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Colorectal cancer (CRC) is the most common gastrointestinal malignancy in the U.S.A. and approximately 50% of patients develop metastatic disease (mCRC). Despite our understanding of long non-coding RNAs (lncRNAs) in primary colon cancer, their role in mCRC and treatment resistance remains poorly characterized. Therefore, through transcriptome sequencing of normal, primary, and distant mCRC tissues we find 148 differentially expressed RNAs associated with Metastasis (RAMS). We prioritize RAMS11 due to its association with poor disease-free survival and promotion of aggressive phenotypes in vitro and in vivo. A FDA-approved drug high-throughput viability assay shows that elevated RAMS11 expression increases resistance to topoisomerase inhibitors. Subsequent experiments demonstrate RAMS11-dependent recruitment of Chromobox protein 4 (CBX4) transcriptionally activates Topoisomerase II alpha (TOP2α). Overall, recent clinical trials using topoisomerase inhibitors coupled with our findings of RAMS11-dependent regulation of TOP2α supports the potential use of RAMS11 as a biomarker and therapeutic target for mCRC.
Colon cancer (CRC) is the most common gastrointestinal malignancy in the United States. At the time of initial diagnosis, 20% of patients present with metastasis, and of those patients with primary disease approximately 50% will eventually develop metastatic disease. Furthermore, the overall 5-year survival rate for patients with metastatic CRC (mCRC) is only 14%–23. Currently, there are numerous therapeutic treatments for patients with mCRC including surgery, cytotoxic chemotherapy, targeted therapy, immunotherapy, radiation, and combination strategies. However, there is little pre-treatment data that can predict response to treatment and development of resistance. While there are promising developments in second-line treatment options for mCRC patients using cytotoxic agents or targeted agents, the mechanisms driving metastatic progression remain poorly characterized thus prohibiting effective drug development. Furthermore, response to second-line treatment is even less effective than first line. These statistics and poor treatment options highlight the critical need for improved biomarker-driven therapies at the time of diagnosis.

CRC research has primarily focused on the deregulation of protein-coding genes to identify oncogenes and tumor suppressors as potential diagnostic and therapeutic targets. While more recent studies have explored the role of microRNAs in CRC,9,10 there is still a lack of studies focusing on long non-coding RNAs (lncRNAs) in mCRC. LncRNAs are typically greater than 200 nucleotides in length, lack coding potential, are transcribed by RNA polymerase II, spliced, 5′ capped, and polyadenylated. LncRNAs are known to have a diverse range of biological functions, including serving as critical regulators in tumorigenesis and metastasis. Furthermore, the clinical significance of lncRNAs can be exemplified by their use as diagnostic, prognostic, predictive biomarkers, and potential therapeutic targets. Therefore, the characterization of lncRNAs, elucidating their function, and assessing their clinical applicability could significantly impact mCRC diagnosis and treatment.

While transcriptome sequencing has provided an unbiased method for discovering lncRNAs, existing large-scale sequencing projects such as The Cancer Genome Atlas Network (TCGA) are comprised of predominantly primary tumors lacking matched metastatic samples. This represents a critical barrier to discovering novel lncRNAs throughout the progression of primary to metastatic disease correlated to treatment response and resistance. To address this, we have conducted a meta-analysis of normal, primary, and distant metastatic tissues from CRC patients across two independent patient cohorts to discover differentially expressed (DE) lncRNAs in metastatic tumors compared with primary tumors, termed RNAs Associated with Metastasis (RAMS). We have prioritized RAMS11 as it was a top up-regulated lncRNA in metastasis and associated with poor disease-free survival across multiple cohorts. We then demonstrate that RAMS11 promotes aggressive phenotypes in vitro and in vivo. While lncRNAs have been shown to promote tumor progression, the understanding of their role in treatment resistance is still unknown. Therefore, we have utilized a drug screen to discover that RAMS11 promotes resistance to topoisomerase inhibitors and provide mechanistic insight into RAMS11-dependent topoisomerase II alpha (TOP2a) regulation to promote mCRC.

Results

LncRNA landscape of mCRC. To identify consistently altered lncRNAs during mCRC, we performed transcriptome sequencing and analysis of 37 patients from two independent cohorts. The first cohort includes ten normal colon epithelium, two primary CRC, and fourteen distant mCRC patient samples collected from Washington University, termed WUSTL cohort. The second cohort is from a previously published transcriptome sequencing study by Kim et al,29 using matched normal, primary, and metastatic samples from 18 CRC patients, termed Kim cohort (Fig. 1a).

To identify lncRNAs altered in the metastatic samples relative to primary and normal samples, we performed a meta-analysis of the WUSTL and Kim cohorts. We identified 148 DE lncRNAs (FDR < 0.05, fold change > 2) in metastasis, termed RAMS (Fig. 1b and Supplementary Data 1). Several previously well-known and characterized lncRNAs known to promote oncogenic phenotypes in CRC or other cancer types were also detected. This includes increased expression of H19, HULC, CCAT4, and TCONS_2_00022545 in mCRC and decreased expression of FENDRR in metastatic samples (Fig. 1b). Overall, this serves as a key meta-analysis from aggressive CRC patient tissues to establish the mCRC lncRNA landscape.

