

## Mixed Lineage Kinase MLK4 Is Activated in Colorectal Cancers Where It Synergistically Cooperates with Activated RAS Signaling in Driving Tumorigenesis

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### Abstract

Colorectal cancers (CRC) are commonly classified into those with microsatellite instability and those that are microsatellite stable (MSS) but chromosomally unstable. The latter are characterized by poor prognosis and remain largely intractable at the metastatic stage. Comprehensive mutational analyses have revealed that the mixed lineage kinase 4 (MLK4) protein kinase is frequently mutated in MSS CRC with approximately 50% of the mutations occurring in KRAS- or BRAF-mutant tumors. This kinase has not been characterized previously and the relevance of *MLK4* somatic mutations in oncogenesis has not been established. We report that *MLK4*-mutated alleles in CRC are constitutively active and increase the transformation and tumorigenic capacity of RAS-mutated cell lines. Gene expression silencing or targeted knockout of MLK4 impairs the oncogenic properties of KRAS- and BRAF-mutant cancer cells both *in vitro* and in xenograft models. In establishing the role of MLK4 in intracellular signaling, we show it directly phosphorylates MEK1 (MAP2K1) and that MEK/ERK (MAPK1) signaling is impaired in MLK4 knockout cells. These findings suggest that MLK4 inhibitors may be efficacious in KRAS- and BRAF-mutated CRCs and may provide a new opportunity for targeting such recalcitrant tumors. *Cancer Res*; 73(6); 1912–21. ©2012 AACR.

### Introduction

The genomic landscape of cancer is highly complex with perhaps the most striking feature being the frequency of previously uncharacterized mutations. What is now acutely required is to define those mutations that play a directive role in tumorigenesis and those that inherently accumulate as a consequence of genetic instability. Such genetic lesions

can be categorized as driver and passenger or "hitchhiker" mutations, respectively. Establishing whether the mutations are fundamental drivers of disease progression or not will enable us not only to understand the basic mechanisms of tumorigenesis and the significance of their acquisition to disease progression but may provide additional therapeutic avenues. This is exemplified by the identification of mutations in kinases, such as ALK, HER2, AKT1, MEK1, and MET in lung cancer proving that genetic lesions that direct pathogenesis can be successfully exploited for cancer treatment (1). Unfortunately, despite the potential clinical relevance of such novel cancer alleles, their rate of discovery far outstrips their rate of functional validation.

Mixed lineage kinase 4 (MLK4) is the second most frequently mutated protein kinase in microsatellite stable (MSS) colorectal cancers (CRC; refs. 2, 3). The latter encompass the vast majority of sporadic CRC and are characterized, compared with microsatellite instability (MSI) tumors, by poorer prognosis (3, 4).

MLKs are a family of serine–threonine kinases thought to control multiple intracellular signaling pathways (5–7). MLKs are characterized by an amino-terminal SRC-homology domain (SH3; ref. 8) followed sequentially by a kinase domain, a leucine-zipper region and a Cdc42/Rac-interactive binding (CRIB) motif. The carboxyl terminus of all MLKs is proline-rich but diverges significantly among different members of the family, suggesting that this region serves different regulatory functions (9, 10).

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Systematic genomic analyses led to the discovery that *MLK4* is mutated in glioblastomas and CRCs suggesting that this kinase plays an important role in tumorigenesis (2, 11, 12). As nothing is presently known about the biochemical and cellular properties of wild-type (WT) and mutant *MLK4*, we analyzed its role in neoplastic cells using forward and reverse genetics as well as biologic and biochemical assays.

## Materials and Methods

### Cell culture

A-549, 293T, SW48, NIH3T3, and LoVo were obtained in 2005 from American Type Culture Collection, which conducts routine cell line authentication testing by single-nucleotide polymorphism (SNP) and short tandem repeat (STR) analysis. HCT116, Colo205, and DLD-1 were obtained in 2005 from NCI60 cell line panel (Wellcome Trust Sanger Institute, Hinxton, United Kingdom). DiFi were provided in 2005 by Prof. Baselga (Vall d'Hebron University Hospital, Barcelona, Spain). All the cell lines were tested by STR analysis (Cell ID System; Promega) to confirm their authenticity once yearly.

A-549, Colo205, and HCT116 were cultured in RPMI-1640 medium (Invitrogen); 293T, SW48, NIH3T3, and DLD-1 were grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen); DiFi and LoVo were cultured in F12 medium (Invitrogen).

### DNA constructs and mutagenesis

Full-length *MLK4* cDNA was subcloned into the pCEV29.1 (13) or into pRRL plasmid (14). Mutants of *MLK4* containing point mutations were constructed using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) with *MLK4* WT plasmid as the template DNA. The presence of appropriate mutations was confirmed by DNA sequencing. The constitutively active RAS expression vector used was pDCR-H-RasV12, kindly provided by Letizia Lanzetti (Torino Medical School, Candiolo, Torino, Italy).

### Targeted deletion of the *MLK4* locus in human cancer cells

Disruption of the *MLK4* exon 1 in CRC HCT116 cells was conducted as previously described (15). Clones were selected after 2 weeks of growth under 0.4 mg/mL geneticin (Invitrogen) selection and then propagated in the absence of selective agents. Homologous recombination events were identified by locus-specific PCR screening.

### Animal studies

All animal procedures were approved by the Ethical Commission of the University of Turin (Turin, Italy) and by the Italian Ministry of Health. Six-week-old immunocompromised *CD1<sup>-/-</sup>* nude athymic female mice (Charles River Laboratories) were injected subcutaneously in right posterior flanks. Tumor appearance was evaluated every 2 days using a caliper. Tumor volume was calculated using the formula  $V = 4/3 \times (d/2) \times (D/2)$ , where  $d$  is the minor tumor axis and  $D$  is the major tumor axis. Superficial pulmonary metastases were contrasted by black Indian ink airway infusion before excision

and were counted on dissected lung lobes using a stereoscopic microscope.

