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# Extracellular Metabolic Energetics Can Promote Cancer Progression

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# EXTRACELLULAR METABOLIC ENERGETICS CAN PROMOTE CANCER PROGRESSION

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# EXTRACELLULAR METABOLIC ENERGETICS CAN PROMOTE CANCER PROGRESSION

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Colon cancer progression is characterized by growth of the primary tumor in the colon followed by metastasis to distant organs. The metastatic cascade involves invasion of cells from the primary tumor into the surrounding tissue, entering into and survival of cancer cells in the circulation, arrival at the end organ and finally colonization of the end organ. The liver is the primary site of colon cancer metastatic colonization, with over 70% of colon cancer patients experiencing liver metastases. Despite current standard-of-care surgical intervention and broad-spectra cytotoxic chemotherapeutics, the survival rate of patients with metastatic disease is less then 5%. A greater understanding of the biology and molecular determinants of liver colonization is therefore of great importance to the scientific and clinical community. This thesis presents unbiased approaches to identify regulators of liver metastasis in colon cancer and the elucidation of the mechanisms involved.

The first part of this thesis describes the identification of two microRNAs, miR-483-5p and miR-551a as suppressors of liver metastasis by human colon cancer cells using two parallel, complementary xenograft models of colon cancer metastasis. The first approach involved a functional library-based *in vivo* screen of 661 microRNAs. The second approach utilized *in vivo* selection of liver-

metastatic colon cancer cell population from poorly metastatic parental population. Functional studies revealed both microRNAs to target a common downstream effector gene, Creatine Kinase Brain (CKB).

CKB was found to promote metastasis and the second part of this thesis present mechanistic studies that describe CKB-mediated modulation of intra- and extra-cellular energetics by colon cancer cells that contributed to colon cancer cell survival in the liver microenvironment, allowing for development of macro-metastases and finally liver colonization. Further investigation identified the membrane transporter SLC6a8 as an important effector of the CKB pathway and also a promoter of colon cancer metastasis.

The final part of this study reveals miR-483-5p, miR-551a, CKB and SLC6a8 to be clinically relevant across multiple patient datasets and archival patient samples. MiR-483-5p and miR-551a were found to be down-regulated in liver metastases of patients relative to primary tumors, while CKB and SLC6a8 were up-regulated. In addition, proof-of-principle therapeutic experiments involving adeno-associated viral delivery of the microRNAs and small molecule inhibition of CKB and SLC6a8 demonstrated the therapeutic potential of targeting this pathway in suppressing colon cancer metastasis.

To my Wife, my Parents and Sister, and my Friends for their unwavering support.

*To Jessica, who has been a great source of comfort as well.*

*In memoria mea avunculus*

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### Chapter II



### Materials and Methods

# CHAPTER I: Introduction

**Colorectal cancer: Epidemiology, staging and prognosis**

Colorectal cancer (CRC) is the third most common type of cancer and one of the leading contributors to cancer mortality in the United States. It is the third most common type of cancer for both genders, behind lung cancers and gender-specific breast and prostate cancers. Every year, there are over 160,000 newly diagnosed cases of colorectal cancer, with about 50,000 colorectal cancer patients dying from the disease (Jemal et al., 2011; Siegel et al., 2014b).

Colorectal cancer progresses through several stages (I-IV) that, without early diagnosis and medical intervention, can lead to metastatic disease, the primary cause of mortality (Fig. 1.1). Staging of colorectal cancer is determined by the extent of primary tumor growth (T), the presence of cancer cells in regional lymph nodes (N) and the presence of distal metastases. Precancerous lesions, otherwise known as polyps, can be removed during colonoscopy. Localized non-invasive tumors that had not grown beyond the muscularis priopia of the colon (stage I) and invasive primary tumors (stage II) without lymph nodes involvement can be surgically resected with high curative rates of 93% and 78% respectively. Disease that had progressed to stage III, which involves spread to regional lymph nodes, is treatable with surgery and adjuvant chemotherapy and is highly curable; stage III patients who undergo treatment have a 64% five-year survival rate. Despite current systemic chemotherapy and targeted therapy, the prognosis for patients with stage IV metastatic disease is poor, with five-year survival rate of approximately 5-7% (Siegel et al., 2014a). There is therefore an urgent need to identify therapeutically targetable pathways that drive colorectal cancer metastasis.

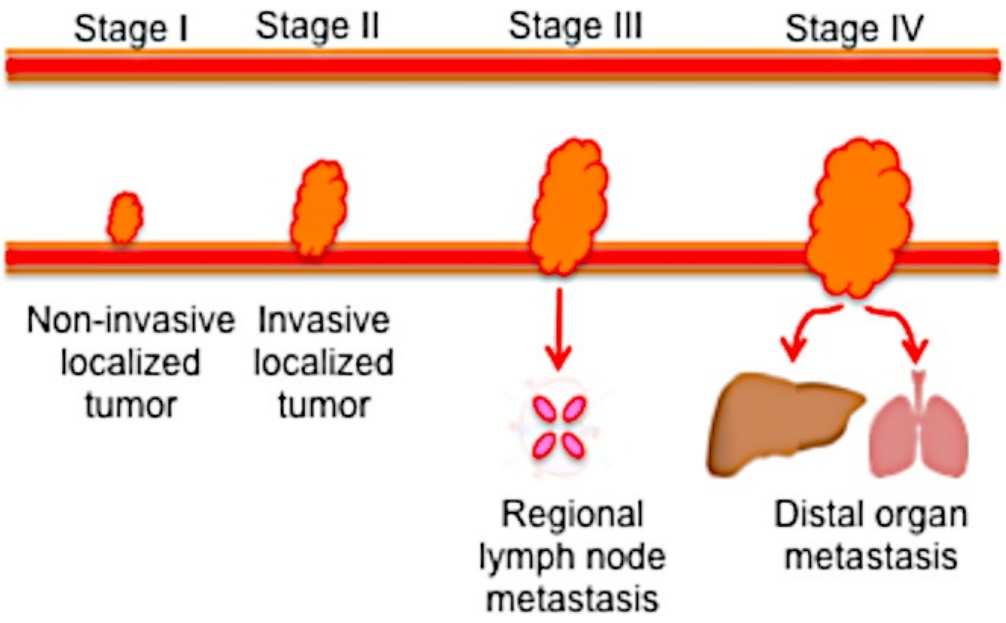



**Figure 1.1 | Stages of colorectal cancer progression.** Localized non-invasive tumors and invasive primary tumors can be cured by surgical resection. Patients with regional lymph node metastases (Stage III) have relatively good prognosis compared to patients with Stage IV diseases, which involves distal organ metastases (<7% five-year survival rate).

**Colorectal cancer: Therapeutic intervention**

The mainstay of current colorectal cancer chemotherapy is 5′-fluorouracil, a fluorinated uracil that acts through inhibition of thymidylate synthase, the rate-limiting enzyme for pyrimidine nucleotide synthesis. In combination with leucovorin, which stabilizes the binding of 5′-fluorouracil to thymidylate synthase, 5′-fluorouracil has been shown to prolong median survival in patients about two-fold, from 6 months to almost a year (Moertel, 1994). Two other cytotoxic drugs, Irinotecan and Oxaliplatin have also been utilized in colorectal cancer chemotherapy. Like 5′-fluorouracil, the two drugs act by perturbing

nucleic acid homeostasis, but through different mechanisms. Irinotecan stabilizes DNA breaks caused by DNA topoisomerase I during DNA replication and transcription. The accumulation of such breaks results in cell death arising from activation of DNA damage checkpoints. Oxaliplatin is a platinum derivative that forms adducts with DNA, which again results in activation of DNA damage response pathways and subsequent cell death. Clinical trials have demonstrated that combinations of the above drugs in therapeutic regimes such as FOLFOX (a regime consisting of 5'fluorouracil, Leucovorin and Oxaliplatin) or FOLFIRI (5'-fluorouracil, Leucovorin and Irinotecan) resulted in patients with metastatic disease having median survival times of approximately 20 months as compared to 6 months without treatment (Meyerhardt and Mayer, 2005).

Regardless of the treatment regime used, patients diagnosed with metastatic colorectal cancer have a poor prognosis; 5-year survival rates for such patients are around 5%. The dependence of patients on chemotherapeutic agents that have been around for decades—5'-fluorouracil was first synthesized in 1957 (Heidelberger et al., 1957), Oxaliplatin in 1976 (Kidani et al., 1976) and Irinotecan in 1987 (Kunimoto et al., 1987)—and yet only provide modest survival benefits that are accompanied by toxic side effects highlight the urgent need for more effective targeted therapies with diminished side effects.

In recent years, identification of epidermal growth factor receptor (EGFR) as a receptor that is over-expressed in colorectal cancer has led to the development of small molecule inhibitors of EGFR (Erlotinib and Gefitinib) as well as inhibitory monoclonal antibodies (Cetuximab and Panitumumab). When such antagonists

of EGFR activity are used in clinical trials with or without Irinotecan or Oxaliplatin, patient response was observed with improved quality of life. Although there is statistically significant increase in overall survival of patients with metastatic disease, the increase in survival is typically modest, between 6 months to a year, and limited to patients with KRAS wild-type disease (Bokemeyer et al., 2009; Van Cutsem et al., 2009).

Given the relative lack of clinical progress in the treatment of metastatic colorectal cancer, there is an urgent need for a better understanding of the pathogenesis of colorectal cancer metastasis to identify targetable nodes in metastatic pathways. As the liver is the main site of colorectal cancer metastasis—over 70% of patients that developed metastatic disease will present with liver lesions, an understanding of the biology that governs colorectal cancer cells metastatic colonization of the liver could lead to the development of therapeutics targeting this important stage of colorectal cancer progression that are more effective then currently available therapies.

**Colorectal Cancer: Origins and progression**

*Pathophysiology*

Colorectal cancer arises from the lower gastrointestinal tract, which is comprised of the cecum, the colon and the rectum and initiates in the mucosa, the innermost lining of the gastrointestinal tract. Colorectal cancer begins as pre-cancerous lesions called polyps that project above the mucosa of the intestinal tract. The majority of polyps in the intestinal tracts are from non-glandular epithelium and are small and hyperplastic and usually do not develop into pre-cancerous lesions.

Adenomatous polyps are the important precursors of colorectal cancer and arise from the glandular epithelium of the colon and rectum. Such polyps are usually larger then ten millimeters in size and if not detected and removed during colonoscopy, can further progress to display severe dysplasia and before progression into adenocarcinoma and subsequent invasion through the mucosa of the colon and into surrounding tissues.

*Molecular determinants of colorectal cancer tumorigenesis*

As with its pathophysiology, the molecular determinants of colorectal cancer tumorigenesis are well understood. Seminal work by Vogelstein and others had identified genes and pathways which, when dysregulated progressively in the colonic epithelium, results in formation of adenomas and subsequently colorectal cancer (Fig. 1.2) (Fearon and Vogelstein, 1990). Key pathways that are deregulated during colorectal cancer tumorigenesis include but are not limited to the APC tumor suppressor pathway (Kinzler and Vogelstein, 1996), the KRAS oncogenic pathway (Fang and Richardson, 2005) and the p53 tumor suppressor pathway (Hollstein et al., 1991).

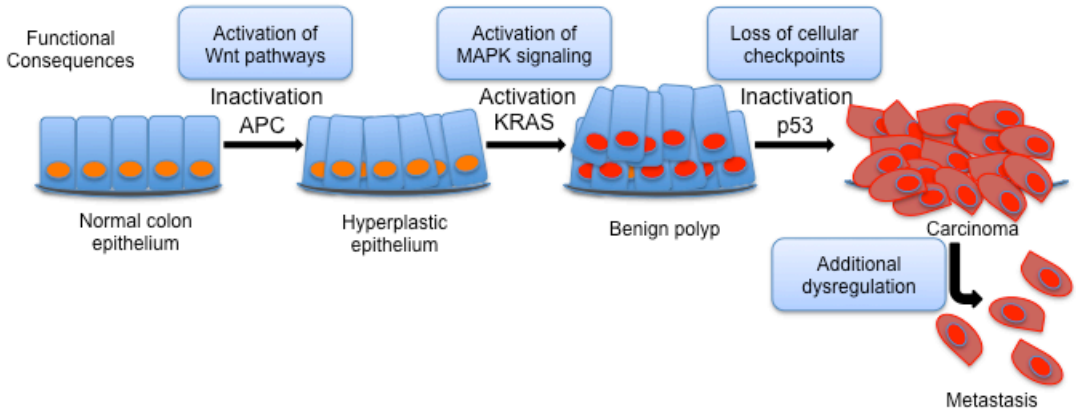



**Figure 1.2 | Molecular basis of colorectal cancer tumorigenesis.** Colorectal cancer tumorigenesis is marked by distinct mutational inactivation of tumor suppressors and activation of oncogenes leading to loss of cellular checkpoints and activation of oncogenic pathways. After tumorigenesis, further dysregulation of pathways contributes to metastatic progression.