RAMS11 is upregulated in mCRC. We prioritized our functional studies on lncRNAs that were highly deregulated and potentially clinically relevant in mCRC. To prioritize all RAMS, we evaluated whether their expression correlated with patient outcome. First, we found that six of the 148 RAMS were associated with disease-free survival using 232 patients from the TCGA CRC cohort (RNA-Seq). Among the six RAMS associated with survival in the TCGA cohort, only RAMS11 was associated with poor survival from a second cohort of 82 patients (Fig. 1a, c) from the Sveen study (GSE24549, exon array). These results indicate that high levels of RAMS11 in primary tumors may serve as an indication of poor patient outcome. Notably, RAMS11 was also a top upregulated lncRNA in metastatic tumors (FPKM = 4.81) as compared with primary tumors (combined p = 2.56 × 10−10 average fold change = 6.1) and normal tissues (combined p = 2.2 × 10−20, average fold change = 12.9) (Fig. 1d and Supplementary Fig. 1a). We further validated the upregulation of RAMS11 by qPCR when comparing matched metastatic patient samples with normal (p = 0.007, two-tailed paired t-test) and primary (p = 0.024, two-tailed paired t-test) patient samples (Supplementary Fig. 1b).

Our de novo transcript assembly using the WUSTL cohort identified RAMS11 as a five-exon transcript of 959 nucleotides, which we confirmed by 5′ and 3′ rapid amplification of cDNA ends (RACE) (Fig. 1d, Supplementary Data 2). Previously, three exons of the RAMS11 transcript were annotated as LINC01564 (NR_125841) through a microarray probe-based analysis. We further characterized RAMS11 expression in a panel of CRC cell lines. RAMS11 was highly expressed in a panel of six primary (more than three-fold increase) and two mCRC cell lines (more than 11-fold increase) compared with CCD18-Co, a normal colon control cell line (Supplementary Fig. 1c). Since the cellular localization of lncRNAs can help decipher their functions, we fractionated LoVo mCRC cells with high endogenous expression of RAMS11. As shown in Supplementary Fig. 1d, RAMS11 is predominantly expressed in the nucleus (89.5%), with only a 10.5% expression in the cytoplasm. Taken together, these results show that RAMS11 is a five-exon, nuclear localized, lncRNA that is highly expressed in primary and mCRC cell lines and absent in normal colon epithelium.

RAMS11 promotes aggressive phenotypes in vitro. To understand RAMS11 functional significance, we created a RAMS11 knockout (KO) model by generating two CRISPR/Cas9 luciferase-tagged cell lines with a genomic deletion of the last four exons of RAMS11 in the LoVo metastatic colon cancer cell line
We confirmed greater than a 99.9% reduction in our RAMS11 CRISPR KO models (clones referred to as CRISPR1 and CRISPR2) relative to wild-type cells (Fig. 2a) and confirmed that the genomic deletion of RAMS11 did not alter the expression of adjacent genes GCLC and KLH31 (Supplementary Fig. 2b, c).

We used these genetically engineered cell lines to determine changes in the invasiveness of cells using Matrigel-coated transwells in a modified Boyden chamber assay. There was more than a 60% decrease in invasiveness of RAMS11 CRISPR KO cells (CRISPR1 \( p = 0.004 \), CRISPR2 \( p = 0.023 \), two-tailed paired \( t \)-test) compared with wild-type cells (Fig. 2c, e). We also conducted a transient
knockdown of RAMS11 in a second colon cancer metastatic cell line, SW620, and observed at least 80% knockdown in two independent siRNAs (Fig. 2a). We saw a 50% decrease of invaded cells relative to control cells that were transfected with scrambled siRNA (p < 0.00005, two-tailed paired t-test, Fig. 2c, e). Conversely, stably overexpressing RAMS11 (Clone1 and Clone2) in HT29 cells, with low endogenous RAMS11 expression (Fig. 2b), resulted in a 53% increase in cellular invasion (Clone1 p = 0.008, Clone2 p = 0.042, two-tailed paired t-test) relative to the empty vector control cell line (Fig. 2c, g). Further, we rescued the number of invaded cells to wild-type levels with transient overexpression of RAMS11. Our CRISPR KO models re-expressing RAMS11 (CRISPR RAMS11 OE) revealed more than a 60% increase of invaded cells relative to the CRISPR KO cell lines (CRISPR1 RAMS11 OE and CRISPR2 RAMS11 OE p < 0.00005, two-tailed paired t-test) (Supplementary Fig. 2d, e). We also observed a 73% decrease in cellular migration in the CRISPR KO cells (CRISPR1 and CRISPR2 p < 0.0005, two-tailed paired t-test) and more than 67% decrease in SW620 RAMS11 silenced cells (p < 0.05, two-tailed paired t-test) (Fig. 2d, f). In addition, there was increased migration in HT29 RAMS11 overexpressing cells (Fig. 2d, h). Taken together, this demonstrates that RAMS11 promotes cellular invasion in CRC.

