediated transcription [44,45,46,47,48]. **RUNX3** has been shown to play important roles in GI epithelial cell development and tumorigenesis. Loss of **RUNX3** predisposes knockout mice to gastric hyperplasia, indicating a tumor suppressor role for this gene [34,49,50,51,52]. In human gastric cancers, hypermethylation of **RUNX3**, hemizygous deletion and truncating point mutations have been observed [34,52,53,54,55,56,57,58]. To test whether **Tle6-like**/**TLE6D** interact with **RUNX3**, we first evaluated sub-cellular localization using immunofluorescence staining in 293 cells co-transfected with **Tle6-like** or **TLE6D** and native **RUNX3** (Figure S3). Using anti-Myc, anti-Xpress and anti-RUNX3 antibodies, we observed that highest levels of **Tle6-like** and **TLE6D** and are in the nucleus overlapping with nuclear **RUNX3** staining. Furthermore, in 293 cells, transiently co-transfected with **Tle6-like** or **TLE6D**, endogenous **RUNX3** co-immunoprecipitated with anti-Myc or anti-Xpress antibodies (Figure S8 and S8), suggesting an interaction between **Tle6-like**/**TLE6D** and **RUNX3**. Similar co-localization and co-immunoprecipitation results were seen in HCT116 and 3T3 cells (data not shown). Finally, to evaluate the functional consequences of **Tle6-like**/**TLE6D** interaction on **RUNX3** transcriptional regulation we used a well characterized **RUNX3** transcriptional reporter construct targeting osteocalcin (OC), fused to a luciferase reporter gene [47]. As expected, transfected **RUNX3** activated luciferase expression in 293, Hela or HCT116 cells (Figure 8C, lane 1 and 2). Co-transfection of **Tle6-like** or **TLE6D** decreased **RUNX3** transcriptional reporter activity in a dose-dependent manner (Figure 8C), whereas **Tle6-like**/**TLE6D** transfection had no effect on promoters lacking **RUNX3** binding sites, such as the TOPFLASH/FOPFLASH system (data not shown). Taken together, these results are consistent with a model whereby **Tle6-like**/**TLE6D** expression antagonizes **RUNX3** GI tumor suppressor mediated target gene transactivation through an interaction between the **Tle6-like**/**TLE6D** and **RUNX3**, providing a selective growth advantage for cell proliferation and migration.

**TLE6D is Highly Expressed in Human CRCs with High RUNX3 Expression Levels**

In gastric cancer, **RUNX3** activity is most commonly reduced through a mechanism involving **RUNX3** promoter hypermethylation and subsequently decreased mRNA expression. However, its expression levels in CRC have not been well characterized. We therefore used qPCR to evaluate **RUNX3** expression in 40 human CRC and matched normal GI epithelial samples, normalized to **GAPDH** expression. In many CRCs, **RUNX3** expression is low, consistent with a role in GI tumor suppression. However, in a subset of CRCs **RUNX3** expression is paradoxically increased (Figure 5B). To test whether elevated **TLE6D** expression is associated with **RUNX3** activation, we used qPCR to analyze **TLE6D** expression levels in the same matched sets of CRCs and normal mucosa. We observed a clear correlation of **RUNX3** and **TLE6D** expression levels (R = 0.723; Figure 5C). However, at the same time no clear correlation was seen for **RUNX3** and **TLE6D** expression levels with regard to MSI-H/MSSS status or for expression levels of the full length **TLE6D** and **RUNX3** (data not shown). Overall, in combination with the functional antagonism of **RUNX3** activity by **TLE6D** observed in colon cancer cells, the correlation of **RUNX3** and **TLE6D** expression in human CRCs suggests that **TLE6D** may interact with the **RUNX3** GI epithelial tumor suppressor and inactivate **RUNX3** in a subset of CRCs independent of MSI status. However, further experiments will be required to analyze the association between **RUNX3** and **TLE6D** expression levels and functional interactions in more detail.