Constitutive activation of the Wnt pathway, achieved through inactivating mutations of APC or activating mutation of its degradative target, β-catenin is widely recognized as the initiating event prior to adenoma formation, with over 80% of sporadic colorectal adenomas and cancers found to have aberrations in this pathway (Bienz and Clevers, 2000).

Another oncogenic pathway that is frequently activated in colorectal cancer tumorigenesis is the mitogen activated protein kinase (MAPK) signaling pathway. Constitutive activation of MAPK signaling in colorectal cancer results from activating mutations in KRAS (Bos et al., 1987; Forrester et al., 1987) or its downstream effector BRAF (Rajagopalan et al., 2002; Wan et al., 2004). Such oncogenic mutations are commonly found in polyps and adenomas and indicate

a substantial role of this pathway in the early stages of colorectal cancer tumorigenesis (Fang and Richardson, 2005).

An important and relatively late step in colorectal cancer formation is the inactivation of the p53 tumor suppressor pathway. The inactivation of the pathway through missense mutations or chromosomal deletion leads to loss of critical cellular checks against oncogenic stresses. This prevents activation of cell-cycle arrest checkpoints or initiation of the apoptotic cascade as a result of cellular stresses, contributing to carcinogenesis (Rodrigues et al., 1990).

In addition to the above-mentioned pathways, mutations in other regulatory pathways such as those mediated by phosphatidylinositol 3-kinase and PTEN (Yuan and Cantley, 2008), as well as the TGF-β tumor suppressor pathway (Wakefield and Roberts, 2002) have been described to occur and contribute in varying frequencies during colorectal cancer development. Besides these classical pathways, the advent of high-throughput sequencing and whole genome sequencing of colorectal cancer samples have led to the identification of additional somatic mutations that are putative drivers of colorectal cancer tumorigenesis (Kandoth et al., 2013; Network, 2012). However, much work remains with regards to functional characterization of putative driver genes identified by whole genome sequencing.

Besides the above-mentioned dysregulated pathways in colorectal cancer tumorigenesis, there is a subgroup of patients who possess deficiencies in DNA

mismatch repair as a result of inactivation of the genes involved. Mismatch repair genes that are commonly inactivated in these patients include *MLH1*, *MSH2* and *MSH6*. The inactivation of these genes results in colorectal cancers that are characterized as microsatellite instable (MSI) in which sizes of repetitive DNA elements in the genome are frequently altered (Thibodeau et al., 1998). It is not uncommon for these patients to have inactivation of other tumor suppressor genes as a result of DNA repair deficiency. *MYH*, another DNA repair gene involved in base-excision repair of nucleotides, is also inactivated in certain colorectal cancer patients (Al-Tassan et al., 2002).

*The metastatic cascade in colorectal cancer*

Metastasis during colorectal cancer progression is a complex multi-step process during which the primary tumor on the surface of the mucosa develops an invasive front that invades through the mucosa and into the underlying submucosa of the colonic tract. The submucosa is richly supplied by blood vessels and is also responsible for the draining of blood carrying nutrients absorbed by the colon into the portal circulation towards the liver. Once the primary tumor invades into the submucosa and surrounding circulatory vessels, shedding of cancer cells from the primary tumor into the circulation will result in dissemination of cancer cells towards distal organs (Fig. 1.3) (Gupta and Massagué, 2006; Nguyen et al., 2009). Upon arrival at the distal organ, the majority of disseminated cancer cells will undergo cell death as a result of inability to adapt to a new microenvironment (Chambers et al., 2002). As the liver is the first major organ encountered by the portal circulation, it is the main organ of colon cancer cell dissemination (Weiss et al., 1986). Cells that survive in

the liver microenvironment might undergo a period of dormancy that can last from several months to years. Clinically, patients have been found to have substantial numbers of circulating tumor cells, as well as disseminated colon cancer cells in the liver. However, only a small fraction of disseminated cells go on to develop macroscopic metastases as majority of cells die without forming macro-metastases (Sugarbaker, 1993). Given such evidence, in colorectal cancer, the rate-limiting step of liver metastases appears to be that of liver colonization. It is therefore of great scientific and clinical value to understand the molecular mechanisms that drive this process.

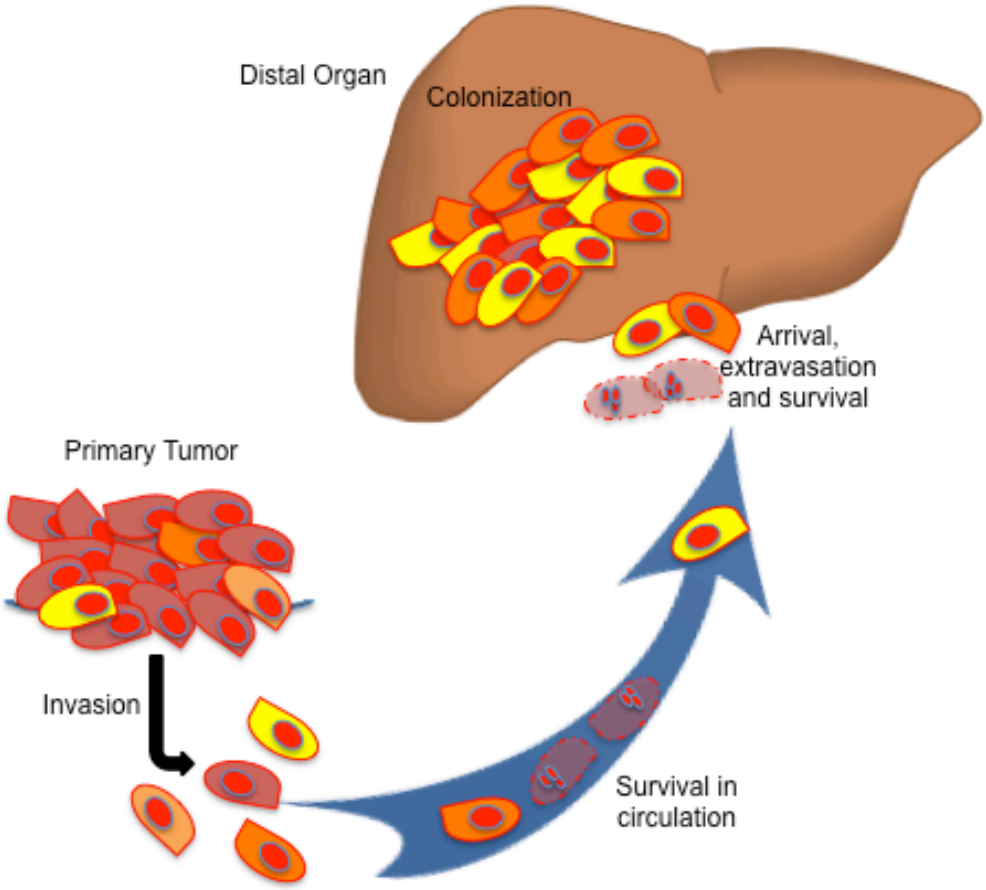



**Figure 1.3 | The metastatic cascade.** During metastasis, cells disseminate from the primary tumors and invade into the circulatory system. Cells that survive dissemination in circulation and arrive at distal organs have to adapt and survive within the foreign microenvironment before they can proliferate and colonize the distal organ. Colonization of distal organs and subsequent organ failure is a significant cause of patient mortality.

*Molecular determinants of colorectal cancer metastasis*

Given the complexity of the metastatic cascade, it is of little surprise that identified pathways involved in colorectal progression from the primary tumor

to distal metastasis are more diverse. Unlike primary tumorigenesis, mutational inactivation of specific metastasis-suppressing genes or activation of metastasis promoting genes is rare and comparative sequencing of matched primary tumors and metastases from the same patient have not identified many mutations responsible for metastatic progression (Brannon et al., 2014; Jones et al., 2008). However, gene expression analyses followed by experimental studies have identified some of the pathways involved in various steps of the metastatic cascade during colorectal cancer metastasis.

Gene expression changes during colorectal cancer progression and metastasis can be brought about through various means, including but not limited to chromatin aberrations such as re-arrangements, amplifications or deletions. DNA hypermethylation or hypomethylation in the promoter region of genes can cause down-regulation or up-regulation of gene expression, respectively, and have been reported in colon cancer (Markowitz and Bertagnolli, 2009). More recently, post-transcriptional regulation of gene expression has been a subject of intense scrutiny (Licatalosi and Darnell, 2010; Schwanhäusser et al., 2011).

A class of short noncoding RNAs known as microRNAs can mediate post-transcriptional modulation of mRNA stability and translation with corresponding changes in gene expression. The biogenesis of mature microRNAs is well understood (Ha and Kim, 2014). A primary microRNA transcript (pri-miRNA) is first transcribed by RNA polymerase II, and occasionally RNA polymerase III. Subsequently, pri-miRNA transcripts are cleaved by the ribonuclease Drosha, to form pre-miRNA stem-loop transcripts that are then

exported into the cytoplasm where they are recognized and cleaved by a second ribonuclease, Dicer, to form short 21-26 nucleotide miRNA-duplexes. These duplexes are loaded onto the RNA-induced silencing (RISC) complex, with the subsequent release of the passenger non-targeting microRNA strand. The RISC complex bearing a mature single-stranded miRNA will bind to target mRNAs bearing sequences complementary to the bound miRNA and recruit effector proteins responsible for initiating the decay or translational repression of the target mRNA. Target sequences of microRNAs are usually present on the 3'-UTR, and occasionally coding sequences (CDS) of the mRNA transcript.

In recent years, as with identification of genes and pathways involved in primary tumorigenesis, microRNAs that regulate tumorigenesis have also been identified and their downstream effector genes and pathways well established (Schickel et al., 2008). In addition to the numerous studies identifying microRNAs that regulate overt tumorigenic phenotypes such as proliferation and apoptosis, and early stages of the metastatic cascade such as invasion and migration, microRNAs involved in later stages of metastasis such as lung colonization by breast cancer and melanoma cells have been described (Pencheva et al., 2012; Png et al., 2012). The relative lack of studies investigating and identifying genes and microRNAs involved in liver colonization is in part due to a lack of defined experimental models that specifically examine liver colonization by colon cancer cells without involving other aspects of the metastatic cascade such as invasion into circulation, survival during dissemination and extravasation into the liver parenchyma.

Given the importance of liver colonization as a rate-limiting step in the culmination of the metastatic cascade, it would be of scientific interest develop a model of colon cancer liver colonization that can be utilized to identify microRNAs that regulate liver colonization. Clinically, identification of microRNAs and downstream pathways that suppress liver metastasis can potentially lead to development of therapeutics that suppress liver metastasis, either by therapeutic delivery of the microRNAs or inhibition of their effector genes. Availability of such therapeutics, alongside currently available drugs can potentially lead to better outcomes for patients.

**Colorectal cancer: *In vivo e*xperimental models**

*Mouse models of colorectal cancer tumorigenesis*

The tumorigenic process of colorectal cancer is well defined and consequently, genetic mouse models of colorectal cancer tumorigenesis with mutations of key oncogenic or tumor suppressor genes have been established (Karim and Huso, 2013).

Considering the significance of APC as a gatekeeper to colon tissue integrity and inactivation of APC as an initiating event in formation of most adenomas, the most common types of genetic mouse models of colorectal cancer tumorigenesis is the APC mutant mouse or the β-catenin mutant mice that regulate the Wnt signaling pathway downstream of APC.