## Results

### MLK4 is mutated in colorectal cancers and glioblastomas

We and others have previously reported the identification of somatic mutations in the *MLK4* gene in CRCs (2, 12). We extended the mutational profiling of the coding region of the *MLK4* gene in additional tumor types including bladder, breast, gastric, glioblastoma (GBM), melanoma, lung, pancreas, and ovary (Supplementary Table S1). The PCR and sequencing primers are listed in Supplementary Table S2. We further established the prevalence of *MLK4* mutations in a large CRC dataset (340 samples). In addition to those previously reported, we found new somatic mutations affecting the *MLK4* gene in CRCs and glioblastomas (Table 1). Some of the mutations had not been previously reported (S322P, R442Q, and K494Q), some were found in independent samples (P843S), others were in close proximity to those previously found (R553STP), and others were identical to those previously identified (R470C; Fig. 1A). Some residues (for example R470) were affected by different aminoacidic changes. Overall, the *MLK4* mutation frequency is 3% (9 of 340) and 2% (2 of 113) in CRCs and glioblastomas, respectively.

We next determined whether the *MLK4* mutations cooccur with mutated *KRAS* or *BRAF* oncogenes, 2 major oncogenic players in CRC. To this end, we assessed the *KRAS* and *BRAF* mutational status in the entire set of CRCs and glioblastomas. We found two cases *KRAS/MLK4*-positive, two cases *BRAF/MLK4*-positive, and seven cases in which *MLK4* mutations occur alone (Table 1). We concluded that *MLK4* mutations can occur both independently but also together with *KRAS* or *BRAF* mutations.

After that, we also investigated a possible association between the occurrence of *MLK4* mutations and certain clinical parameters. To this effect, we found a correlation between *MLK4* mutations and metastatic stage ( $P = 0.0358$ ). Further analysis on a larger sample dataset is warranted to further define this correlation.

### Mutant *MLK4* cooperates with Ras in driving cellular transformation

Next, we sought to establish the role of *MLK4* mutations on cellular phenotypes associated with tumorigenesis using both forward and reverse genetic strategies. We initially used the standard NIH3T3 focus-forming assay to assess the transforming potential of WT and mutated *MLK4* alleles. A kinase-inactive *MLK4* mutant, K151A, was used as a control. Neither WT nor mutant *MLK4* triggered focus formation, whereas as expected, RAS G12V readily transformed NIH3T3 cells (Fig. 1B). This indicates that WT and mutant *MLK4* alleles are unable, *per se*, to sustain full cellular transformation. Given the cooccurrence of *MLK4* mutations with *KRAS* and *BRAF* in CRCs, we assessed whether mutant *MLK4* may cooperate with oncogenic RAS (G12V). A striking synergistic effect was observed (Fig. 1C). Importantly, while *MLK4* mutants increased the transforming potential of RAS G12V, the WT and the kinase dead did not have the same effect.

**Table 1.** Somatic mutations in the *MLK4* gene in CRCs and glioblastomas

Tumor type	Nucleotide (cDNA)	Amino acid (protein)	Residue properties	Exon	Functional domain	Mutation type	KRAS	BRAF	MSI/MSS
CRC	T964C	S322P	C, K	2	STY kinase	Missense	WT	WT	MSS
CRC	G1326A	R442Q	C	5	CC	Missense	WT	V600E	MSI
CRC	G1409A	R470H	C	5	CC	Missense	WT	WT	MSI
CRC	C1408T	R470C	C	5	CC	Missense	G13D	WT	MSS
CRC	A1480C	K494Q	—	5	None	Missense	WT	WT	MSS
CRC	C1657T	R553Stp	C	6	None	Nonsense	WT	V600E	MSI
GBM	C1663T	R555Stp	C	6	None	Nonsense	WT	NA	NA
CRC	1700delA	FS	—	7	None	Indel	WT	WT	MSI
CRC	1702insT	FS	—	7	None	Indel	WT	WT	MSI
CRC	C2788T	P843S	—	9	None	Missense	G12D	WT	MSS
GBM	C2788T	P843S	—	9	None	Missense	WT	NA	NA

NOTE: The table lists mutations in the *MLK4* gene that were confirmed to be somatic, the tumor type where they were identified, the KRAS/BRAF status and MSI/MSS status of the corresponding sample. Domains were defined according to the HPRD database (31). Abbreviations: C, residue is evolutionarily conserved; CC, coiled coil; GBM, glioblastoma; indel, insertion/deletion; K, residue is within the kinase domain; STY, serine threonine tyrosine kinase domain.

#### MLK4 mutants display increased kinase activity

To assess whether the mutations were activating in nature, we measured how they affect the kinase activity of the MLK4 protein. A mutation at position H261 was selected for subsequent studies as it is located in a region (HRDLK) highly conserved among mitogen-activated protein kinase (MAPK), and it was observed to be mutated twice (H261Y and H261Q). The G291E mutation was chosen as it is located in a region corresponding to the BRAF V600E oncogenic mutation found in multiple tumor types (16). R470C was characterized as this variant has been identified in 2 independent CRC samples. Finally, the R555STP change was selected to investigate the impact of a truncating mutation on the catalytic activity of MLK4. Lentiviral vectors were designed to transiently stably express MLK4 WT and the corresponding mutant alleles (H261Y, G291E, R470C, and R555STP; Fig. 2A). To compare the enzymatic activity of WT and mutated MLK4 proteins, we used an *in vitro* biochemical assay. The recipient cells used for these experiments was a CRC cell line in which the *MLK4* gene is genetically inactive to dissect the activity of transduced proteins in the absence of the endogenous MLK4 protein (see paragraphs later and Fig. 3B for full details on the MLK4 knockout cells). The kinase activity of WT and mutant MLK4 proteins was measured using a luminescent kinase assay. Mutated MLK4 proteins displayed increased kinase activity, albeit at different levels, as compared with WT (Fig. 2B). These results indicate that the MLK4 mutations identified in the previous analysis are indeed activating.