**Discussion**

Because **APC** is a common mutation target in MMR-deficient CRC, we created novel mouse models combining different mutations in these genes to analyze their roles in MMR-deficient GI carcinogenesis and progression. The observation that **MA** mice have increased tumor multiplicity but no accelerated tumor progression or decreased survival vs. **ApCA** mice suggests a primary role for the Mlh1–Mlh3 heterodimer in suppression of GI tumor initiation. While previous in vitro studies have alternatively suggested that Mlh1–Mlh3 participates in IDL repair [13] and SBR[15,59], our study provides the first in vivo evidence that **Mlh3** deficiency significantly increases IDL mutation frequency. This type of mutation occurred both at repetitive and non-repetitive **ApC**
sequences, implicating its role in repair of both types of IDL (Figure 2). Previous studies of Pms2-/-;Apc1638N mice have shown a primary role for Mlh1-Pms2 in GI tumorgenesis suppression but not tumor progression[60]. We therefore combined these mutations to create MPA mice. Like Mlh1-/-;Apc1638N mice, MPA mice have significantly increased GI tumor multiplicity, accelerated tumor progression and decreased overall survival[61] . MPA tumors harbor proportionally more C:G→T:A (at either CpG or CpNpG sites) transition mutations than MA tumors, showing recurrence in certain arginine codons, one of which was at Apc codon 854, a SBR hotspot that was also previously seen in Mlh1-/-;Apc1638N mice.

In addition to Mlh1-Pms2 and Mlh1-Mlh3, several lines of evidence from our study suggest a potential role for Mlh1-Pms1 in suppression of GI tumorgenesis. First, MPA mice have later mean GI tumor onset compared to previous studies of Mlh1-/-;Apc1638N mice[32]. Second, the multiplicity of GI tumors is decreased vs Mlh1-/-;Apc1638N mice. Third, two Apc insertion/deletion muta-
tion hotspots seen in Mlh1<sup>−/−</sup>; Apc<sup>TG53N</sup> mice have not been detected in MPA tumors. These data are consistent with previous studies of yeast Mlh2p (orthologue of mammalian PMS1) that demonstrate a minor role for this protein in IDL repair [62].

Because the combination of Mlh3, Pms2 and Apc mutations accelerates tumor progression, we searched MPA GI tumor specific genetic changes associated with progression using high-resolution aCGH. MPA tumors contained a recurrent 5-Mb amplicon with a critical interval containing a novel, poorly characterized member of the TLE family of transcriptional corepressors, Tle6-like. Unexpectedly, this MPA recurrent amplification hotspot is not detected by aCGH in GI tumors from Mlh1<sup>−/−</sup>; Apc<sup>1638N</sup> mice (data not shown). The reason for this difference is unclear, but again suggests that Mlh1-Pms1 may play a role in causing chromosomal instability.

**TLE** genes are the mammalian homologues of *Drosophila* groucho that play critical roles in a wide range of developmental and cellular pathways [40]. TLE proteins are transcriptional corepressors for specific families of DNA-binding transcription factors, including RUNX proteins[48]. In addition, Tle1/Grg1 has been shown to act as a lung-specific oncogene in a transgenic mouse model [63]. Mouse Tle6/Grg6 has been shown to synergize with the E2A-HLF oncoprotein in antagonism of Runx1 transactivation in murine pro-B cells, causing acute leukemogenesis [64]. Tle6/Grg6 also participates in developmental mechanisms of neurogenesis [43]. Here, we provide data that a previously uncharacterized TLE family member containing only the WD repeat domain, Tle6-like, has amplified gene copy number, mRNA and protein levels in GI epithelial tumors from MMR deficient/Apc mutant mice, and is associated with accelerated tumor progression. Consistent with this observation, in functional studies Tle6-like/TLE6D enhances cell proliferation, colony-formation, migration and xenograft tumorigenicity. While TLE family members have previously been shown to repress Wnt/β-catenin signaling [42,65,66,67], we were unable to demonstrate any Tle6-like/TLE6D protein-protein interactions with β-catenin or effect of Tle6-

**Table 3. Amplifications of Chromosome 12.**

| Chromosome | Change | Start Position | Start Band | End Position | End Band | Frequency |
|------------|--------|----------------|------------|--------------|----------|-----------|
| 12         | Gain   | 109,044,957    | 12 q F1    | 109,106,314  | 12 q F1  | 8 /12 (66.7%) |
| 12         | Gain   | 109,106,314    | 12 q F1    | 109,348,509  | 12 q F1  | 10 /12 (83.3%) |
| 12         | Gain   | 109,348,509    | 12 q F1    | 109,556,449  | 12 q F1  | 9 /12 (75%)   |