APC mutant mice with various inactivating mutations of the APC tumor suppressor genes have been generated. Loss of APC activity results in formation of polyps. However the polyps themselves do not progress to aggressive adenocarcinoma (Moser et al., 1990). Similarly, activating stabilizing mutations of β-catenin downstream of APC regulation results in the formation of polyps that do not progress to malignancy. Based on the work of Vogelstein and others in defining the molecular progression of colorectal cancer tumorigenesis, APC or β-catenin mutant mice with mutations in other oncogenic and tumor suppressors genes have also been generated.

APC mutant mice with genetic alterations in other genes have been observed to exhibit progression of colonic polyps to adenomas and adenocarcinomas. In particular, abrogation of the TGF-β tumor suppressor pathway through mutation of SMAD4 in APC mutant mice resulted in development of invasive adenocarcinomas (Takaku et al., 1998).

In addition to genetic mouse models developed through abrogation of APC tumor suppressor pathways and synergistic mutation of oncogenes and/or suppressors, genetic mouse models that involve perturbation of the mismatch repairs system were also developed which also resulted in the onset of adenomas in the gastrointestinal tracts of the mice (de Wind et al., 1999).

Despite the numerous genetic mouse models of colorectal cancer tumorigenesis, a common caveat for all of the above-mentioned models is the lack of cancer

progression beyond the primary site. It is not surprising that these genetic mouse models rarely develop distal metastases (Nandan and Yang, 2010), given the shorter lifespan of the above-described mice due to heavy primary tumor burden.

As metastasis to distal organs such as the liver and lungs are responsible for the majority of colon cancer deaths in human patients, *in vivo* mouse models of metastasis that are complementary to available genetic models of tumorigenesis have to be developed and utilized to study the metastatic cascade and potential therapeutic applications (Francia et al., 2011).

*Mouse models of colorectal cancer metastasis*

In order to study colon cancer metastasis *in vivo* with human colon cancer cells, xenograft mouse models in which cells are implanted or injected into immunodeficient mice are developed. Depending on the stage(s) of the metastatic cascade that are under investigation, investigators have transplanted or inoculated cells into mice at various sites.

To model the progression of colon cancer cells from the primary site to metastasis of distal organs, intra-cecal injections of colon cancer cells or implantation of small tumor fragments onto the cecum of nude mice have been performed (Morikawa et al., 1988a). Mice inoculated with colon cancer through these means went on to develop metastases at local and regional lymph nodes as well as distal organs such as the liver and lungs, demonstrating the recapitulation of the entire metastatic cascade.

Besides inoculation of colon cancer at orthotopic sites, direct introduction of colon cancer cells into the circulation have been performed through portal vein injection or intrasplenic injection, both of which result in dissemination of colon cancer cells into the portal circulation and into the liver parenchyma (Morikawa et al., 1988b). Compared to intra-cecal injection, these procedures bypass the earlier stages of the metastatic cascade such as invasion of the primary tumor and intravasation into the circulation. Instead, survival of colon cancer cells in the circulation and later stages are examined.

Regardless of which stage of the metastatic cascade is under investigation, xenograft models of colon cancer metastasis have proven of great utility in delineating the mechanisms involved in metastasis as *in vivo* modeling of colorectal cancer progression can provide insights that are not readily apparent in *in vitro* systems. A criticism of xenograft models of human colon cancer cell metastasis is that immunodeficient mice are used, which do not allow for study of the interactions between the immune system and cancer cells. To address this concern, it is possible to utilize xenograft models using mouse cancer cells implanted into syngeneic mice. However, currently available mouse colon cell-lines are limited to those derived from chemical carcinogenesis which might not necessarily represent the common etiology of human colon cancers (Griswold and Corbett, 1975). Eventually, derivation of mouse cell-lines from primary tumors arising from genetic mouse models of colorectal cancer for use in xenograft experimental systems can allow for better interrogation of the metastatic cascade in an immuno-competent setting.

*In vivo selection of metastatic phenotypes*

*In vivo* selection of cancer cells is a technique pioneered by Fidler and colleagues in 1973 (Fig. 1.4) (Fidler, 1973). It can be utilized to enrich for specific cell populations with desired phenotypes from more heterogeneous populations. Within the context of cancer biology, *in vivo* selection had been utilized to select for breast cancer cells with enhanced capacity for lung, bone and brain metastasis. Transcriptomic profiling and mechanistic studies comparing the parental heterogeneous breast cancer population and the *in vivo* selected organ-metastatic derivatives had resulted in the identification of genes that regulate metastasis to the respective organs by breast cancer cells (Kang et al., 2003; Minn et al., 2005; Png et al., 2012; Tavazoie et al., 2008). More recently, *in vivo* selection was performed with melanoma cells and mediators of melanoma metastasis to the lungs were identified (Pencheva et al., 2012). Similar cellular phenotypes were identified in breast and melanoma cancer cells that were *in vivo* selected for lung colonization capacity, although different genes and pathways were utilized in breast cancer and melanoma respectively (Pencheva et al., 2012; Png et al., 2012). This highlights the possibility that with regards to organ colonization, there might be common phenotypes that are selected for irrespective of cancer type.

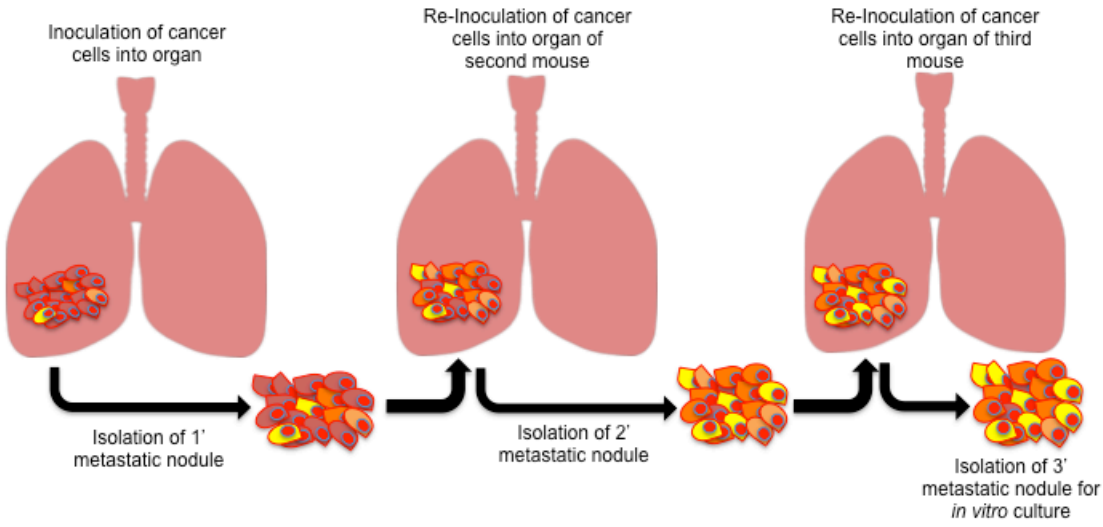



**Figure 1.4 | *In vivo* selection.** During *in vivo* selection, a heterogeneous population of cancer cells is inoculated into specific organs (such as lung) in a mouse. The first generation metastatic nodule that forms can be excised, dissociated into cells and the process repeated. Over several iterations, a more metastatic population of cells can be selected for.

With regards to colorectal cancer, an *in vivo* selection model for liver colonization, the final step of metastasis has not been yet been demonstrated. Given the utility of *in vivo* selection for identifying molecular mediators of breast and lung cancer metastasis, novel mediators of colorectal cancer metastasis can be identified with an appropriate model of liver colonization and *in vivo* selection.

**Specific aims**

This thesis aims to identify molecular determinants of colorectal cancer metastasis to the liver, the primary site of colorectal cancer metastasis. The first part of the thesis will describe two complementary approaches used to identify microRNAs that suppress liver colonization and metastasis by colon cancer cells.

The first approach is an *in vivo* microRNA library screen using multiple colon cancer cell-lines with distinct mutational spectrum. The second approach harnesses *in vivo* selection of colon cancer cells to identify endogenous microRNAs that suppress colon cancer liver colonization. The complementary approaches led to the identification of miR-483-5p and miR-551a as suppressors of colon cancer metastasis and colonization (Chapter II). Functional characterization of both microRNAs was performed and an organotypic slice culture system was developed to allow for phenotypic characterization of the microRNAs *in vitro*.

Following the identification of miR-483-5p and miR-551a, unbiased transcriptomic profiling approaches were used to identify downstream targets and effectors of both microRNAs. One gene, Creatine Kinase Brain (CKB), was identified to phenocopy the effects of the microRNAs and further mechanistic dissection of CKB-mediated pathways identified it as a pro-survival factor in the hypoxic liver microenvironment of colon cancer metastases through modulation of intra- and extra-cellular energetics. Other components of the creatine kinase axis during colon cancer metastasis to the liver are also examined, leading to the identification of SLC6a8, a membrane channel as another critical effector in the creatine kinase mediated metastatic axis (Chapter III).

In the third part of this thesis, clinical evidence is provided to support the clinical significance of these findings to colorectal cancer metastasis in patients (Chapter IV). Publicly available datasets as well as archival materials from primary tumors and liver metastases were interrogated to reveal the relevance of the microRNAs,

CKB and SLC6a8 to colon cancer liver metastasis. Given the clinical relevance of the pathway described, proof-of-principle therapeutic delivery of the microRNAs and inhibition of CKB and SLC6a8 was investigated as well. The final chapter of the thesis discusses the findings of this thesis and presents future directions (Chapter V).

# Chapter II: Identification of Novel Molecular Determinants of Colorectal Cancer Metastasis to the Liver

As a first step to identify molecular determinants of colorectal cancer metastasis to the liver, this chapter describes two complementary approaches used to identify microRNAs that regulate colon cancer cell metastasis. The first approach employed a library-based gain-of-function *in vivo* screen of 661 microRNAs to identify microRNAs that when over-expressed, suppressed liver colonization by colon cancer cells. The second approach utilizes *in vivo* selection to select for highly metastatic colon cancer cells to identify endogenous microRNA suppressors of colon cancer metastasis. MicroRNAs that were identified and common to both approaches were further characterized and studied. An *in vitro* organotypic slice culture system was also developed to allow for phenotypic study of processes regulated by the microRNAs.

### *In vivo* screening of microRNA library identifies suppressors of liver colonization by colon cancer cells

Library-based screening approaches have been previously employed to identify regulators of cancer progression (Mohr et al., 2010). However, most of the earlier studies have been performed in an *in vitro* setting that might not result in identification of modulators that are active in an *in vivo* and more physiologically relevant setting.

To identify microRNAs that when over-expressed, suppress colon cancer liver colonization in a physiological context, a lentiviral expression library of 661 microRNAs was used (Fig. 2.1). Heterogeneous colon cancer cell-line populations were infected with the lentiviral library at a low multiplicity-of-infection (MOI), such that each individual cell likely over-expressed a single microRNA.

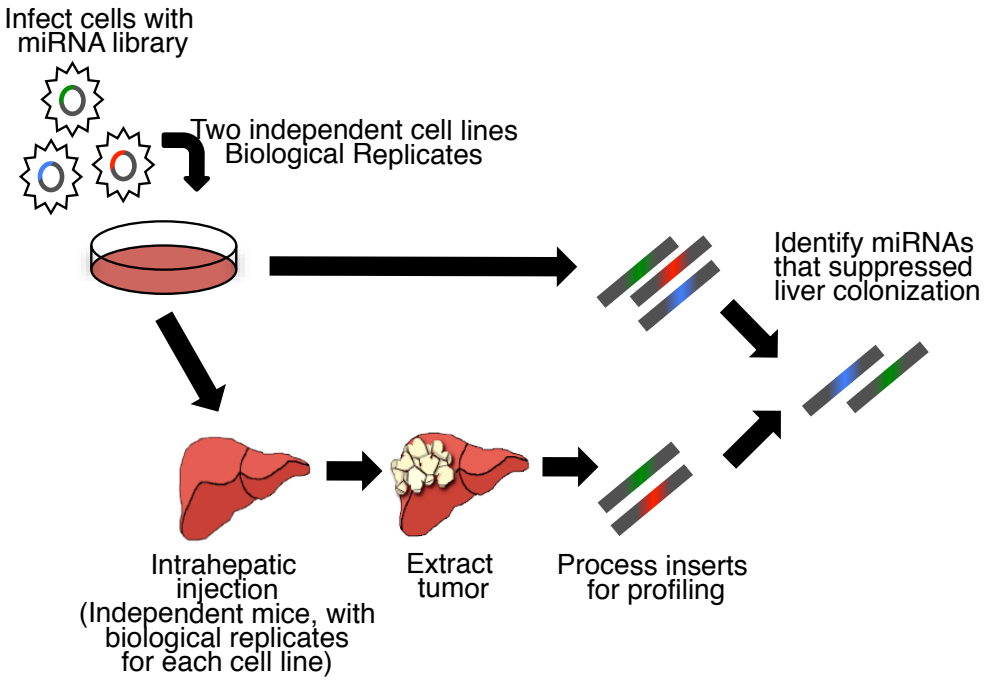



**Figure 2.1 | A lentiviral-based microRNA library screen is used to identify microRNAs that are potential suppressors of liver metastasis by colorectal cancer cells.**

Two colon cancer cell-lines, SW620 and WiDR, were used and were selected in part for their different known driver mutational spectrum. This allowed for identification of microRNAs that can suppress colon cancer liver colonization irrespective of their mutational backgrounds.