#### Expression of mutant MLK4 enhances the tumorigenicity of cancer cell lines *in vivo*

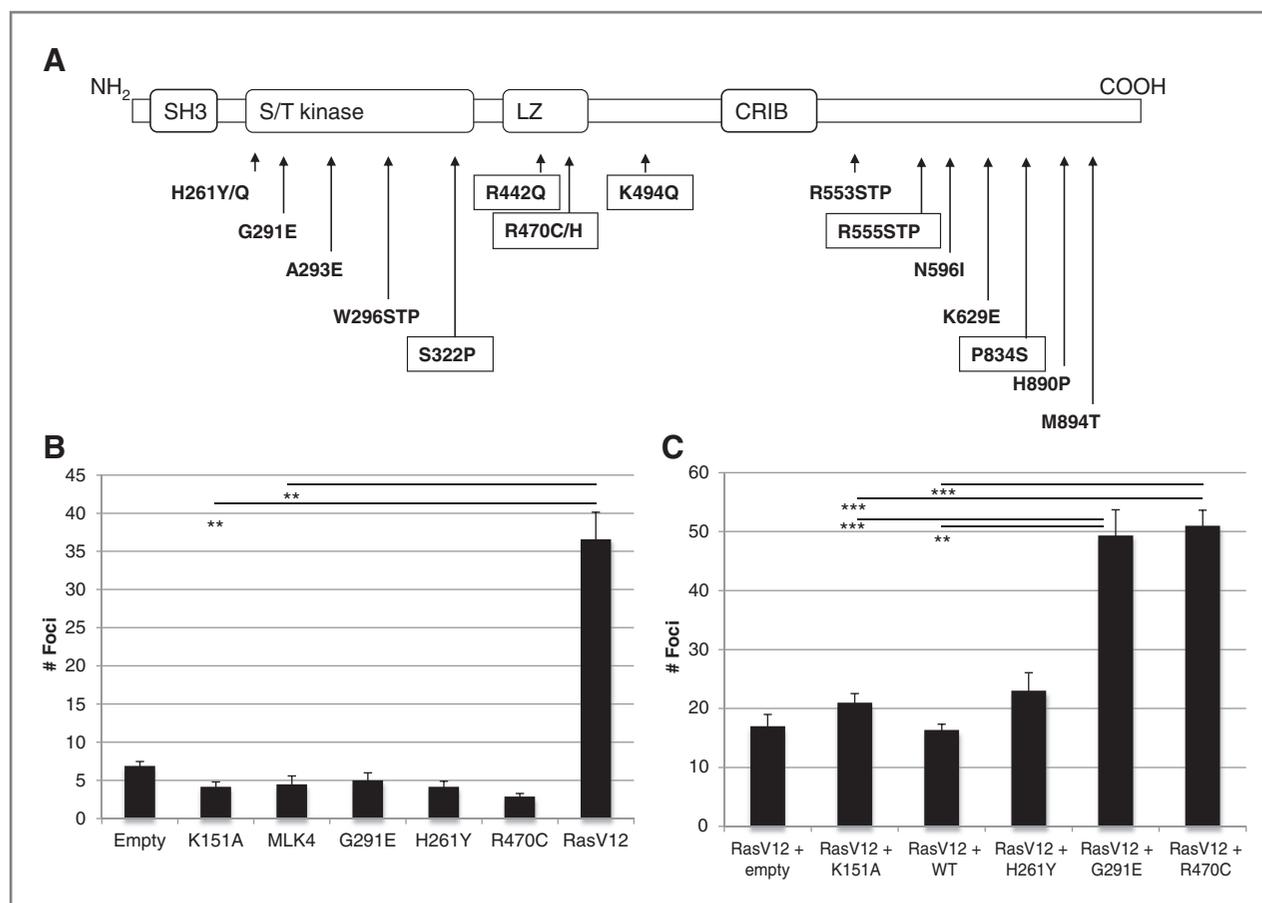
We next assessed the oncogenic potential of mutated MLK4 in the neoplastic process. We checked whether ectopic expression of WT or mutated MLK4 affected the tumorigenic prop-

erties of DLD-1, a KRAS-mutant CRC cell line. DLD-1 cells expressing WT MLK4, G291E, or R470C mutants were subcutaneously injected into immunocompromised mice. Cells expressing the empty vector were used as controls. DLD-1 cells transduced with mutant MLK4 gave rise to larger tumors compared with control cells (Fig. 2C). We then extended the experiments to another tumor type, the lung cancer cell line A549, which also harbors a KRAS mutation. Results were comparable with those obtained with the DLD-1 CRC cell lines (Supplementary Fig. S1A). Metastasis formation in the lungs of mice bearing MLK4 overexpressing tumors was subsequently quantified. We found that the number of metastases increased in MLK4 mutant expressing tumors versus controls in both DLD-1 (Fig. 2D) and A549 (Supplementary Fig. S1B) cellular models. Altogether, these observations support a role for MLK4 mutations in increasing the tumorigenicity of cancer cells carrying KRAS mutations.

#### Generation of knockdown and knockout cellular models of MLK4

As a complementary approach, reverse genetics was used to evaluate how reduced expression or deletion of the *MLK4* gene affected the tumorigenic properties of cancer cells. We first identified short hairpin RNA (shRNA) that efficiently targeted the *MLK4* sequence leading to effective and stable downregulation of its expression. Two independent MLK4 shRNAs were selected on the basis of their ability to reduce MLK4 expression in multiple cell lines derived from colon and lung cancers (Fig. 3A).

To exclude any potential off-target effects of the shRNA approach, we also established an isogenic cell line in which the *MLK4* gene was disrupted, and hence not expressed. The CRC cell line HCT116 was selected for gene targeting as it contains a KRAS mutation. Furthermore, this cell line is diploid at the *MLK4* locus and is amenable to gene targeting



**Figure 1.** Genetic and functional analysis of MLK4 mutations. **A**, schematic representation of MLK4 structure and its functional domains. The position of the somatic mutations is reported. Previously found mutations are reported in black; novel mutations are framed. **B**, focus forming assay in NIH3T3 cells transfected with WT and mutant *MLK4* alleles. Ras (V12) is used as a positive control. \*\*,  $P \leq 0.01$ ; error bars represent SD. **C**, focus forming assay in NIH3T3 cells cotransfected with mutant Ras (V12) and WT/mutant *MLK4* alleles. \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; error bars represent SD.