**Table 4. Primers Used in Real-Time PCR.**

| Primer | Sequences |
|--------|-----------|
| Crip-F | GGCTGCCACATTGAAAGAAT |
| Crip-F | TCACTGCAAGACACAGAT |
| Cdc5L-F | TGGCAATAATGCTGCTTGAGG |
| Cdc5L-F | TGCCCTTCCCTAAAGCTCT |
| Myef2L-F | CATGGTCAAGCCCATTACAA |
| Myef2L-F | GACCTCTCTGTGTCTGAGT |
| Tle6_F | ACACTATTTAGGCCCTAAGTTCCTC |
| Tle6_R | AGCTATGCCATAGCTGACAGT |
| Adam6-F | CACCTGCAATCTGTTAAA |
| Adam6-R | GACATGGCCATGACAGG |
| Igh-a_F | AGCGAGTCGAGTCGTGAGACAGG |
| Igh-a_R2 | TGCTCTCAGAAGGTTTTAGT |
| U110086690_F | ATGGAAAGTGGTGGGTTTC |
| U110086690_R | TTGTCATCGGAGACCCGT |
| Vipr2_F | GTGACGACAGCTGACCAG |
| Vipr2_R | CTCTTCGTATTGCCGTTGG |
| Alkbh-F | GTAAGGCTCCCCAGAGTGC |
| Alkbh-R | CTGTCGAGCTGGTGAAATTG |

**Figure 4. TLE Family.** Schematic diagram of TLE protein family members. (A) Numbers indicate amino acids: Q, glutamine rich domain; GP, glycine/praline rich domain; CcN, domain containing putative phosphorylation sites and putative nuclear localization signal; SP, serine/praline rich domain; WD repeats, domain containing series of tandem repeats of tryptophan and aspartic acid residues. (B) Diagram of **TLE6** RNA and alternative splice form **TLE6D** is indicated. **TLE6A** is the full length mRNA transcript. Orange bars indicate position of primer sets for qPCR. Arrows indicate exon-exon junctions.

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**Table 4.**
like/TLE6D overexpression on β-catenin reporter gene activity using TOPFlash in transient transfection in colon cancer cell lines (data not shown), suggesting that Tle6-like/TLE6D might not be involved in canonical Wnt pathway.

RUNX family genes regulate lineage and stage specific gene transcription by direct binding to DNA promoters and enhancer elements [44,45]. Loss of Runx3 in the mouse results in the development of gastric mucosal hyperplasia, decreased apoptosis and attenuated TGF-β anti-proliferative signaling. Consistent with previous observations of interactions between RUNX3 and TLE family members mediated through the Runt and WD repeat domains, respectively [46,48], we detected an interaction between RUNX3 and Tle6-like/TLE6D by co-immunoprecipitation. Furthermore, we demonstrated that Tle6-like/TLE6D antagonized RUNX3 regulated transcriptional targets. However, while these experiments show an association between RUNX3:TLE6D interactions and tumor progression, they do not demonstrate mechanistically the functional importance of this interaction in accelerating tumor progression.

Figure 5. TLE6D and RUNX3 mRNA expression levels in human colorectal cancers. (A,B) Dotplots of TLE6D and RUNX3 expression levels for each of the 40 samples. Expression is shown as fold elevation vs. accompanying normal adjacent mucosa. (C) Superimposed plot of TLE6D and RUNX3 expression levels by sample (Pearson correlation 0.724; Pvalue<0.001). (D) A scatterplot of log-transformed RUNX3 by TLE6D expression levels along with the least squares estimate of the regression of RUNX3 on TLE6D. Expression levels were log-transformed in the regression analysis due to heavy skewness. Based upon the regression, it was estimated that the geometric mean of RUNX3 expression increased 0.525 with a 2-fold increase in TLE6D expression (95% CI: 0.165, 0.563; p Value<0.001).