We next investigated the ability of RAMS11 to promote anchorage-independent growth as another indication of aggressive oncogenic phenotypes. Using a soft agar colony formation assay, we detected more than a 66% reduction in colony formation in the RAMS11 CRISPR KO cell lines relative to the wild-type LoVo cell line (Fig. 2i, j). Conversely, there was a 30% increase in colony formation in the RAMS11 overexpressing cells (Fig. 2i, k). These combined data show that decreased expression of RAMS11 in genetically modified and transient knockdown cell lines mitigates...
aggressive phenotypes, while overexpressing RAMS11 promotes aggressive phenotypes.

As another hallmark of aggressive phenotypes, we next assessed the effect of RAMS11 expression on cellular proliferation. We observed a 27% decrease in proliferation in our CRISPR KO cell lines (p < 0.05, two-tailed paired t-test) (Supplementary Figs. 2f, g). Taken together, our in vitro data demonstrate that RAMS11 can promote multiple oncogenic phenotypes.

Next, we evaluated whether RAMS11 is broadly deregulated across cancer types, which would suggest a critical conserved oncogenic role in cancer progression, which we refer to as an onco-lncRNA.37 We conducted a pan-cancer analysis of 6984 tissues comprised of matched and unmatched normal and primary tumors across 22 different cancer types studied within the TCGA. This analysis revealed that RAMS11 had elevated expression in primary tumors compared with normal tissue of origin in colorectal adenocarcinoma (p < 0.00001), four additional cancer types including lung adenocarcinoma (p < 0.00001), lung squamous cell carcinoma (p < 0.00001), head and neck squamous cell carcinoma (p < 0.00001) and kidney renal papillary cell carcinoma (p < 0.00001) (Supplementary Fig. 3a).

Last, since we found that RAMS11 is an onco-lncRNA upregulated across cancer types, we determined if RAMS11 also promoted oncogenic phenotypes in additional cancer types. Therefore, we silenced RAMS11 expression and assessed invasion in two different histologies of non-small cell lung cancer, lung squamous (HCC95) and lung adenocarcinoma (A549), cell line models. We found that silencing RAMS11 expression in both cancer cell lines caused a decrease in cellular invasion (HCC95 p < 0.05; A549 p < 0.005, two-tailed paired t-test) (Supplementary Fig. 3b–g). These results indicate that increased RAMS11 expression promotes oncogenic phenotypes in multiple cancer types.

**RAMS11 promotes tumor growth and metastasis in vivo.** Since our RAMS11 CRISPR KO lines had a significant decrease in cellular proliferation in vitro (Supplementary Fig. 2f, g), we evaluated tumor growth by injecting RAMS11 CRISPR KO cells into NOD/SCID immunocompromised mice. Twenty-five days after subcutaneous injection of LoVo luciferase-tagged wild-type and our luciferase-tagged RAMS11 CRISPR KO cells we found a significant decrease (p < 0.0005, two-tailed paired t-test) in both tumor volume and size in mice injected with RAMS11 CRISPR KO cells compared with wild-type cells (Fig. 3a–d). These results indicate that RAMS11 may indeed induce tumor formation and promote oncogenesis in vivo.

Next, to assess the contribution of RAMS11 to cause metastasis in vivo, we used two mouse models of metastasis: a tail vein injection model to study the development of lung metastases and a hemi-splenectomy model to study the development of liver metastases. For the tail vein model, we injected LoVo luciferase-tagged wild-type and our luciferase-tagged RAMS11 CRISPR KO cells into the tail vein of 5-week-old NOD/SCID mice. We monitored the mice at day 0, within 30 min to 1 h post injection, and weekly for metastasis formation with bioluminescence imaging (BLI). All mice were injected successfully showing similar luminescence levels determined by BLI at baseline day 0 (Fig. 4a). Further, images from day 7 showed no detectable signal indicating the internalization of circulating cells throughout the mouse. There was little or no lung metastasis in mice injected with RAMS11 CRISPR KO cell lines by day 35 (p = 0.02, two-tailed paired t-test) as compared with wild-type cells (Fig. 4a, b). We continued to monitor lung metastasis for 91 days and saw significantly less lung metastasis in RAMS11 CRISPR KO cell-injected mice compared with wild-type cell-injected mice (p < 0.05, two-tailed paired t-test) (Fig. 4b, c). We also detected less lung metastasis ex vivo in mice injected with RAMS11 CRISPR KO cells compared with mice injected with wild-type cells (Fig. 4d). In addition, extracted lungs had little to no tumors detected by hematoxylin and eosin (H&E) stain and lower levels of Ki67 staining from RAMS11 CRISPR KO cell-injected mice compared with wild-type cell-injected mice (Fig. 4e).