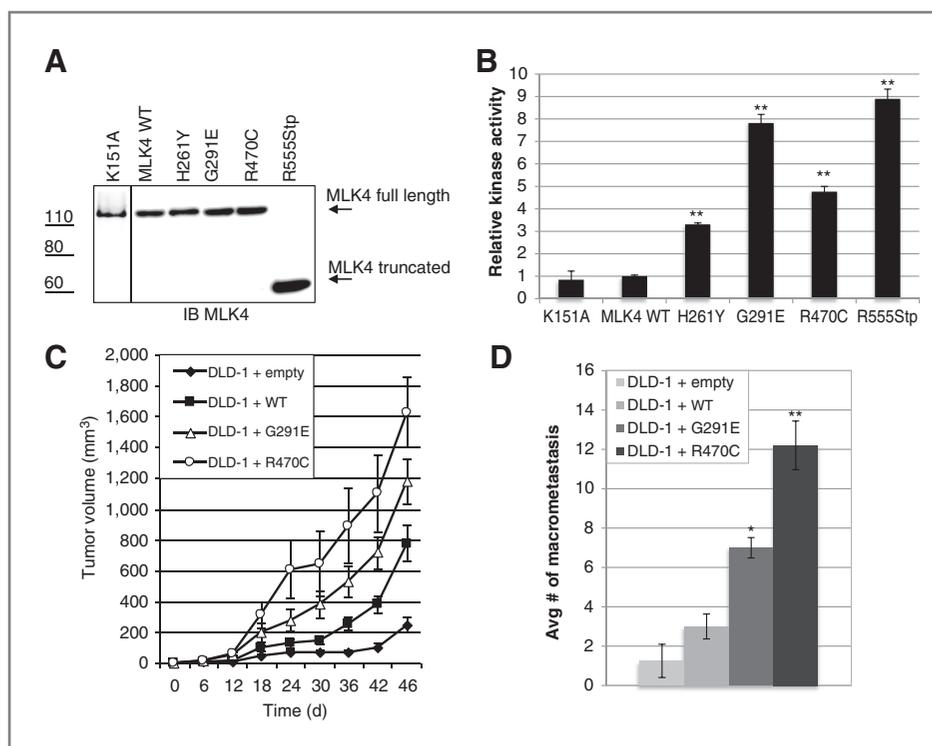
through homologous recombination (17). AAV-mediated homologous recombination was exploited to delete the first exon of *MLK4*, which encodes the kinase domain (Fig. 3B). A 2-step genetic strategy was used to obtain first heterozygous and then homozygous HCT116 cells in which both alleles of the *MLK4* locus were targeted. Two independent *MLK4*<sup>-/-</sup> clones (HCT116 *MLK4*<sup>-/-</sup> a and HCT116 *MLK4*<sup>-/-</sup> b) were identified by PCR using primers specific to the targeting vector and to adjacent genomic sequences. Targeted (knock-out) cells were viable yet lacked MLK4 expression as confirmed by immunoblotting with the anti-MLK4 antibody (Fig. 3C).

#### Gene expression silencing or genetic inactivation of MLK4 impairs the tumorigenic properties of cancer cells

We initially investigated the effect of transcriptional down-regulation of MLK4 on anchorage-independent growth, a key feature of the neoplastic phenotype. As a model system we used multiple cancer cell lines. The panel included cell lines either WT or carrying mutations in KRAS or its effector BRAF. In two cell models, SW48 and DiFi, both WT for KRAS and BRAF, the reduction of MLK4 expression exerted limited or no

effect. When MLK4 expression was knocked down in cancer cells carrying activating mutations in KRAS or BRAF (A549, Colo-205, DLD-1, and HCT116) anchorage-independent growth was markedly affected. In the HCT116 cell line, growth was almost completely impaired (Fig. 4A). To further assess the effect of MLK4 on cell growth, we investigated proliferation rates in tissue culture plates and in soft agar. *MLK4*<sup>-/-</sup> clones grew at a lower rate than WT cells on plastic (Supplementary Fig. S2A) and this difference was also observed when the cells were assessed for anchorage independence (Fig. 4A). To provide further evidence for the role of mutated MLK4 in the growth of CRC cells, we evaluated the effects of MLK4 down-regulation in an additional CRC cell line (LoVo) that we found to carry one of the *MLK4* heterozygous mutations (R470C) increasing the *MLK4*-transforming potential (Supplementary Fig. S2B). MLK4 knockdown reduced the growth rate of LoVo cells on plastic (Supplementary Fig. S2C), and this difference was even more evident when the assay was conducted in low adherence or in anchorage-independent conditions (Supplementary Fig. S2D and S2E).

Next, we determined the effect of reduced or abrogated MLK4 expression on the tumor-forming ability of CRC cells



**Figure 2.** MLK4 mutants cooperate with oncogenic RAS in transformation and tumor formation. A, Western blot analyses of MLK4 proteins, WT, and different mutants. HCT116 cells lacking endogenous MLK4 were transduced with indicated vectors and MLK4 expression levels were assessed by immunoprecipitation (Ab anti-N MLK4). The WT or mutant MLK4 proteins (K151A, H261Y, G291E, R470C, and R555Stp) are indicated. B, relative kinase activity of MLK4 WT and mutant alleles in cells lacking endogenous MLK4. Detailed description of the MLK4 knockout cells is reported in Fig. 3. Kinase activity was calculated as the ratio between total MLK4 protein and the amount of ATP consumption. Results were normalized using the activity of WT MLK4 as a reference. \*\*,  $P \leq 0.01$ ; error bars represent SD. C, tumor formation by DLD-1 cancer cells expressing WT or two MLK4 mutants in xenograft mouse models. Cells were injected into the side of nude mice and tumor growth was measured at the indicated time points. Error bars represent SEM. D, DLD-1 CRC cells expressing WT or mutant MLK4 were injected into nude mice. At the end of the experiment, the animals were sacrificed and the lungs were labeled by airway perfusion using Indian ink. Metastases (colonies of cells growing on the surface of the lungs) were counted using a stereoscopic microscope. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; error bars represent SEM.