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Alternative mRNA splicing allows multiple gene products to be produced from a single coding sequence, and through this mechanism a higher diversity of mammalian genes is generated [68]. Several distinct TLE/Grg gene alternative splice forms, such as Grg-1s, QD of TLE4, and Grg3b [42,69,70], have been reported. While the human genome does not encode a TLE6-LIKE ortholog, a structurally equivalent protein, TLE6D, is generated through alternative splicing. The observation that GI adenocarcinomas from both humans and mice use two very distinct mechanisms to amplify Tle6-like/TLE6D activity suggests a strong growth advantage and selective pressure for this TLE isoform in tumor progression. Similarly, the correlation between TLE6D and

![Figure 6. Tle6-like and TLE6D Enhance Cell Proliferation, Colony Formation, and Cell Migration.](image)

(A) HCT116 Cell proliferation MTT assay. (B) Representative picture of plates of colony formation assay on MEFs transfected with vector, TLE6D, and Tle6-like. (C) Plot of number of colonies from colony formation assay. (D) In Vitro Cell Mobility Assay. "Wound" was generated by razor blade, clearing the adherent cells on the right side of the slides. Black lines indicate the edge of the "wound". Representative pictures from HCT116 cells transfected with vector, TLE6D, and Tle6-like are shown.

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RUNX3 expression in human CRC suggests a model whereby RUNX3 inactivation by TLE6D could be an important factor driving this growth advantage in both MSI-H and MSS CRC. Future studies will be required to understand the mechanistic implications of the interaction between these two proteins in CRC progression in more precise detail.
Figure 8. Tle6-like and TLE6D Antagonize RUNX3 Gene Target Transcription. (A and B) Tle6-like/TLE6D interacts with endogenous RUNX3 in 293 cells. Left panel represents the immunoblot of protein extracts before immunoprecipitation. Right panel represents the immunoblot after the immunoprecipitation. (C) Luciferase reporter assay. Cells were transient transfected with indicated plasmids and relative luciferase activities were determined the next day. (Error bars indicate the standard deviation of the mean).

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Materials and Methods

Mouse Lines and Survival Analyses, Tumor Analysis, and Apc Mutation Analysis

Wild-type (Wt), Pso2+/− and Mlh3+/− mice were maintained on the 129 Sv/Ev genetic background and intercrossed to generate Mlh3+/−;Pso2+/− mice as described before [13]. Ape1/+ mice were backcrossed four times to 129 Sv/Ev and subsequently intercrossed with Mlh3+/−;Pso2+/− to generate Mlh3+/−;Ape1/+ and Mlh3+/−;Pso2+/−;Ape1/+ mice. Kaplan-Meier survival curves were generated and statistical significance between genotypes was determined using the Log Rank test as previously performed [13]. All lines of mice were necropsied when they became moribund or moribund. Sacrificed mice were surveyed for tumors and suspicious masses were histology analyzed as previously performed. Statistical analyses of tumor onset and incidence among the different mouse lines were performed using the Mann-Whitney test as previously described [23,29,33,55,71,72,73,74,75,76]. Tumors from stomach, small intestine, and colon were cut into two parts. One part of the tumor was processed for histopathological analysis and the other part was used for DNA/RNA extractions. Genomic DNA samples were extracted using Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN) and subjected to mutational analysis of Apc gene between codons 677–1674 as previously described [33].

Array Comparative Genomic Hybridization Analysis

Genomic DNAs were isolated from tumor tissue and tail tissue from each mouse using PUREGENE DNA Isolation kit (Gentra Systems, Minneapolis, MN). DNAs were digested with PstI and subsequently purified using the QIAquick PCR Purification kit (Qiagen). The quality of the DNA samples was evaluated using the Agilent 2100 BioAnalyzer. The purified fragmented DNA samples were random-prime labeled with either Cy5 or Cy3 and hybridized as previously described [77].