We assessed if RAMS11 promoted liver metastasis using the hemi-splenectomy model. LoVo luciferase-tagged wild-type and luciferase-tagged RAMS11 CRISPR KO cells were injected into 8-week-old NGS mouse spleens.38 We detected liver metastasis by day 7 in mice injected with wild-type cells (Fig. 5a) and detected significantly lower levels of bioluminescence in RAMS11 CRISPR KO cell-injected mice compared with wild-type cell-injected mice by Day 21 (CRISPR1 p = 0.016, CRISPR2 p = 0.008, two-tailed paired t-test, Fig. 5a, b). We excised all mouse livers and validated the decrease of liver metastasis (Fig. 5c), decreased liver weights (CRISPR1 p = 0.0000026, CRISPR2 p = 0.00069, two-tailed paired t-test, Fig. 5d), and decrease in overall liver metastasis area (CRISPR1 p = 0.000021, CRISPR2 p = 0.00018, two-tailed paired t-test, Fig. 5e) in RAMS11 CRISPR KO cell-injected tumors. Decreased tumor burden and proliferation in RAMS11 CRISPR KO cell livers were further determined by H&E and Ki67 staining (Fig. 5f). Overall, our cell models manipulating RAMS11 expression demonstrate the ability of RAMS11 to promote invasive phenotypes both in vitro and in vivo.

Drug screen reveals RAMS11 resistance to TOP2a inhibitors. To implicate RAMS11 in specific biological processes and establish its clinical importance, we conducted a high-throughput viability assay using 119 FDA-approved anticancer drugs from the NIH Developmental Therapeutics Program (Approved Oncology Drugs Set VI). The FDA-approved anticancer panel included multiple classes of drugs such as kinase inhibitors, alkylating agents, antineoplastic antibiotics, anthracycline antibiotics, and antineoplastic agents (topoisomerase inhibitors). The HT29 RAMS11 overexpressing and control cells were treated for 72 h to assess cellular viability upon drug treatment (Supplementary Fig. 4a and Supplementary Data 3). The RAMS11 overexpressing cells were resistant to nine drugs as demonstrated by a greater than three-fold increase in cellular viability when compared with the empty vector control cell line with the greatest resistance observed with gemcitabine and floxuridine (FUDR) (Supplementary Fig. 4b, c). FUDR, a 5-FU derivative, is commonly used to treat mCRC, while gemcitabine is used in refractory mCRC.39–42 Due to 5-FU commonly used to treat mCRC, we further determined if RAMS11 expression altered drug sensitivity in treated cells. In our RAMS11 CRISPR KO lines we found a 1.7-fold and 5.8-fold increase in drug sensitivity in CRISPR1 and CRISPR2, respectively, compared with wild-type cells (Supplementary Fig. 5a). Similarly in SW620 cells with transient silencing of RAMS11 there was a greater than 1.5-fold increase in drug sensitivity in both siRNAs (siRNA1 fold > 1.53, siRNA2 fold > 1.59) relative to scrambled control wild-type treated cells (Supplementary Fig. 5b). 5-FU, irinotecan (topoisomerase I inhibitor (TOP1)), and oxaliplatin (new-generation platinum compound) are currently used as first-line active chemotherapy options individually or in combination for patients with metastatic disease.43,44 We did not see a significant effect of cell viability for irinotecan or oxaliplatin using our HT29 RAMS11 overexpressing cells or LoVo RAMS11 CRISPR KO cells (Supplementary Data 3, Supplementary Fig 5c–e). SW620 cells with silenced RAMS11 also did not have a significant effect of cellular viability for oxaliplatin treatment, but we did detect an increase in...
drug sensitivity to irinotecan (siRNA1 fold > 3.17 and siRNA2 fold > 11.8, Supplementary Fig 5f).

Interestingly, half of the topoisomerase inhibitors assessed caused at least a two-fold increase in drug resistance in the HT29 RAMS11 overexpressing cells including the TOP1 topotecan hydrochloride (HCl) and four TOP2α inhibitors (doxorubicin HCl, epirubicin HCl, daunorubicin HCl, and idarubicin HCl (Fig. 6a, b). To further support our observation that RAMS11...
overexpression promoted resistance to topoisomerase inhibitors, we narrowed our focus on clinically relevant drugs that selectively target the DNA topoisomerase TOP2α, doxorubicin and epirubicin. Measuring cellular viability in LoVo RAMS11 CRISPR KO cells we showed a 1.5-fold increase in drug sensitivity with 0.5 μM doxorubicin or 0.7 μM epirubicin treatment compared with wild-type treated cells (p < 0.05, two-tailed paired t-test, Fig. 6c).