Following transduction, colon cancer cell-populations were intra-hepatically injected into immunodeficient mice to allow for selection of cells capable of colonizing the liver and forming metastatic liver nodules 4-6 weeks after injection. Genomic DNA was extracted from the resulting nodules and PCR amplification, reverse transcription and microRNA profiling of microRNA inserts allowed for quantification of changes in library-derived microRNA

representation in the context of liver colonization compared to a reference pool obtained from cell-populations that had not undergo the selective pressure of liver colonization (Fig. 2.1). Biological replicate screenings were performed for both cell-lines and significant correlation was found between pairs of biological replicates, indicating that the screen was reproducible (for SW620, $r=0.7344$, $p<0.001$; WiDR, $r=0.8066$, $p<0.001$). MicroRNAs that displayed reduced representation in the context of liver colonization were identified in both cell-lines (Table 2.1) and considered to be putative suppressors of liver colonization.

**Table 2.1 | List of microRNAs depleted after liver colonization by SW620 and WiDR cells transduced with the microRNA library.** *In vivo* library screen was performed in duplicate for both cell-lines and compared to reference samples comprising of cell populations transduced with the library, but not injected into mice.

| miRNA | Fold Change vs. Reference | | miRNA | Fold Change vs. Reference | |
|---|---|---|---|---|---|
| | SW620 | WiDR | | SW620 | WiDR |
| hsa-let-7e | -1.92 | -92.73 | hsa-miR-384 | -3.57 | -5.63 |
| hsa-miR-009-1 | -3.47 | -3.79 | hsa-miR-409-3p | -3.27 | -8.58 |
| hsa-miR-023a | -6.11 | -1.61 | hsa-miR-412 | -2.51 | -1.92 |
| hsa-miR-092a | -1.99 | -2.91 | hsa-miR-483-5p | -1.71 | -2 |
| hsa-miR-099a | -4.24 | -1.99 | hsa-miR-509-1 | -1.98 | -11.25 |
| hsa-miR-099b | -1.7 | -6.35 | hsa-miR-542-3p | -2.09 | -1.64 |
| hsa-miR-107 | -2.43 | -3.05 | hsa-miR-545 | -2.17 | -1.61 |
| hsa-miR-1203 | -1.79 | -2.18 | hsa-miR-551a | -7.14 | -8.07 |
| hsa-miR-1243 | -1.61 | -1.95 | hsa-miR-551b | -12.7 | -8.97 |
| hsa-miR-1244 | -3.52 | -1.61 | hsa-miR-557 | -1.92 | -5.27 |
| hsa-miR-1288 | -2.61 | -1.81 | hsa-miR-563 | -1.78 | -2.43 |
| hsa-miR-1297 | -1.74 | -3.62 | hsa-miR-574 | -8.09 | -4.33 |
| hsa-miR-133a | -1.75 | -2.8 | hsa-miR-574-5p | -3.18 | -5.18 |
| hsa-miR-134 | -1.99 | -2.5 | hsa-miR-590-5p | -1.86 | -2.38 |
| hsa-miR-138-2 | -2.84 | -2.99 | hsa-miR-591 | -1.93 | -3 |
| hsa-miR-143 | -2.58 | -2.2 | hsa-miR-592 | -2.4 | -5.36 |
| hsa-miR-149* | -2.74 | -2.05 | hsa-miR-638 | -2.1 | -1.95 |
| hsa-miR-181a-2* | -1.65 | -1.66 | hsa-miR-656 | -2.02 | -2.51 |
| hsa-miR-192 | -1.67 | -3.8 | hsa-miR-769-5p | -2.17 | -1.61 |
| hsa-miR-196a-1 | -1.92 | -19.33 | hsa-miR-877 | -1.83 | -1.87 |
| hsa-miR-218-1 | -29.13 | -17.78 | hsa-miR-9* | -1.96 | -7.84 |
| hsa-miR-363 | -2.37 | -3.68 | hsa-miR-92a-2* | -1.64 | -6.14 |
| hsa-miR-369-3p | -1.83 | -2.03 | | | |

***In vivo* selection for liver metastatic colon cancer cells identifies endogenous microRNAs that suppressed liver metastasis**

As a complementary approach to library-based screening, which identified microRNAs that when over-expressed suppressed liver colonization by colon cancer cells, *in vivo* selection was used to identify endogenously modulated microRNAs that suppress liver metastasis. *In vivo* selection was performed on the LS174T human colon cancer line, which displays MSI, for enhanced liver colonization activity through iterative intra-hepatic injection of cancer cells into immunodeficient mice followed by surgical resection of metastatic liver nodules and dissociation of cells (Fig. 2.2).

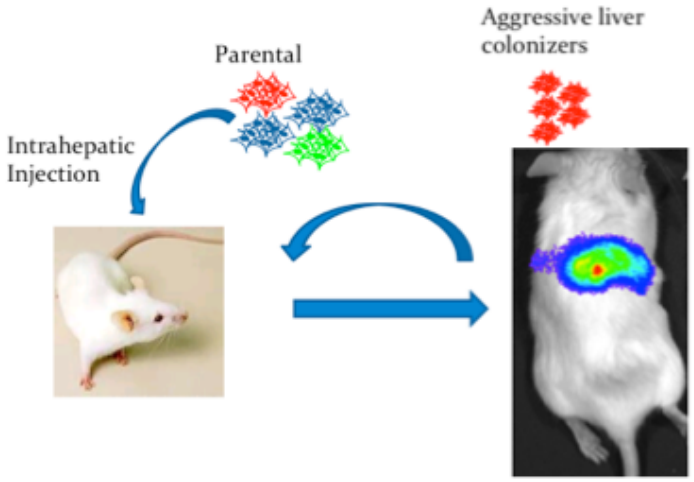



**Figure 2.2 | *In vivo* selection of LS174T human colon cancer cells.** LS174T parental cell populations expressing a luciferase reporter for bioluminescent imaging were injected into the liver of immunodeficient NOD-SCID mice. Bioluminescent imaging was used to monitor the development of metastatic nodules in mice. After a period of 3-4 weeks, mice were euthanized and established nodules excised and dissociated into cells. Dissociated cells were injected into mice. After three iterations of the process, two independent third-generation liver colonizers cell populations were derived and tested for enhanced liver colonizing potential.

Independently derived third-generation liver colonizers LS-LvM3a and LS-LvM3b displayed significantly enhanced (>50 fold) capacity for liver colonization (Fig. 2.3A). Importantly, these derivatives also displayed dramatically enhanced (>150 fold) liver metastatic capacity upon portal circulation injection in experimental metastasis assay—revealing the acquisition of liver colonization capacity to be sufficient for imparting enhanced liver metastasis activity (Fig. 2.3B). These *in vivo* selected metastatic derivatives were found to display reduced expression of endogenous microRNAs that could be suppressors of metastasis (Table 2.2).

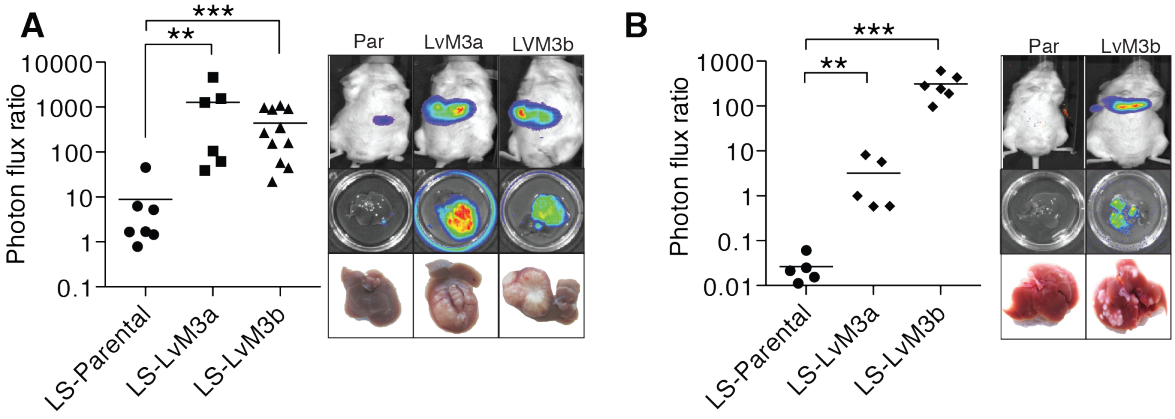



**Figure 2.3 | Liver colonization is a rate-limiting step during colorectal cancer metastasis to the liver.** A, Bioluminescence plot of liver colonization by 5 X $10^5$ LS-Parental, and *in vivo*-selected LvM3a and LvM3b cells after direct intrahepatic injection (n>5). Each data-point represents the measurement from one mouse. Mice were imaged at day 21 after injection and livers extracted for *ex vivo* imaging and gross morphological examination. B, Liver metastasis in mice injected intra-splenically with 5 X $10^5$ LS-Parental, LvM3a and LvM3b cells. Metastatic progression was measured by bioluminescence imaging (n>5). Mice were imaged at day 21 after injection and livers extracted for gross examination. p values are based on one-sided Mann-Whitney test for non-Gaussian distribution. **p<0.01; ***p<0.001.

**Table 2.2 | List of miRNAs depleted in highly metastatic LS Derivatives compared to LS174T parental cell-line.**

| miRNA | Fold Change in Metastatic Derivatives |
|---|---|
| hsa-miR-483-5p | -11.74 |
| hsa-miR-429 | -7.43 |
| hsa-miR-218-1* | -6.015 |
| hsa-miR-551a | -5.03 |
| hsa-miR-146a* | -3.8 |
| hsa-miR-423-3p | -3.64 |
| hsa-miR-499-3p | -3.55 |
| hsa-miR-1246 | -3.2 |
| hsa-miR-148a | -3.065 |
| hsa-miR-20b* | -3.065 |
| hsa-miR-155 | -2.775 |
| hsa-let-7d* | -2.715 |
| hsa-miR-215 | -2.585 |
| hsa-let-7d* | -2.465 |
| hsa-miR-7-1* | -2.435 |
| hsa-miR-377 | -2.31 |
| hsa-miR-2115 | -2.265 |
| hsa-miR-7 | -2.165 |
| hsa-miR-1231 | -2.135 |
| hsa-miR-16-2* | -2.09 |
| hsa-miR-200b* | -2.08 |
| hsa-miR-211 | -2.02 |

**Mir-483-5p and miR-551a are robust suppressors of liver metastasis by colon cancer cells**

Two microRNAs, miR-483-5p and miR-551a were identified by the overlap of the library-based screening approach and *in vivo* selection to be putative suppressors of liver metastasis (Fig. 2.4).

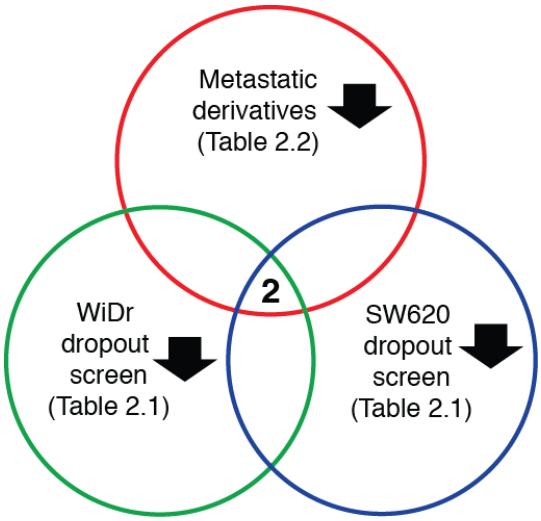



**Figure 2.4 | Integrative approach to identification of miR-483-5p and miR-551a as putative suppressors of colon cancer metastasis.** microRNAs that had a loss of representation of at least 1.6 fold in both SW620 and WiDR cell-lines in the miRNA library screen (Table 2.1) and silenced at least 2 fold in metastatic derivatives (Table 2.2) were considered potential suppressors.