Briefly, for each labeling reaction, 2 µg of purified digested DNA were used. Each sample was dye-swap labeled for hybridization to mouse 70-mer oligonucleotide microarrays (Agilent Technologies, Palo Alto, CA) containing 20,281 clones. After hybridization, the arrays were scanned using an Agilent Microarray DNA scanner (Agilent Technologies) and the spot intensity was extracted from slide images using Agilent Feature Extraction Software 7.0. The data were further analyzed using the procedures of Automatic Data Analysis Pipeline (ADAP). Only spots with fluorescence intensities statistically different from the surrounding background (P<0.001) were considered reliable, taking up >85% of total spots on the chip. For further analysis the fluorescence intensity values of reliable spots were transformed to log2. To minimize the effect of the variations, the log2 intensity ratios of remaining spots were subjected to normalization by Lowess fitting. Gene copy number changes for each sample was calculated by taking the median of the normalized log2 intensity ratios of dye-swatched chip experiments for the corresponding sample. The gene copy numbers were ordered along chromosomes by the map positions of corresponding genes. To eliminate systematic noise, gene copy number changes (log2Ratios) along the chromosomes were smoothed by taking a moving median of symmetric 5-nearest neighbors, followed by Lowess fitting (f = 0.2). The mean and standard deviation (SD) of smoothed log2Ratios for all genes in the all samples were calculated. The copy number profiles of at least 5 consecutive genes that deviated significantly above mean+3SD were interpreted as regional gains, below mean-3SD as regional losses. The threshold for whole chromosomal gain/loss was mean±2SD. The ideograms of chromosomal aberrations were drawn using mapping information of cytogenetic bands to the mouse genome (NCBI Mapview Build 32).

cDNA Preparation and Real-Time PCR

For RNA extractions, Trizol reagent (Invitrogen) was used to isolate total RNA. RNA were further digested with RNase-free DNaseI (Promega) and cleaned with RNeasy Mini kit (Qiagen). High Capacity cDNA Archive kit from Applied Biosystems was used to make cDNA from the RNA samples. Real-time quantitative PCR was performed with either SYBRGreen PCR master mix or Taqman PCR master mix (Applied Biosystems) following the manufacturer’s protocol on ABI 7900 machine. Primers used for SYBR Green assays are listed in Table 1. Each gene was normalized to the internal control gene Gapdh and then compared to a known single copy gene Alkbh, which is located on non-amplified region on chromosome 12 D3 in the MPA tumors.

Generation of Tle6-Like Antibody

The whole Tle6-like gene (encoded 240 amino acids) was cloned into pET28b vector and Tle6-like protein was induced and purified from E. coli. Rabbit anti-serum was raised against Tle6-like protein. The anti-serum was further purified using affinity column, in which Tle6-like protein was covalently bound to CNBr-activated Sepharose 4B (Sigma). The purified antibody was used in immunoblotting at 1:100 dilutions.

Cell Culture

HCT116, 293, Hela or 3T3 cells were maintained in DMEM with 10%FBS and transfected using Lipofectamine 2000 (Invitrogen). The human isomorf TLE6D cDNA clone was purchased from Invitrogen (Full-length Human Clones CS0DC017YC05; Accession number BX375733). Tle6-like was cloned from cDNA samples from MPA mice. We subcloned Tle6-like and TLE6D into either Xpress-epitope-tagged pcDNA6/HisA vector (Invitrogen) or Myc-tagged pCS2+MT vector. Cells were transfected with following plasmids: pcDNA6/HisA, pcDNA6/HisA-Tle6-like, pcDNA6/HisA-TLE6D, pCS2+MT, pCS2+MT-Tle6-like, pCS2+MT-TLE6D. Stable cell lines from each transfectant were generated with the selection medium containing 10 µg/ml blasticidin (Calbiochem) for 10 days. The pooled populations of cells that survived were used in the experiments for MTT assay and cell mobility assay. The transient-transfected cells were used for colony formation assay, immunoprecipitation, and reporter assay.

MTT Cell Proliferation Assay and Colony Formation Assay

For the cell proliferation assay, 4000 cells were plated in 96-well plates and MTT assay were used to determine the cell numbers in a time-course experiment. Briefly, cells were washed with PBS and treated with 5 µg/ml MTT ([3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide]Sigma, St. Louis, MO) for 5 hours. After removal of MTT, DMSO was added to dissolve the dark purple formazan crystals in the viable cells and absorbance of 600 nm were determined by a multiwell scanning spectrophotometer. The cell numbers were calculated with a control standard curve. For colony-formation assay, MEF cells were seeded in 6 well plates and transient-transfected with 1 µg of the respective plasmids in the next day. After 24 h, cells were trypanized, transferred to 10-cm plates and allowed to grow with the selection medium containing 10 µg/ml blasticidin for 2 weeks. Survived cells were fixed in 30% ethanol and stained with 0.25% methylene blue. Colonies containing more than 30 cells were counted. Both assays were repeated three times in three independently-derived cell lines.
In Vitro Cell Mobility Assay