*in vivo*. To this end, we took advantage of 2 cellular models: DLD-1 and HCT116. In DLD-1, MLK4 expression was down-regulated by shRNA, in HCT116 it was ablated by genetic targeting of the *MLK4* locus. The cells and their parental controls were injected into immunocompromised mice. DLD-1 cells rapidly formed tumors whereas tumor formation in MLK4 knockdown DLD-1 cells was significantly delayed (Fig. 4B). Most notably, HCT116 cells lacking MLK4 expression were virtually unable to form subcutaneous tumors, whereas the corresponding isogenic WT cells rapidly grew forming large tumor masses (Fig. 4C).

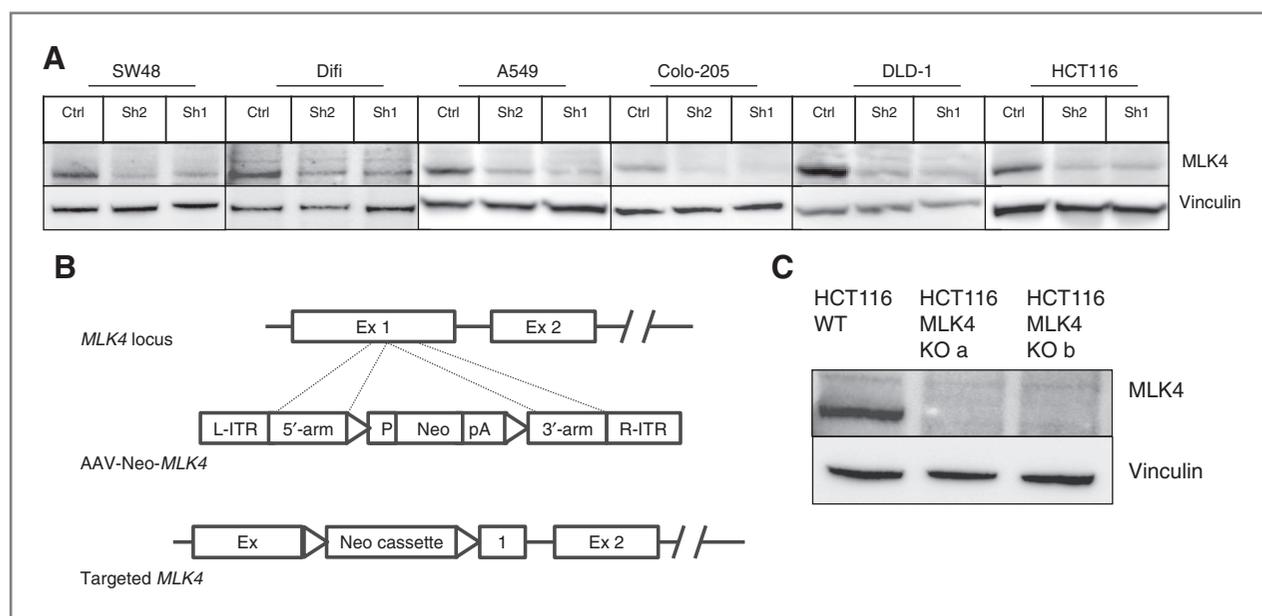
#### MLK4 phosphorylates MEK1 on Ser217/221

The data presented earlier suggest that MLK4 acts within the KRAS/MAPK pathway. We therefore hypothesized that MLK4 may turn on (directly or indirectly) mitogen-activated protein/extracellular signal-regulated kinase (MEK) or extracellular signal-regulated kinase (ERK), two MAPK activated by phosphorylation. The kinase domain of MLK4 (aa A98 to L451) was expressed as a glutathione *S*-transferase (GST) fusion protein in insect cells using the baculovirus system and purified. We then assessed whether the MLK4 protein could

directly phosphorylate MEK1 and ERK2. Considering that the latter are also kinases, we used their catalytically inactive versions in which the lysine, which coordinates ATP-binding, is mutated (MEK1-K97M and ERK2-K54R). We found that MEK1 but not ERK2 were readily phosphorylated by MLK4 (Fig. 5A). To assess which residue of MEK1 was phosphorylated by MLK4, we conducted Western immunoblotting with anti-MEK1 phospho-specific antibodies. These experiments showed that MEK1 is phosphorylated by MLK4 on Ser217/221 (Fig. 5B). Overall, the *in vitro* phosphorylation experiments are consistent with a role of MLK4 as a MEK (or MAP3) kinase, and we propose that MLK4 modulates this pathway by direct phosphorylation of MEK, which in turn, activates ERK.

#### MLK4 modulates MEK/ERK signaling in cancer cells

We then sought to better define the role of MLK4 in the MEK/ERK signaling pathway. Ligand-mediated activation of receptor tyrosine kinases (RTK) is considered the initial event in this pathway. We focused on the EGF receptor (EGFR) tyrosine kinase, a well-characterized receptor implicated in CRC progression. As model systems, we used the SW48 and HCT116 cell lines, which harbor WT and mutant KRAS,



**Figure 3.** Generation of knockdown and knockout cellular models of MLK4. **A**, two independent shRNAs targeting the *MLK4* gene were used to downregulate MLK4 expression in cell lines derived from CRC and lung tumors. Expression levels of the MLK4 protein were assessed by Western blotting. **B**, schematic representation of the vector used to knockout the *MLK4* genes from the genome of HCT116 CRC cells. A sequential targeting strategy was used to knockout both *MLK4* alleles. L-ITR, left-inverted terminal repeats; P, neomycin promoter; Neo, neomycin resistance cassette; pA, polyadenylation sequence; R-ITR, right-inverted terminal repeats. **C**, expression of the MLK4 protein assessed by Western blot analysis in parental and two independent clones of MLK4 knockout HCT116 cells.

respectively. Parental and derivative cells, in which MLK4 expression was reduced or abrogated, were compared side by side. Cells were treated with EGF and activation of the ensuing signaling pathways was analyzed. Ligand-mediated receptor tyrosine phosphorylation was unaffected by abrogation of MLK4 expression indicating that MLK4 does not directly modulate activation of RTKs (Supplementary Fig. S3A and S3B).