Consistent with a suppressive role for these microRNAs in liver metastasis, over-expression of miR-483-5p or miR-551a robustly suppressed metastasis by LS-LvM3b cells introduced into the portal circulation (Fig. 2.5A), while inhibition of endogenous miR-483-5p or miR-551a in poorly metastatic colon cancer cell-lines, LS-174T and SW480 significantly enhanced liver metastatic colonization (Fig. 2.5B, C).

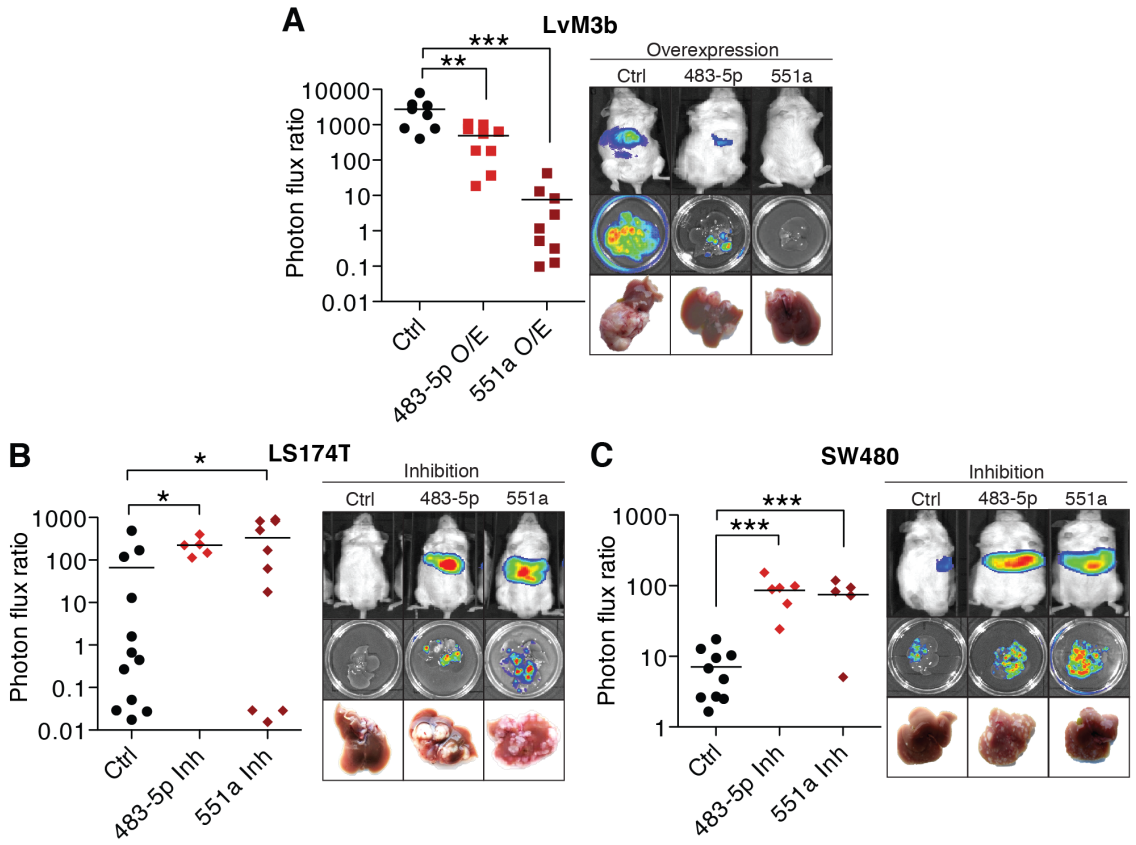



**Figure 2.5 | miR-483-5p and miR-551a are endogenous microRNAs that suppress liver metastasis.** A, Liver metastasis of mice injected with 5 X $10^5$ LvM3b cells over-expressing either a control hairpin, miR-483-5p or miR-551a (n>5). Mice were imaged at day 21 after injection and livers extracted for *ex vivo* imaging and gross morphological examination. B, Metastatic progression in NOD-SCID mice injected with 5 X $10^5$ poorly metastatic LS174T parental cells, whose endogenous miR-483-5p or miR-551a activity was inhibited (n>5). Mice were imaged at day 28 and euthanized. C, Metastatic progression in mice injected with 5 X $10^5$ poorly metastatic SW480 cells, whose endogenous miR-483-5p or miR-551a was inhibited using LNA targeting miR-483-5p and miR-551a (n>5). Mice were imaged at day 28 after injection. p values are based on one-sided Mann-Whitney test for non-Gaussian distribution. *p<0.05; **p<0.01; ***p<0.001.

The effects of these microRNAs on metastatic progression were not secondary to modulation of intrinsic proliferative capacity since miR-551a inhibition did not affect *in vitro* proliferation, while miR-483-5p inhibition minimally increased proliferation (10%)—an order of magnitude less than its effect on metastasis (Fig. 2.6A). Importantly, over-expression of either microRNA did not suppress primary tumor growth (Fig. 2.6B).

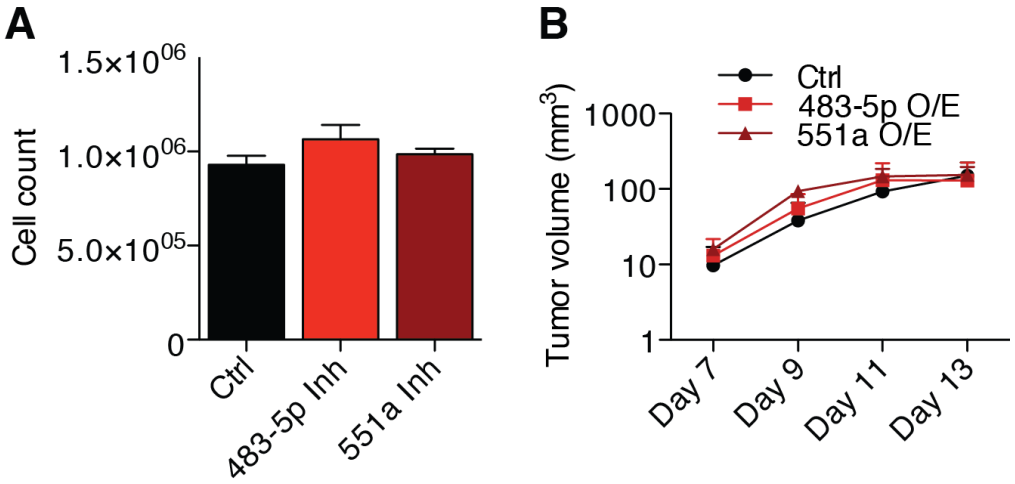



**Figure 2.6 | Mir-483-5p and miR-551a do not regulate *in vitro* proliferation and *in vivo* primary tumor growth.** A, $1x10^5$ SW480 cells whose endogenous miR-483-5p and miR-551a activity was inhibited were seeded onto a 6-well plate in triplicate. Cells numbers were counted after 5 days (n=3). B, 1 X $10^6$ LvM3b cells over-expressing a control hairpin, miR-483-5p or miR-551a was injected into the subcutaneous flanks of NOD-SCID mice. Tumor volumes were measured over time (n=4). Error bars, s.e.m; p values are based on one-sided Student's t-tests. *p<0.05; **p<0.01; ***p<0.001.

**Mir-483-5p and miR-551a suppress colon cancer cell survival in the liver microenvironment**

The liver microenvironment is a complex milieu and liver-specific interactions of colon cancer cells within the liver might not be easily identified with *in vitro* phenotypic assays that do not recapitulate the liver microenvironment. To better investigate the mechanism(s) by which miR-483-5p and miR-551a exert their anti-metastatic effects, an *in vitro* liver organotypic slice culture system to study events during liver metastasis was developed.

The organotypic slice culture system involved the inoculation of colon cancer cells labeled with fluorescent dyes or expressing fluorescent proteins into the portal circulation of immunodeficient mice through intrasplenic injection, allowing for dissemination to the livers (Fig. 2.7A). Following inoculation into mice, the animals were euthanized and livers excised. Organotypic slices were prepared from the livers and were cultured prior to two-photon microscopy imaging of colon cancer cells within the liver slice cultures (Fig. 2.7B).

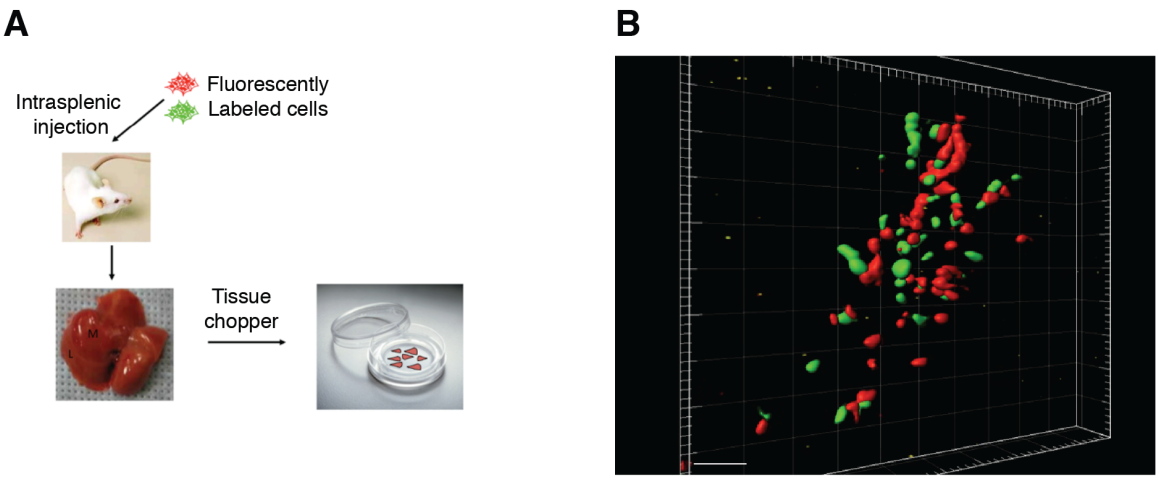



**Figure 2.7 | Organotypic slice culture system for *in vitro* studies of colon cancer cells interactions within the liver microenvironment.** A, Schematic of the hepatic organotypic slice culture system for studying events during liver colonization by colorectal cancer cells. Colon cancer cells were labeled with cell-tracker dye and introduced into the livers through intrasplenic injection. The livers were then excised and cut into 150um slices and plated onto cell culture inserts before imaging by multi-photon microscopy. B, Representative image of two colorectal cancer cell populations within the liver microenvironment. Scale bar represent 50μm.

Consistent with prior clinical and experimental studies, which revealed a significant selection on cell survival during metastatic colonization (Morikawa et al., 1988a; Sugarbaker, 1993), the highly metastatic LvM3b colonizer cells were significantly better at persisting in the liver microenvironment than their poorly metastatic parental line (Fig. 2.8); consistent with a key role for intrahepatic persistence in metastatic progression (Gupta and Massagué, 2006; Talmadge and Fidler, 2010).