To further support our drug panel findings, we evaluated whether RAMS11 regulated TOP2α protein expression. We observed that RAMS11 overexpressing cell lines had elevated TOP2α protein expression, whereas our CRISPR KO cells displayed a decrease in TOP2α protein levels (Fig. 6d). The decrease in TOP2α expression in our CRISPR KO cells was rescued by re-introducing RAMS11 expression in these cells (Fig. 6d).Transient silencing of RAMS11 in SW620 cells also decreased TOP2α protein and mRNA levels relative to our scrambled control (Supplementary Fig. 6a, b). To demonstrate the specificity of RAMS11 regulation of TOP2α, we confirmed there was only a decrease of TOP2α mRNA expression by making primers specifically targeting TOP2α and not TOP2β in the LoVo CRISPR KO and SW620 silenced cell lines (Supplementary Fig. 6b, c). Lastly, we assessed downstream targets of TOP2α in our CRISPR KO cell lines and observed a decrease in MLH1 and ERCC2 mRNA levels supporting RAMS11 regulation of TOP2α (Supplementary Fig. 6d). Overall, our high-throughput drug panel established that RAMS11 expression impacted cellular sensitivity to topoisomerase inhibitors, specifically inhibitors targeting TOP2α, which led to the discovery that RAMS11 overexpression increased TOP2α protein expression in colon cancer cell lines. These data highlight the potential for RAMS11 expression to serve as an important biomarker to select mCRC patients that may potentially benefit from topoisomerase inhibitor treatment.

**RAMS11 binds to chromobox protein 4 (CBX4) to regulate TOP2α.** Due to the nuclear localization of RAMS11 we hypothesized that it may transcriptionally regulate TOP2α expression to increase protein levels and promote topoisomerase resistance. Notably, a recent study found that CBX4 bound to the promoter of Top2α. Since CBX4 is known to possess both activation and repressive activities, and has been found to interact with IncRNAs, we hypothesized that it could interact with RAMS11.
and transcriptionally regulate TOP2α expression through interaction with CBX4. We found an 83-fold and 16-fold enrichment of RAMS11 bound to CBX4 in LoVo and SW620 cells, respectively, as determined by an RNA immunoprecipitation (RIP) coupled with qPCR (Fig. 7a, b). To orthogonally validate these findings, we conducted a RNA pull-down assay utilizing a 5′ Bromo-UTP full-length RAMS11 sense labeled probe and a negative control antisense probe to pull-down proteins that may be bound to RAMS11. We found that the RAMS11 sense probe was bound to CBX4 protein compared with the antisense probe (Fig. 7c) by Western blot of nuclear lysates. In order to identify the regions of RAMS11 that bind to CBX4, we conducted in vitro RNA pull down in the LoVo cell line using four truncated RAMS11 fragments (Supplementary Fig. 7a). We re-validated our previous findings that full-length RAMS11 binds to CBX4 and revealed that nucleotides 600–959 of RAMS11 interact with CBX4 protein (Supplementary Fig. 7b). These orthogonal methods support RAMS11 binding to CBX4 protein.

We next evaluated if CBX4 interacts with the TOP2α promoter and whether this was dependent on RAMS11 expression. Binding of CBX4 to the promoter of TOP2α was confirmed by chromatin immunoprecipitation (ChIP) coupled with qPCR in LoVo colon cancer cells. Silencing CBX4 led to a 78% decrease in CBX4 occupancy at the TOP2α promoter in LoVo cells (p = 0.021, two-tailed paired t-test, Fig. 7d). Further, we demonstrated this binding is dependent on RAMS11 expression since there was a greater than 68% decrease in CBX4 occupancy in the TOP2α promoter in our RAMS11 CRISPR KO models (p = 0.005, two-tailed paired t-test, Fig. 7d). We also observed a decrease in tri-methylation of lysine 4 on the Histone H3 protein subunit (H3K4me3), a modification commonly associated with active transcription, in CBX4 siRNA treated cells and our RAMS11 CRISPR KO models (CRISPR1 p = 0.001 and CRISPR2 p = 0.0008, two-tailed paired t-test, Fig. 7e). Decreased CBX4 occupancy and H3K4me3 at the TOP2α promoter was further confirmed in the SW620 cell line with transiently silenced CBX4 or RAMS11 (CBX4 p = 0.004, RAMS11 siRNA p < 0.05, two-tailed paired t-test, Fig. 7f, g). In addition to demonstrating endogenous binding of RAMS11 to CBX4 in LoVo and SW620 cells, we found more than 15000-fold enrichment of CBX4 binding to RAMS11 in our HT29 RAMS11 overexpressing cells compared with empty vector (Fig. 7h). In addition, the HT29 RAMS11 overexpressing cells had increased occupancy of CBX4 (p = 0.0005 and p = 0.004, two-tailed paired t-test) and increased occupancy of H3K4me3 (p = 0.01 and p = 0.001, two-tailed paired t-test) at the TOP2α promoter (Fig. 7i, j). Further, we rescued CBX4 (Fig. 7k) and H3K4me3 (p = 0.005, two-tailed paired t-test, Fig. 7l) occupancy by re-introducing RAMS11 expression into the LoVo CRISPR KO cells. The decrease in TOP2α protein expression was confirmed by Western blot in RAMS11 CRISPR KO and CBX4 silenced cells (Fig. 7m). We also observed a decrease in cellular invasion when CBX4 or TOP2α were transiently silenced in LoVo cell lines (Supplementary Fig. 8a, b). Collectively, these data demonstrate RAMS11-dependent CBX4 binding to the TOP2α promoter. Taken together, we provide evidence of RAMS11-dependent CBX4 regulation of TOP2α to induce the metastatic phenotype in CRC (Fig. 8).