Further investigation of the MAPK signaling cascade revealed that lack of MLK4 slightly reduced ligand-dependent phosphorylation of MEK and ERK kinases only in the KRAS-mutated cells (Supplementary Fig. S3B). Considering the striking phenotype, we had previously observed when cells lacking MLK4 were grown in the absence of anchorage (Fig. 4A), we assessed whether MLK4 affected MAPK signaling under this experimental condition. MLK4 knockout cells were seeded and allowed to grow for various periods of time in the absence of anchorage using ultra-low attachment surface flasks. As previously reported (18), when the parental cells were grown in the absence of anchorage the expression of E-cadherin, an epithelial surface marker, was increased. At the same time, MAPK activation, as measured by MEK and ERK phosphorylation, was observed (Fig. 6). Under these conditions, activation of MEK1 was evidently lower in the absence of MLK4. Levels of active ERK, the most common MEK1 substrate, were also decreased in *MLK4*<sup>-/-</sup> cells. The correlation between MLK4 downregulation and MEK-ERK phosphorylation was also assessed in one additional CRC cellular model (DLD-1) in which MLK4 expression was downregulated by shRNAs, as described earlier. As shown in Supplementary Fig. S4, the

reduction of MEK-ERK phosphorylation was consistently observed also in DLD-1 knockdown cells.

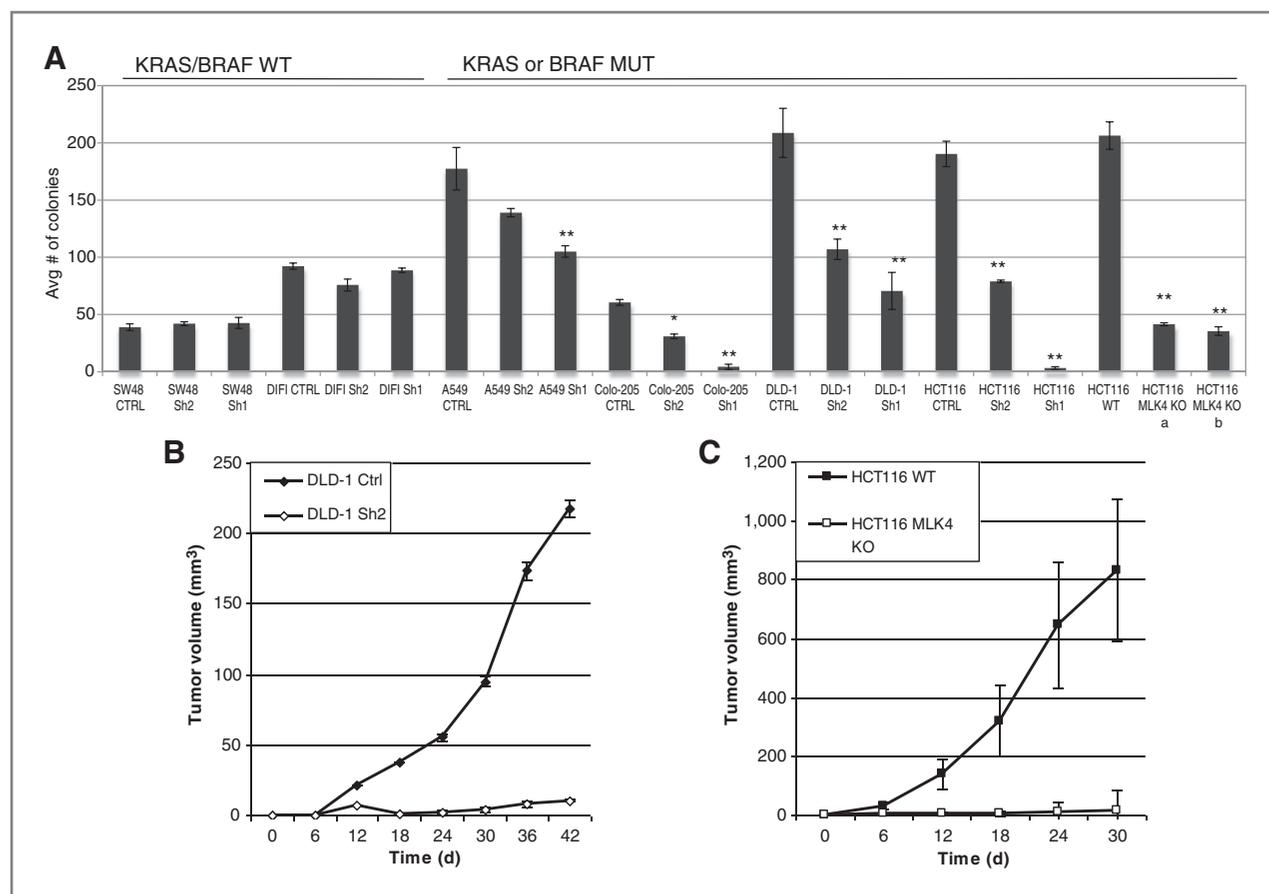
## Discussion

In the past five years, the mutational profile of multiple tumor types including colon, lung, breast, glioblastomas, pancreas, and prostate have been completed. With few exceptions, these studies revealed that there are very few genes mutated at high frequency. Among those, the ones that are constitutively activated (oncogenic) and considered pharmacologically "druggable" are just a handful. MLK4 is the second most frequently mutated protein kinase in MSS CRC (2, 3). Among the 340 CRC samples we analyzed 4 of 9 MLK4-mutated tumors are MSS. Recent data from The Cancer Genome Atlas (TCGA) consortium (2), indicate that MLK4 (KIAA1804) mutations are present in 4% of nonhypermutated (MSS) CRCs.