The monolayer “wounding assay” was used to demonstrate the in vitro cell migration. Human colon cancer HCT116 cells stably expressing corresponding plasmids were plated on glass microscope slides and cultured to confluence. A “wound” was generated by scratching the slide with a razor blade, clearing a portion of adherent cells on the slide. Photo documentation was taken at day 4 and the migration of cells from the cut edge of the monolayer into the clear portion of the slides was assessed. Two independently-derived stable cell lines for each plasmid were used in this assay.

Antibodies, Immunoprecipitation, and Immunoblotting

Transient-transfected 293 cells in 10-cm plate were lysed with 1 ml of NP-40 lysis buffer and prepared as described before [13]. Five hundred μl of lysates were pre-cleared with 50 μl ProteinA/G agarose beads (Santa Cruz) for 1 h. After spinning down the ProteinA/G beads, the collected supernatants were incubated with 5 μg anti-Xpress or anti-myc monoclonal antibody (Invitrogen) and 50 μl ProteinA/G beads overnight at 4°C. The next day, the beads were washed with NP-40 buffer 5 times and incubate with 4 x protein loading dye (Invitrogen) 10 min at 95°C to elute the binding proteins. These samples were resolved by SDS-PAGE and the immunoblotting was used as previously described to detect the corresponding proteins. The antibodies used in immunoblotting are: mouse monoclonal anti-Xpress and anti-myc (1:2000, Invitrogen), rabbit anti-RUNX3 (1:1000, Abcam) and goat anti-β-actin (1:10000, Santa Cruz Biotechnologie).

Luciferase Reporter Assay

293, Hela or 3T3 cells were transient-transfected accordingly with the Flag-RUNX3 (a kind gift from Dr. Yoshiaki Ito) and rat Osteocalcin promoter fused to luciferase reporter construct (OC-Luci, a kind gift from Dr. Gary Stein), and plasmids as described above. Luciferase activities were determined using Dual-Luciferase reporter assay systems kit (Promega) on the luminometer.

Tumor Growth in Nude Mice

Female 6-week-old nude mice (Charles River Laboratories, Wilmington, MA) were divided into four experimental groups, five

for each. One million HCT116 cells stably transfected with vectors (pCS2+MT or pCDNA6/HisA), pcS2+MT-Tle6sh, or pCDNA6/HisA-TLE6D were injected subcutaneously in the flanks of each mouse. Mice were monitored daily for palpable tumors. Because of rapid growth, tumors were dissected out 3 weeks after injection and were analyzed.

Supporting Information

Figures S1 Array Comparative Genome Hybridization (aCGH) analysis of GI tumors. (A) Display of aCGH signal genome wide from a representative Apc1638N tumor. (B) Display of aCGH signal genome wide from a representative Mlk3−/−;Apc1638N tumor. (C) Display of aCGH signal genome wide from a representative Mlk3−/−;Pms2−/− tumor. Found at: doi:10.1371/journal.pgen.1000092.s001 (0.07 MB PPT)

Figure S2 Protein sequences of TLE family. Found at: doi:10.1371/journal.pgen.1000092.s002 (1.95 MB PNG)

Figure S3 Cellular localization of endogenous RUNX3 and transfected Myc-epitope tagged Tle6-like in 293cells. Mouse monoclonal anti-myc and rabbit anti-RUNX3 were used. Secondary FITC-conjugated anti-mouse and Cy3-conjugated anti-rabbit antibodies were used respectively. DAPI (4’,6-diamidino-2-phenylindole) staining indicates the nuclear location. Found at: doi:10.1371/journal.pgen.1000092.s003 (0.19 MB PPT)

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Author Contributions

Conceived and designed the experiments: PC SL. Performed the experiments: PC MK. Analyzed the data: PC MK KY RE DG SL. Contributed reagents/materials/analysis tools: PC JV YW WE RK SL. Wrote the paper: PC SL.

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