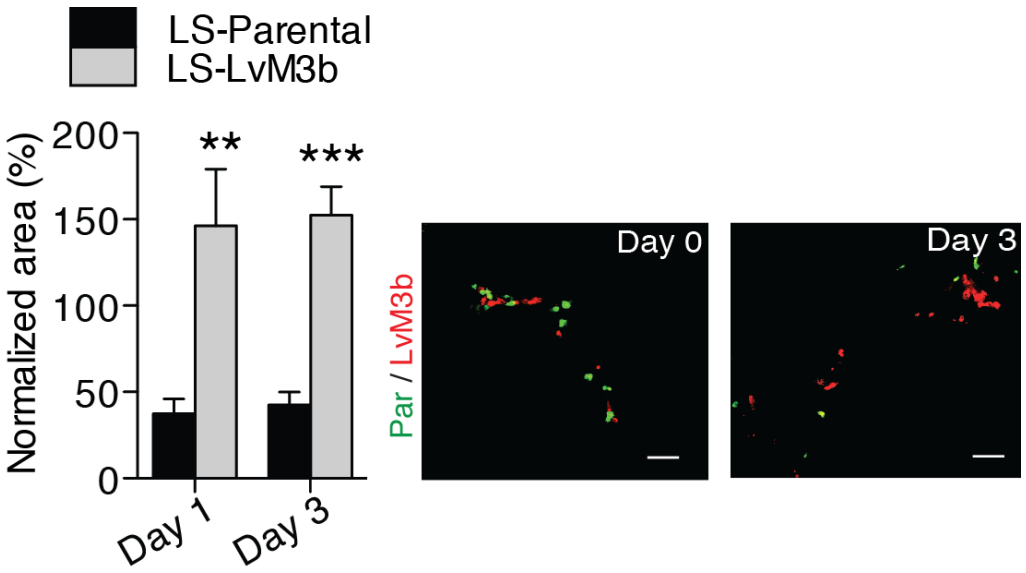



**Figure 2.8 | Highly metastatic LvM3b colon cancer cells display enhanced intrahepatic persistence.** 5 X $10^5$ cells were labeled with cell-tracker green (LS-Parental) or cell-tracker red (LvM3b) and introduced into the liver. Survival of the cells in organotypic cultures was monitored for up to 4 days (n=8). Dye-swap experiments were performed to exclude effects of dye bias. Representative images at day 0 and day 3 are shown. Scale bar represent 50μm. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. **p<0.01; ***p<0.001.

To determine whether the enhanced capacity of metastatic cells to persist in the hepatic microenvironment could be regulated by miR-483-5p or miR-551a, gain-of- and loss-of-function studies were performed using the organotypic slice culture system. Over-expression of miR-483-5p or miR-551a in highly metastatic LS-LvM3b cells suppressed colon cancer persistence in the hepatic microenvironment (Fig. 2.9A, B), while inhibition of either microRNA significantly enhanced persistence of poorly metastatic SW480 cells (Fig. 2.9 C, D).

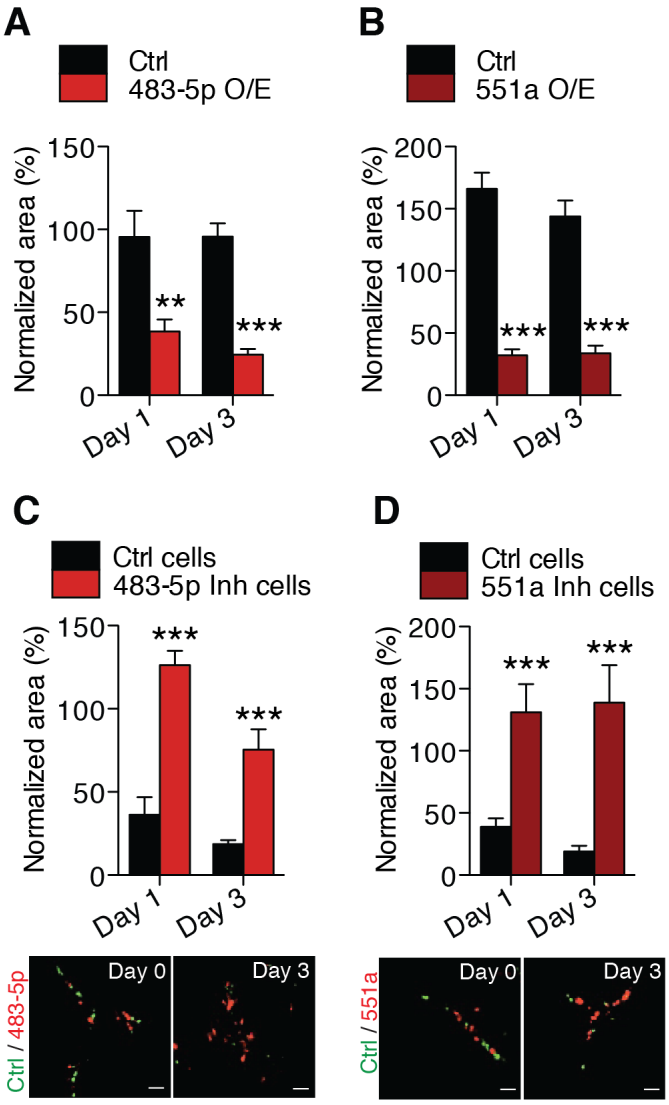



**Figure 2.9 | Mir-483-5p and miR-551a regulate intrahepatic persistence by colon cancer cells organotypic slice cultures.** A-B, Intrahepatic persistence of of LvM3b cells over-expressing miR-483-5p (A) or miR-551a (B) cells in organotypic liver slices (n=8). C-D, Intrahepatic persistence of SW480 cells whose endogenous miR-483-5p (C) or miR-551a (D) were inhibited by pre-treatment with LNAs (n=8). 5 X $10^5$ cells were labeled with cell-tracker green (control LNA) or cell-tracker red (microRNA targeting LNA) and introduced into the livers prior to slice culture. Representative images at day 0 and day 3 are shown. Dye-swap experiments were performed to compensate for dye bias. Scale bar represent 50µm. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. **p<0.01; ***p<0.001.

In agreement with the phenotype of hepatic persistence observed via the organotypic slice culture assay, colon cancer cells whose endogenous microRNAs were inhibited were able to out-compete control cells in the liver as early as 24 hours after injection of cells into the portal circulation for hepatic metastatic colonization assay (Fig. 2.10). Given that neither of the microRNAs regulated proliferation *in vitro* and *in vivo*, the ability of the microRNAs to suppress cancer cell survival during metastatic progression *in vivo* was investigated.

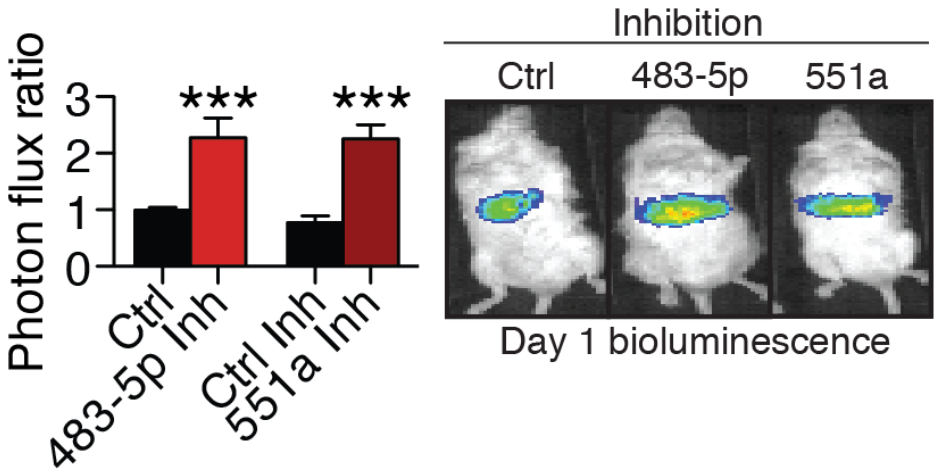



**Figure 2.10 | Mir-483-5p and miR-551a regulate early intrahepatic persistence by colon cancer cells *in vivo*.** Bioluminescent metastatic signal from mice (n=5) injected with 5 X $10^5$ SW480 cells whose endogenous miR-483-5p or miR-551a activities were inhibited. Images and measurements were taken 24hr after tumor cells inoculation. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. ***p<0.001.

To quantify colon cancer cell death *in vivo,* a bioluminescent-based luciferin reporter of caspase-3/7 activity was utilized (Hickson et al., 2010). MicroRNA

inhibition significantly reduced *in vivo* caspase activity in colon cancer cells during the early phase of hepatic colonization, revealing cancer cells survival to be the phenotype suppressed by these microRNAs (Fig. 2.11). Decreased cancer cell apoptosis during the early stages of cancer progression has been demonstrated to be critical for successful cancer progression (Scabini et al., 2011). These *in vivo* findings provide corroboration and a mechanistic basis for the organotypic slice culture observations and reveal miR-483 and miR-551a to suppress liver metastatic colonization through suppression of metastatic cell survival in the liver—a phenotype exhibited by highly metastatic colon cancer cells.

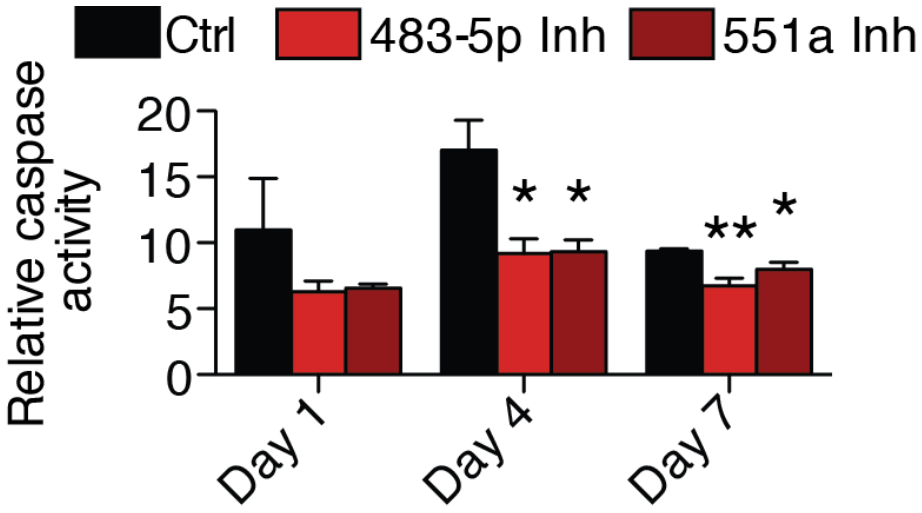



**Figure 2.11 | Mir-483-5p and miR-551a regulate colon cancer cell survival within the liver.** Relative *in vivo* caspase activities of SW480 cells whose endogenous miR-483-5p or miR-551a were inhibited and subsequently introduced into the liver of immunodeficient mice by intrasplenic injection (n=3). Caspase activity was monitored using a caspase-3/7 activated DEVD-luciferin and normalized with signal from regular luciferin. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. *p<0.05; **p<0.01.

**CKB is a common direct target and effector of miR-483-5p and miR-551a**

To delineate the pathways through which miR-483-5p and miR-551a regulate colon cancer cell survival in the liver microenvironment, transcriptomic profiling was performed to identify mRNA transcripts that were down-regulated upon over-expression of each microRNA and that contained 3'-UTR or coding sequence elements complementary to the microRNAs. Interestingly, Creatine Kinase Brain-type (CKB) was identified as a putative target of both microRNAs that could be experimentally validated. As both microRNAs exhibited similar phenotypes in the *in vitro* organotypic slice culture assay, as well as *in vivo* upon inoculation into the liver, CKB could be a common target gene and downstream effector. Indeed, endogenous miR-483-5p and miR-551a were found to suppress CKB protein levels (Fig. 2.12A) in colon cancer cells, and quantitative PCR validation also revealed suppression or up-regulation of CKB transcript levels upon over-expression or inhibition of the microRNAs, respectively (Fig. 2.12B, C).