The role of MLK4 and its somatic variants in CRC is presently unknown. In light of its potential as a novel therapeutic target, we have assessed the biochemical and functional properties of mutant MLK4 and the role of the *MLK4* gene in sustaining the transformed phenotype in CRC cells.

We report that *MLK4*-mutant alleles display increased kinase activity as compared to WT indicating that MLK4 mutations are activating like those affecting other kinases, such as BRAF, MEK, ALK, EGFR, and MET.

MLK4 mutations can be found independently or can cooccur with mutated KRAS in CRCs. The cooccurrence of KRAS and MLK4 mutations in some tumors is evocative of the pattern of mutations in the lipid kinase phosphoinositide 3-



**Figure 4.** Genetic deletion or downregulation of MLK4 impairs transforming potential of human cancer cells carrying mutated KRAS. **A**, anchorage-independent growth (soft agar) assay conducted on parental and MLK4 knockdown/knockout cells. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; error bars represent SD. **B**, control and MLK4 knockdown DLD-1 cancer cells were injected into the side of nude mice, and tumor growth was measured at the indicated time points. Error bars represent SEM. **C**, control and MLK4 knockout HCT116 cancer cells were injected into nude mice, and tumor growth was measured at the indicated time points. Error bars represent SEM.

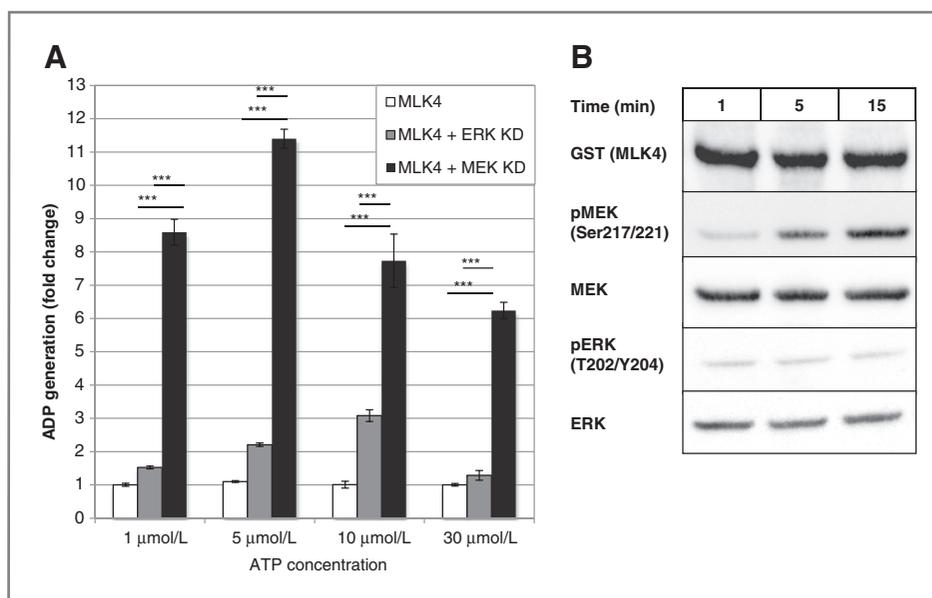
kinase- $\alpha$  (PI3K- $\alpha$ ), which is also mutated either alone or in concomitance with KRAS. Notably, we report that mutant MLK4 enhances RAS-promoting cell transformation. This behavior is reminiscent of other cancer genes (such as *Pokemon*) whose transforming potential becomes detectable only when assessed in cooperation with the *RAS* oncogenes (19). Our results are therefore consistent with a role for MLK4 in the RAS pathway just like that of the PI3K- $\alpha$  (20). In fact, the genetic removal of either WT PIK3CA (20) or MLK4 (as shown in this study) affects the oncogenic potential of RAS resulting in impaired tumor growth and metastasis formation.

Our biochemical analysis suggests that the MLK4 kinase may act within the RAS-MAPK signaling pathway. Indeed, MLK4 can phosphorylate MEK1 on serine 217 and 221, which are located in the activation loop and are known to induce MEK activation. MLK4 may therefore trigger MAPK signaling, just like the RAF kinases, acting as a MAP3K. Notably, unlike RAF proteins, MLK4 does not contain a Ras-binding domain (RBD), making it unlikely that MLK4 could be directly activated through binding to active RAS.

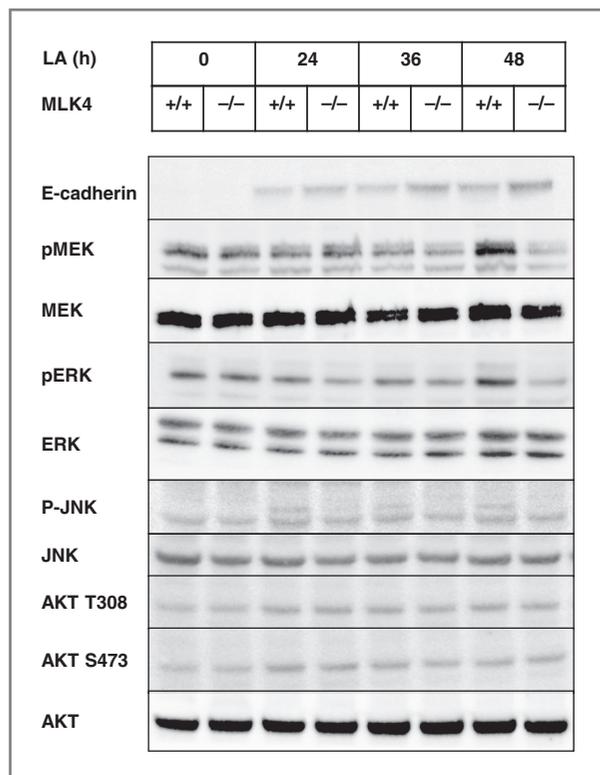
While in this work we have established at least one of the downstream pathways in which MLK4 is involved, the