Discussion

In the current study we performed transcriptome sequencing of matched normal, primary, and distant metastatic patient samples to identify lncRNAs associated with metastatic progression that
could serve as a resource for further functional characterization and biomarker studies. To exemplify this, we prioritized RAMS11 since it was overexpressed in primary and mCRC tumors and its expression correlated with poor disease-free survival. This demonstrates the potential utility of RAMS11 expression as a marker to stratify high-risk patients. Supporting our clinical findings, we were able to confirm that RAMS11 promoted oncogenic phenotypes in vitro and in vivo in several cancer types.

To assess the clinical potential of RAMS11 and elucidate its regulatory mechanism for promoting aggressive phenotypes we used a high-throughput drug assay. We found that antimetabolites promoted resistance to more than half of the topoisomerase inhibitors screened. Currently, the elevated expression of RAMS11 in HT29 RAMS11 overexpressing cells. Since it was overexpressed in primary and mCRC tumors and its expression correlated with poor disease-free survival. This demonstrates the potential utility of RAMS11 expression as a marker to stratify high-risk patients.

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RAMS11 directly affects mCRC biology, including promoting an aggressive phenotype and correlating with treatment response and resistance.

Methods

Patient samples and RNA sequencing. Patients were enrolled at Washington University School of Medicine in St. Louis and informed consent was obtained under an IRB-approved protocol. Adjacent normal, primary, and liver metastasis tissues were resected from mCRC patients and fresh frozen prior to RNA extraction (Supplementary Data 4). PolyA DNA libraries were constructed using NuGen Ovation Kit V2, and paired-end sequencing was performed on Illumina HiSeq 2000. RNA-Seq data from Kim cohort was downloaded from NCBI GEO (GSE50760). TCGA RNA-Seq pre-aligned bam files were downloaded from the Cancer Genomics Hub (http://cghub.ucsc.edu/).

RNA-Seq data analysis. The human reference genome assembly version GRCh38/hg38 and the corresponding gene annotations were used in RNA-Seq analysis. Gene annotations were combined from Gencode v2362, RefSeq downloaded from hg38 and the corresponding gene annotations were used in RNA-Seq analysis.

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Exon array data analysis. We repurposed the Affymetrix exon array for IncRNA analysis by realigning the probe set sequences against the human transcript sequences using SeqMap 1.0.127 allowing one mismatch. Only probe sets consisting of probes that were uniquely aligned to transcripts from the same gene were retained. Exon array expression was processed and normalized using Affymetrix Power Tool 1.18 (https://www.thermofisher.com).

Survival analysis. Survival analysis was performed using the Cox proportional hazard model with R survival package 2.37-7 2014 (https://CRAN.R-project.org/package=survival). The median expression of RAMS11 within a cohort was used to stratifying patients into low and high RAMS11 expression groups. Kaplan–Meier curves were plotted using the R survplot package 0.0.7 2014 (http://www.cbs.dtu.dk/~eklund/survplot/).

Cell culture. Colon cancer cell lines CCD18-Co and SW480 were a kind gift from Dr David Shalloway at Cornell University. All other colon cell lines (HT29, HT-15, DLD1, SW620, Caco-2, HCT-116, and RKO) were a kind gift from Dr A. Craig Lockhart at Washington University. LoVo cell lines were purchased from ATCC (ATCC CCL-229). HCC95 and A549 cell lines were a kind gift from Dr Lauren Michel and Dr Brian Van Tine, respectively, from Washington University. SW620 cells were grown in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Sigma, St. Louis, MO), and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) complete media. LoVo cells were grown in DMEM/F12 (Invitrogen) with 10% fetal bovine serum, and 1% penicillin/streptomycin complete media. HT29, HT-15, DLD1, and Caco-2 cells were grown in McCoy’s (Invitrogen) with 10% fetal bovine serum, and 1% penicillin/streptomycin complete media. LoVo cells were grown in RPMI (Invitrogen) with 10% fetal bovine serum, and 1% penicillin/streptomycin complete media.

Rapid amplification of cDNA ends (RACE). 5′ and 3′ RACE was done using the GeneRacer Kit (Invitrogen) according to the manufacturer’s instructions. RACE PCR products were obtained with Platinum Taq High Fidelity (Invitrogen) using the GeneRacer primer (supplied) and a gene-specific primer found in Supplementary Data 5. Products were visualized on a 2% agarose gel and purified by gel extraction (Qiagen, Germantown, MD). This product was then cloned into pCR4-TOPO vector (Invitrogen) and grown in TOP10 E. coli. Clones were sequenced with the M13 forward primer at the Protein and Nucleic Acid Chemistry Laboratory at Washington University.