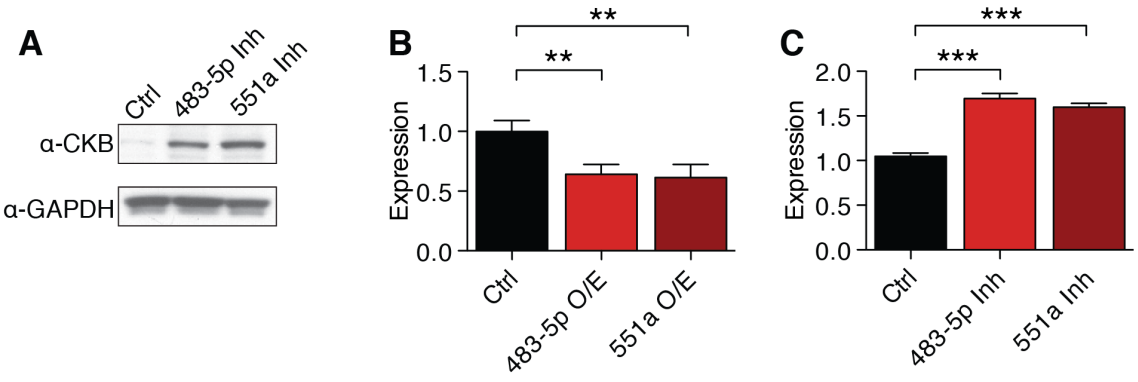



**Figure 2.12 | CKB expression is regulated by miR-483-5p and miR-551a.** A, Protein levels of CKB in SW480 cells whose endogenous miR-483-5p or miR-551a was inhibited with LNAs. B, CKB transcript levels in colorectal cancer cells over-expressing miR-483-5p or miR-551a (n=3). C, CKB transcript levels in colorectal cancer cells whose endogenous miR-483-5p and miR-551a were inhibited by LNA (n=3). Error bars, s.e.m; all P values are based on one-sided Student's t-tests. **p<0.01; ***p<0.001.

In order to confirm that the CKB transcript is a direct target of both microRNAs, luciferase-reporter assay was performed in which the coding region and 3′-UTR of the CKB transcript were cloned downstream of a luciferase reporter. The luciferase reporter assay revealed miR-483-5p to directly target the 3′-UTR (Fig. 2.13A) and miR-551a to target the coding region of CKB (Fig. 2.13B). Mutagenesis of putative microRNA recognition sites abrogated the regulation by the microRNAs and confirmed CKB to be direct target of the microRNAs.

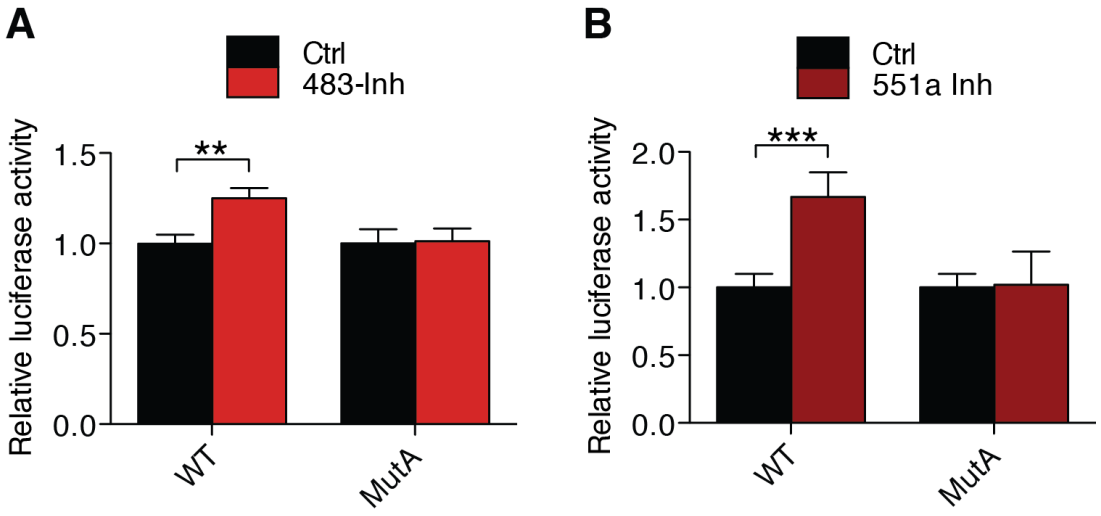
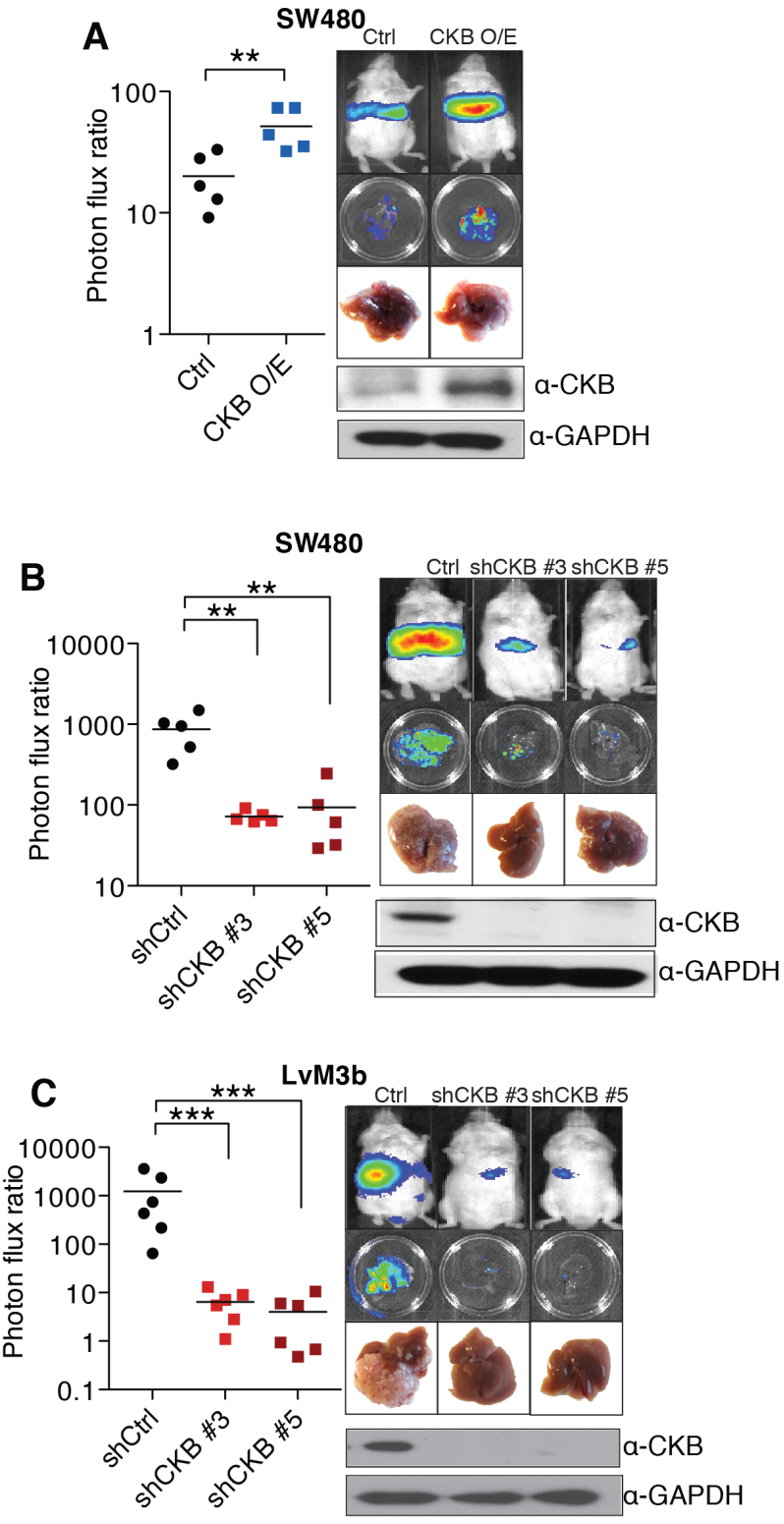



**Figure 2.13 | The CKB transcript is directly targeted by miR-483-5p and miR-551a.** A, B, Luciferase reporter assays of CKB coding sequence and 3′-UTR. A, miR-483-5p and B, miR-551a targeted sequences in the 3′-UTR and coding sequence of CKB respectively. Inhibition of endogenous miR-483-5p and miR-551a resulted in increased expression of luciferase reporter. Mutagenesis of putative target sites of miR-483-5p and miR-551a on the 3′-UTR and CDS respectively abrogated regulation of luciferase reporter by the microRNAs. Independent assays were performed as described above at least 3 times. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. **$p<0.01$; ***$p<0.001$.

Given that CKB is a potential promoter of colon cancer metastasis that is regulated by miR-483-5p and miR-551a, gain-of- and loss-of-function experiments were performed. Over-expression of CKB in poorly metastatic SW480 cells was sufficient to significantly enhance liver metastasis (>3-fold; Fig. 2.14A), while CKB knockdown in metastatic LS-LvM3b and SW480 cells, through the use of two independent shRNA hairpins for each cell-line, robustly suppressed liver metastatic colonization (>5 fold, Fig. 2.14B, C). These results reveal that CKB is indeed a *bona fide* promoter of colon cancer liver metastasis.

**Figure 2.14 | CKB is a promoter of colorectal cancer metastasis.** A, Liver metastasis in mice injected intrasplenically with 5 X $10^5$ poorly metastatic SW480 cells and CKB over-expressing SW480 cells (n=5). Mice were euthanized at 28 days after injection and livers excised for *ex vivo* bioluminescent imaging. B, Liver metastasis in mice injected with 5 X $10^5$ SW480 cells transduced with two independent CKB shRNAs (n=5). Mice were euthanized at 28 days after injection. C, Liver metastasis in mice injected intrasplenically with 5 X $10^5$ highly aggressive LvM3b expressing a control hairpin or two independent shRNA hairpins targeting CKB (n=6). Mice were euthanized 21 days after injection. Western-blot was performed to control for CKB expression levels. All P values are based on one-sided Mann-Whitney test for non Gaussian distribution. *p<0.05; **p<0.01; ***p<0.001.


A
SW480
Ctrl
CKB O/E
**
100
10
1
Photon flux ratio
Ctrl
CKB O/E
α-CKB
α-GAPDH
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SW480
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shCKB #3
shCKB #5
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**
10000
1000
100
10
Photon flux ratio
shCtrl
shCKB #3
shCKB #5
α-CKB
α-GAPDH
C
LvM3b
Ctrl
shCKB #3
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***
10000
1000
100
10
1
0.1
Photon flux ratio
shCtrl
shCKB #3
shCKB #5
α-CKB
α-GAPDH

In an independent experiment, CKB expression was examined in liver metastases that eventually formed from CKB knockdown cells and it was observed that recovered tumors were 'escapers' with restored CKB expression (Fig. 2.15), demonstrating a strong selective pressure for CKB up-regulation in liver metastases.