upstream activators of MLK4 are presently speculative. In this respect, we find that MLK4 modulates the activation of the MEK-ERK cascade when tumor cells are under stressful circumstances, such as in nonadherent conditions. We speculate that in the absence of anchorage, MLK4 could be activated by a RAS effector, such as Cdc42. Intriguingly, it has been shown that both Cdc42 and Rac play a role in preventing detachment-induced apoptosis (anoikis) in transformed epithelial cells (21, 22). Furthermore, previous work indicates that Cdc42 is a physiologic activator of the closely related kinase MLK3 (7). Cdc42 induces membrane targeting and activation by autophosphorylation of MLK3, which is dependent upon an intact CRIB motif (7). Importantly, MLK4 also carries a CRIB domain, and it is possible that Cdc42 or other small GTP-binding proteins (such as Rac) could act as upstream activators of MLK4 through a similar mechanism. In their recent work, Seit-Nebi and colleagues describe a role for MLK4 in inflammatory cytokine production and the negative effect of MLK4 on lipopolysaccharide (LPS)-induced ERK and *c-jun*-NH<sub>2</sub>-kinase (JNK) activation in a murine-derived macrophage cell line (23). It is therefore possible that the MLK4 cellular function (s) are cell and tissue specific. This discrepancy in MLK4

**Figure 5.** MLK4 phosphorylates MEK1. A, MLK4-mediated phosphorylation of kinase inactive ERK2 (K45R) and MEK1 (K97M) at different ATP concentrations. \*\*\*,  $P \leq 0.001$ ; KD, kinase dead. B, residue-specific phosphorylation of ERK2 and MEK1 by purified MLK4 was assessed by Western blot analysis with the indicated phospho-specific antibodies.



function could be explained by the different type of cells analyzed, given that the MLK4 cancer phenotype we report is mainly found in low adherence conditions, a peculiar characteristic often associated with advanced tumor progression.



**Figure 6.** Inactivation of MLK4 impairs MAPK signaling initiated by loss of anchorage. Parental and MLK4 knockout HCT116 cells were seeded and allowed to grow for the indicated times in flasks with an ultra-low attachment surface to prevent cell adhesion. Cells were harvested and protein lysates were analyzed by Western blot analysis with the indicated antibodies.

Moreover, the functional output of MLK4 activation in cancer progression, immunity and inflammation may rely upon different (cell-type specific) pathways. For example, it has been documented that multiple pathways, including MAPK, are activated in response to LPS to promote the expression of many proinflammatory mediators, such as interleukin (IL)-1, -8, TNF, and prostaglandin E (PGE)<sub>2</sub> in monocyte cell lines. At the same level the MAPK axis, upon extracellular stimulation (RTKs), also plays a critical role in the modulation of cancer growth. On the other hand, most CRC cell lines, are known to be either low or not responsive to LPS compared with monocyte lines (24). Bearing in mind all these considerations, we suggest that the inhibitory effect seen by Seit-Nebi and colleagues (23) is related to the specific cellular models they used and to the peculiar MLK4 functions that were analyzed (intracellular interactions and TNF production upon LPS stimulation).

Besides this, the finding that transcriptional silencing or genetic inactivation of the *MLK4* gene severely reduces or abrogates the tumorigenicity of cancer cells carrying KRAS or BRAF mutations is noteworthy. We and others have previously inactivated multiple oncogenes including *MET* (25), *KRAS* (17), and *PIK3CA* (26) in CRC cells. While targeting of these oncogenes drives distinct biologic phenotypes, only the deletion of mutated *KRAS* or *MLK4* results in abrogation of tumorigenesis.

We also reported that *MLK4*-mutant alleles are unable *per se* to promote transformation, but synergistically cooperate with activated RAS to drive tumorigenesis. Such synergy between MLK4 and RAS is further enhanced under low adherence conditions.

We then investigated the relationship between MLK4 mutations and tumor stage and subsequently found a correlation between mutation status and metastatic stage ( $P = 0.0358$ ). However, further analysis on a larger sample dataset is needed to clarify this intriguing observation.

Such observations suggest that MLK4 mutations play a key role at a specific, later point in tumorigenesis.

Activating KRAS mutations are present at high frequency in multiple tumor types, such as CRC, lung, and pancreatic cancer. KRAS-mutated tumors are characterized by poor prognosis and lack of response to therapies. As the Ras protein has proven hard, if not impossible, to pharmacologically inhibit directly, recent efforts have been focused on identifying "druggable" targets that could be synthetically lethal with oncogenic Ras (27–30). MLK4 has never been reported as a "hit" in mutant KRAS synthetic lethality screenings using genome-wide shRNA suppression libraries. These screens have mainly relied on proliferation assays in 2D format as biological readouts. We found that under these conditions, the effect of MLK4 is not apparent. We therefore suggest that similar approaches should be conducted using anchorage-independent conditions, as this may be critical to identify additional mediators of oncogenic RAS.

In conclusion, this work provides genetic and functional evidence that MLK4 mutants are constrictively active and that genetic inactivation of *MLK4* suppresses the growth of KRAS-mutant CRCs. As the MLK4 kinase activity is amenable for direct pharmacologic inhibition, the development of MLK4 inhibitors is warranted and may prove relevant for the treatment of KRAS-driven tumors.

#### Disclosure of Potential Conflicts of Interest

Alberto Bardelli and Miriam Martini have licensed the commercial rights relating to certain MLK4 inventions to Horizon Discovery Limited. Patent, PCT/IB2010/052684. Alberto Bardelli is a shareholder of Horizon Discovery Limited. No potential conflicts of interest were disclosed by the other authors.

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## Mixed Lineage Kinase MLK4 Is Activated in Colorectal Cancers Where It Synergistically Cooperates with Activated RAS Signaling in Driving Tumorigenesis

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