Generation of RAMS11 silenced and overexpression cells. LoVo RAMS11 CRISPR KO cell lines were generated through the Genome Engineering and iPSC center at the Washington University. CRISPR/Cas9 was used to create a genomic deletion of the last four exons of RAMS11 in LoVo metastatic colon cancer cells (Supplementary Fig. 2a). We used two different cell clones for the described experiments. In addition, we silenced expression of RAMS11 using custom siencer select RNAs (siRNAs) targeting RAMS11, CBX4, TOP2a, or a negative scrambled control (Invitrogen). siRNA sequences are listed in Supplementary Data 5.

Full-length RAMS11 transcript was PCR amplified from LoVo cells and cloned into the pCFG5-IEGZ vector (a kind gift from Dr Ron Bose, Washington University). Full-length RAMS11 inserts were confirmed with Sanger sequencing at...
the Protein and Nucleic Acid Chemistry Laboratory at Washington University. Retroviral infection of cancer cells was performed according to Kauri et al. 3 Briefly, the amphotropic murine cell line was transfected with 10 µg of pCGS-RAMS11 or empty vector control by calcium phosphate precipitation and incubated for 24 h. Viral supernatants were harvested after an additional 24-h incubation. Virus was added to cells seeded in six-well dishes in the presence of 8 µg/mL polybrene (Sigma). Cells were centrifuged at 300 x g for 90 min and fresh media was then added to the plate. After 14 days of infection (in vitro), selected colonies were used for assays. HT29 colon cells that had low endogenous expression of RAMS11 were infected with virus expressing RAMS11 for 48 h and selected with 100 µg/mL Zeocin.

**Quantitative real-time PCR.** Total RNA was isolated for each CRC cell line using Takara Bio NucleoSpin RNA (Takara, Mountain View, CA). Total RNA was then transcribed to cDNA using SuperScript III First strand cDNA synthesis system (Invitrogen) and quantified using Fast Sybr Green Master Mix (Invitrogen) as per the manufacturer’s protocol. Primer sequences are available in Supplementary Data 5.

**Protein detection by Western blot.** Western blots were conducted by placing 250,000 representative cancer cells in a six-well dish. For transient knockdown experiments, the next day cells were transfected at 6.25–25 nM with two independent custom designed siRNAs or a negative scramble control to silence Lipofectamine RNAiMax (Invitrogen) for 72 h. Cells were then lysed with Tris lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 131 mM NaCl, 1 mM sodium orthovanadate, 10 mM Na3P04, 10 mM NaF, 1 mM EDTA, 10 mM Tris-HCl (invitrogen) and transferred to nitricollidine membrane (BioRad, Hercules, CA). Blots were then probed overnight at 4˚C with respective antibodies directed against TOP2A, CBX4, and ACTIN, then washed with TBST buffer, and then applied with secondary goat anti-rabbit HRP-linked or goat anti-mouse HRP-linked antibodies (Thermo Fisher, Waltham, MA). Blots were then washed, visualized with Clarity Western ECL Substrate (Bio-Rad), and imaged using the ChemiDoc XRS+ System (Bio-Rad). Bands were quantified from the digital image in ImageJ and are shown normalized to the control lane for each target. Raw Western blots are shown in Supplementary Fig. 9. All antibodies and concentrations are listed in Supplementary Data 6.

**RNA immunoprecipitation.** RIP coupled to qPCR assays were conducted by isolating nuclear lysates from ten millions of LoVo or SW620 cells following the NER-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher). Nuclear lysates were then incubated overnight with 5 µg CBX4 antibody or IgG antibody isotype control in RIPA wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM MgCl2, 1% NP40, 0.5% Na-deoxycholate, 0.05% SDS, 1 mM EDTA) and SUPERase-in RNAse inhibitor (Invitrogen). The next day 50 µL of Invitrogen Dynabeads Protein G were added to the antibody lysate/mixture and were rotated for 1 h at 4˚C. Next, beads were subsequently washed twice with RIPA wash buffer using a magnetic bead separator. Protein was then digested with proteinase K buffer (RIPA buffer, 10% SDS, 10 mg/mL proteinase K), at 55˚C for 30 min shaking. RNA was phenol/chloroform/isooamyl alcohol extracted following the general protocol (Thermo Fisher). Last, gDNA was removed from RNA using ArcticZymes Heat and Run gDNA removal kit following the manufacturer’s protocol (Tromso, Norway). cDNA was made using SuperScript III First strand cDNA synthesis system as indicated above and qPCR was run with Fast Sybr Green Master Mix and SYBR Green I. Primers were available in data file. Band intensities were quantified from the digital image in ImageJ and statistical significance was determined by a Student’s two-tailed t-test. Four to seven images were taken per transwell membrane at ≥20 magnification. Assays were repeated two to three times.

**Soft agar assays.** HT29 cells overexpressing RAMS11 and LoVo RAMS11 CRISPR KO cell lines were reseeded at 75,000 cells in 0.4% Difco soft agar (BD Bioscience, Franklin Lakes, NJ) and seeded onto a 5% Difco base agar. Cells were given fresh media every day for around 2 weeks, and once colonies were visible by eye, cells were stained with 0.5% Crystal Violet (Sigma) for 3 h. Plates were then imaged using the ChemiDoc XRS+ (Bio-Rad) and counted with ImageJ software. Average cell counts were used for comparison and statistical significance was determined by a Student’s two-tailed t-test. Assays were repeated three times.

**Drug treatments.** The NIH approved oncology library of 119 drugs (AODE plate 4825-1 and AOD6 plate 4826) was received from the NIH National Cancer Institute DTP Developmental Therapeutics Program. Drugs were diluted in DMSO to 1 mM and the well assignments were rearranged so drugs were confined to the inner 60 wells of 96-well plates. HT29 RAMS11 overexpressing Clone1 and Clone2 cell lines were seeded at 5000 cells per well in a 96-well plate. The next day serial diluted drug was added to pre-seeded plates in media containing 1% DMSO vehicle, using a Robbins Hydra 96 microdispenser. Two 96-well plates of cells were used as vehicle controls. The plates were incubated for 3 days. Percent viability was scored by incubating cells for 3 h with resazurin sodium (0.023 mg/mL, Sigma R7017). The reaction was stopped by the addition of SDS (1% final concentration). Fluorescence Ex/Em 540/590 was read in a Biotek Synergy H1 plate reader (Winooski, VT). The fluorescence values for the vehicle plates were averaged and percent viability was determined by the formula: Percent viability = (average vehicle – value)/average vehicle – average resazurin in media blank) x 100. We removed drugs that were undetectable, or out of range, by resazurin assay, leaving 118 drugs to assay in the study (Supplementary Data 3). Values with more than a 1.5-fold change in both RAMS11 Clone1 and Clone2 overexpressing cell lines were used to determine significance. Individual drug IC50 assays were done in a similar manner as described above with CRISPR KO cells. Assays were repeated more than three times.

**In vivo models.** The animal studies were reviewed and approved by the Washington University’s Institutional Animal Care and Use Committee protocol. For subcutaneous injections, 26 LoVo wild-type, RAMS11 CRISPR1, or RAMS11 CRISPR2 luciferase-tagged cells were injected subcutaneously in ten NOD/SCID mice per group. Weekly tumor size was determined by caliper measurements capturing length x width x height x 0.5. For post-analysis lung tissues and subcutaneous tumors tissues were removed and formalin fixed and paraffin embedded. This experiment was repeated twice times.

In the lung metastasis mouse model, 26 LoVo wild-type and CRISPR luciferase-tagged cell models were injected into the lateral tail vein of twelve 5-week-old NOD/SCID mice (Jackson Laboratories, Bar Harbor, MA) per group using 30-gauge needles. In weekly intervals mice were imaged with the Olympus...
OV100 Small Animal Imaging System (IVIS Spectrum, Caliper, Hopkinton, MA) in conjunction with the Small Animal Imaging Core (SAIC) at Washington University. Mice were imaged for 1 min with sequential 5 s exposures. Luminescence was quantified using the Living Image Software 3.2 (Caliper). All micrometastases were imaged at day 0 (30 min to 1 h) post operation and weekly for 12 weeks. At the conclusion of the study, mice were sacrificed and examined visually and with bioluminescence for lung metastases in vivo and ex vivo. Lungs were dissected and formalin fixed and paraffin embedded for histological analysis with H&E and Ki67 staining. This experiment was repeated twice.

For the hemisplenectomy mouse model, 26×LoVo wild-type and CRISPR luciferase-tagged cells in 50 µL of PBS were injected into the spleen of 6–8-week-old NCr BALB/c (Jackson Laboratories) (WT n = 18, CRISPR n = 11, CRISPR2 n = 11) using 30-gauge needles during open laparotomy. Cell injections were followed with a 50 µL PBS flush. Incisions were closed with sutures and surgical clips. In weekly intervals mice were imaged with the Olympus OV100 Small Animal Imaging System (IVIS Spectrum) in conjunction with SAIC. Mice were imaged for 10 s to 1 min exposures. Luminescence was quantified with the Living Image Software 3.2 (Caliper). All micrometastases were imaged at day 7, day 14, and day 21 post operation. At the conclusion of the study, mice were sacrificed and examined visually and by bioluminescence for liver metastases in vivo and ex vivo. Livers were dissected, weighed, and formalin fixed and paraffin embedded for histological analysis with H&E and Ki67 staining. This experiment was repeated three times.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The RNA-Seq data generated in this study (WUSTL cohort) have been deposited in the dbGaP database under the accession code phs001722. The Kim et al.'s data29 referenced during the study are available in a public repository from the NCBI Gene Expression Omnibus (GEO) under the accession code GSE50760. The source data underlying all figures are provided as a Source Data File. All the other data supporting the findings of this study are available within the article and its Supplementary Information files and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file.

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**References**


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Author contributions

Competing interests
The authors declare no competing interests.

Additional information
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