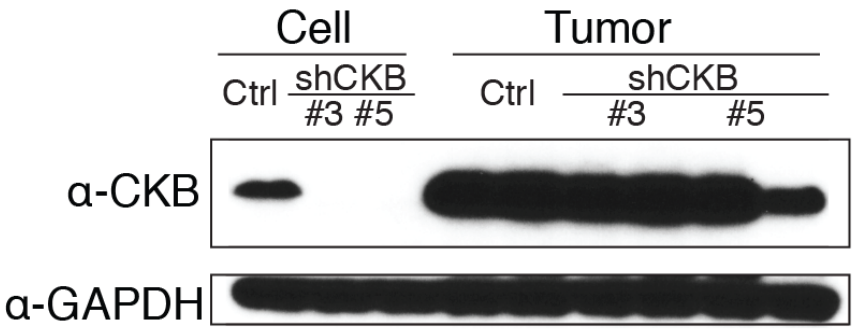
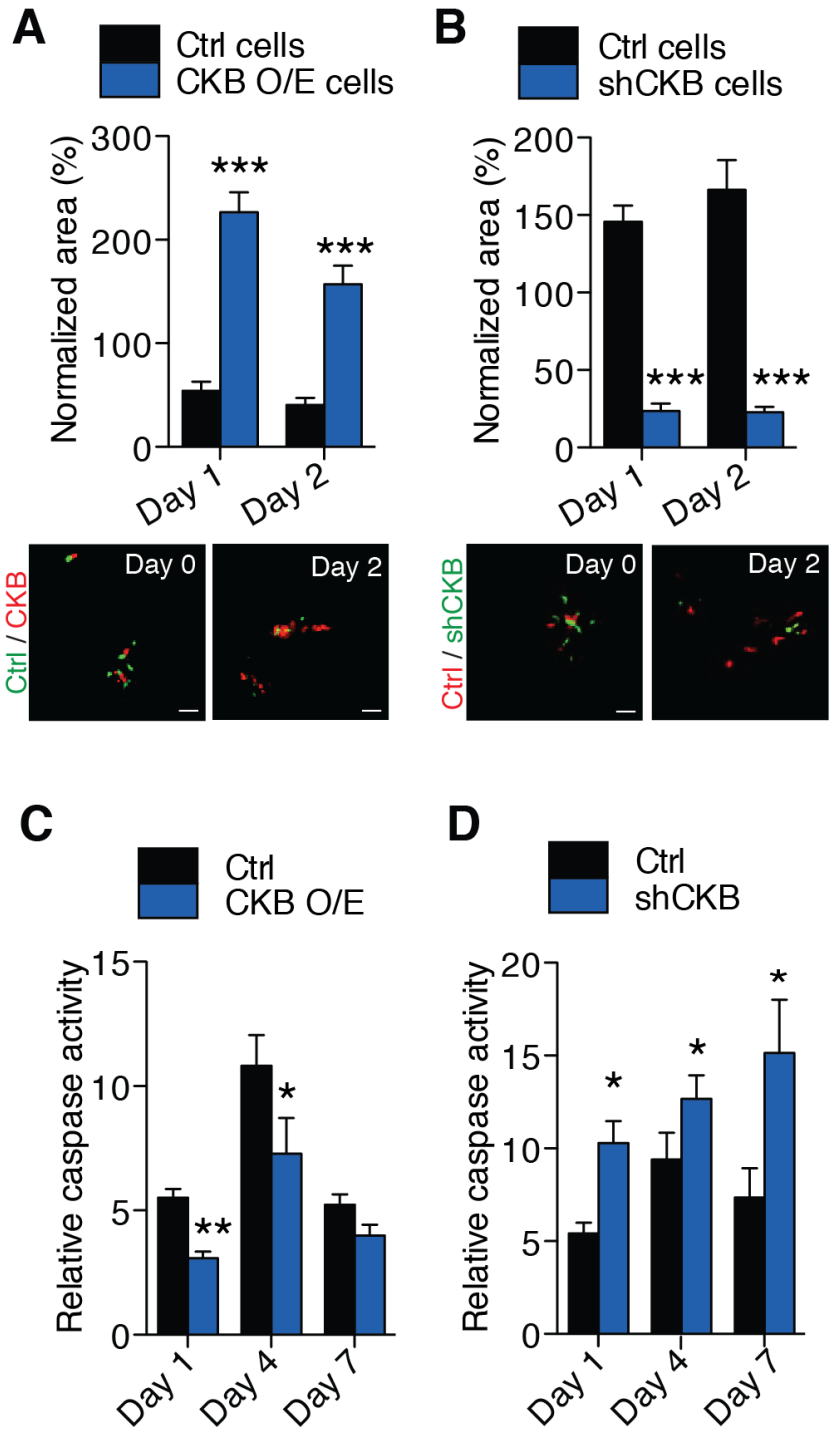



**Figure 2.15 | CKB expression is restored in escaping tumors arising from shRNA-mediated CKB-depleted colon cancer cells.** CKB protein levels in control LvM3b and CKB-knockdown cells, control tumors and escaped CKB knockdown tumors from two independent hairpins. Two escaper tumors were extracted for each CKB-knockdown cell-line and CKB protein levels were examined by western-blot.

The effects of CKB were also consistent with the phenotypic effects of its regulatory microRNAs. CKB over-expression was sufficient to significantly enhance the ability of colon cancer cells to persist in the liver microenvironment and enhanced their representation in the liver (Fig. 2.16A), while CKB knockdown substantially reduced intra-hepatic persistence (Fig. 2.16B). Consistent with this, CKB over-expression significantly reduced (Fig. 2.16C),

while CKB knockdown significantly increased (Fig. 2.16D), *in vivo* caspase-3/7 activity in colon cancer cells during the initial phase of hepatic colonization.

**Figure 2.16 | CKB regulates intrahepatic persistence of colon cancer cells by promoting cancer cells survival within the liver microenvironment.** A, Intrahepatic persistence of control SW480 and CKB over-expressing SW480 cells in organotypic liver slices (n=8). B, Organotypic slice cultures of LvM3b cells expressing a control hairpin or hairpin targeting CKB (n=8). Representative images at day 0 and day 2 are shown. C, Relative *in vivo* caspase activity of SW480 cells expressing control or CKB expression vectors in livers of mice measured by bioluminescence using a caspase-3 activated DEVD-luciferin substrate and normalized to bioluminescence signal from generic luciferin (n=3). D, Relative *in vivo* caspase-3 activity of SW480 cells expressing a control hairpin or hairpin targeting CKB and introduced into the livers of mice through intrasplenic injection. Caspase activity was measured on day 1, 4 and 7 after injection (n=3). Error bars, s.e.m; all P values are based on one-sided Student's t-tests. *$p<0.05$; **$p<0.01$; ***$p<0.001$.


A
Ctrl cells
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300
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As CKB displayed *in vitro* and *in vivo* phenotypes that were consistent with miR-483-5p and miR-551a, epistasis experiments were performed to confirm CKB as a direct downstream effector of both microRNAs. Loss-of-function epistasis experiments in which CKB was depleted in cells displaying endogenous miR-483-5p or miR-551a inhibition prevented the enhanced metastasis effect seen with miR-483-5p or miR-551a silencing (Fig. 2.17A). Conversely over-expression of CKB was sufficient to rescue the suppressed liver metastatic phenotypes of cells over-expressing miR-483-5p or miR-551a (Fig. 2.17B).

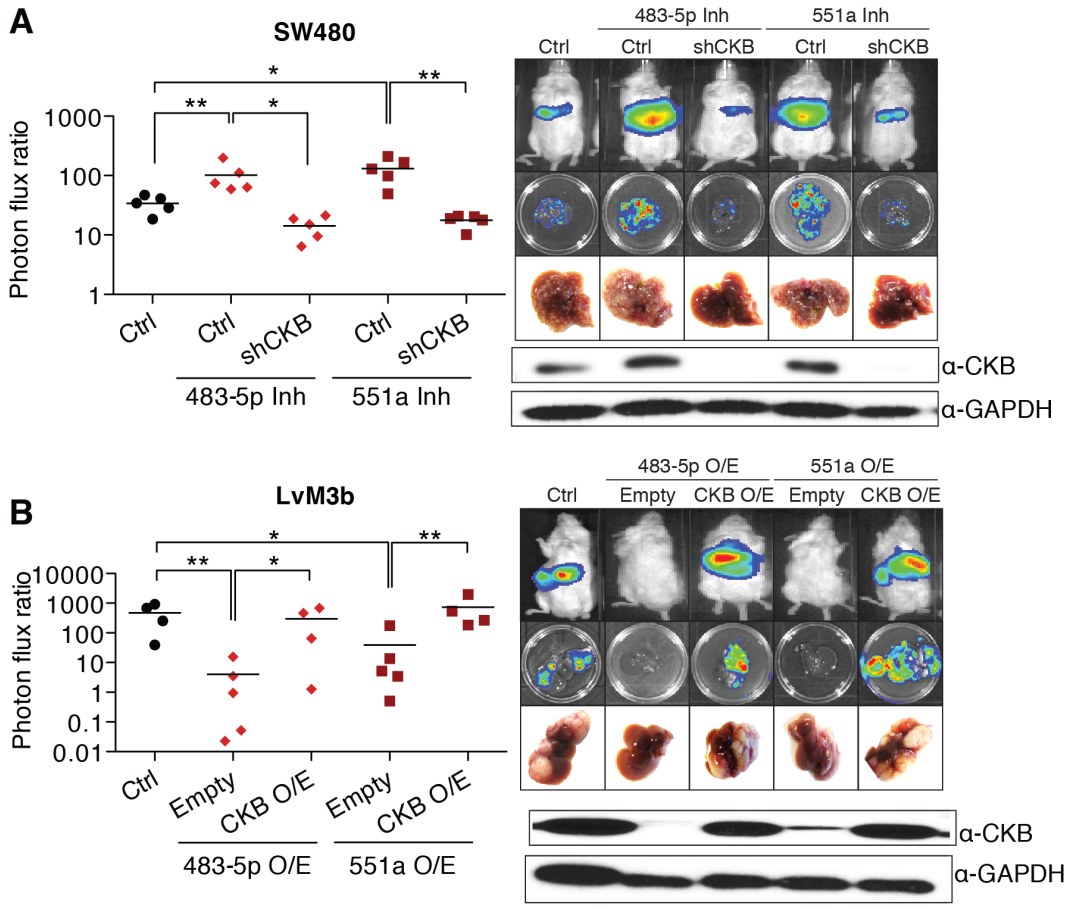


**Figure 2.17 | CKB is a direct downstream effector of miR-483-5p and miR-551a.** A, Liver metastasis in mice injected with 5 X $10^5$ SW480 cells whose endogenous miR-483-5p or miR-551a was inhibited by LNA, with and without CKB knockdown (n=5). Mice were euthanized after 28 days and livers were excised for *ex vivo* bioluminescence imaging. B, Metastatic progression in mice injected with 5 X $10^5$ LvM3b cells over-expressing miR-483-5p or miR-551a, with and without CKB over-expression (n=5). P values are based on one-sided Mann-Whitney test for non-Gaussian distribution. *p<0.05; **p<0.01.

The results of the above mutational, gain-of-, loss-of-function and epistasis analyses confirmed CKB to be a direct downstream effector of miR-483-5p and miR-551a and revealed CKB to be a promoter of colon cancer survival during metastatic colonization in the hepatic microenvironment. The restoration of CKB levels in tumors that 'escaped' from CKB depletion by RNAi also highlighted a strong selective pressure for CKB expression in liver metastases.

**Summary of Chapter II**

Chapter II describes two complementary approaches used to identify microRNAs that suppressed colon cancer metastasis. The first approach employed a lentiviral microRNA library screen in two independent cell-lines, SW620 and WiDr, to identify microRNAs that when over-expressed, suppressed liver colonization. A third independent cell-line, LS174T, was subjected to *in vivo* selection to select for sub-lines with enhanced metastatic colonization capacity of the liver. Two microRNAs, miR-483-5p and miR-551a were identified through both approaches. *In vivo* and *in vitro* phenotypic assays revealed both microRNAs to suppress liver metastasis by colon cancer cells through suppression of colon cancer cells survival in the liver microenvironment.

Transcriptomic analysis followed by gain-of- and loss-of-function validation experiments, as well as luciferase reporter assays identified CKB as direct target of miR-483-5p and miR-551a. Functional CKB over-expression and knockdown experiments using *in vivo* metastasis assays and *in vitro* liver organotypic slice culture assays demonstrated CKB to regulate colon cancer metastasis and cell survival in the liver, consistent with its regulatory microRNAs. CKB was further

confirmed to be a direct downstream effector of miR-483-5p and miR-551a through *in vivo* epistasis experiments. The following chapter shall present efforts to mechanistically dissect the pathways regulated by the microRNAs and CKB that mediate colon cancer metastasis to the liver (Chapter III).

# Chapter III: Mechanistic Studies of the Creatine Kinase Axis During Colorectal Cancer Metastasis to the Liver

CKB is a member of the creatine kinase family of metabolic kinases that are known to regulate the reservoir of rapidly mobilized high-energy phosphates in tissues with high energetic demands such as the brain, muscles and heart by catalyzing the transfer of a high-energy phosphate group from ATP to the metabolite creatine, yielding phosphocreatine (Wallimann et al., 1992; Wyss and Kaddurah-Daouk, 2000). As CKB was demonstrated to be regulator of colon cancer metastasis in Chapter II, it is possible that the metabolic reaction catalyzed by CKB is also relevant to colon cancer metastasis. This chapter describes efforts to delineate the pathways regulated by CKB in colon cancer cells in relation to the physiological context of the liver microenvironment.

**CKB modulates cellular energetics of colon cancer cells**

The maintenance of intracellular ATP levels is critical for cell survival under energetic stress. In normal tissues, cells with potentially high energetic demands maintain high levels of phosphocreatine stores in order to buffer against low ATP states, since phosphocreatine's high-energy phosphate can be transferred to ADP to generate ATP (Wallimann et al., 1992). Consistent with the role of CKB in modulating phosphocreatine and ATP levels within cells, over-expression and knockdown of CKB in colon cancer cells increased and decreased, respectively, intracellular phosphocreatine levels (Fig. 3.1A) and CKB depletion resulted in decreased ATP levels that could be rescued by phosphocreatine supplementation (Fig. 3.1B), consistent with what was previously reported for creatine kinase expressing tissues (Li et al., 2012; Prabhakar et al., 2003).

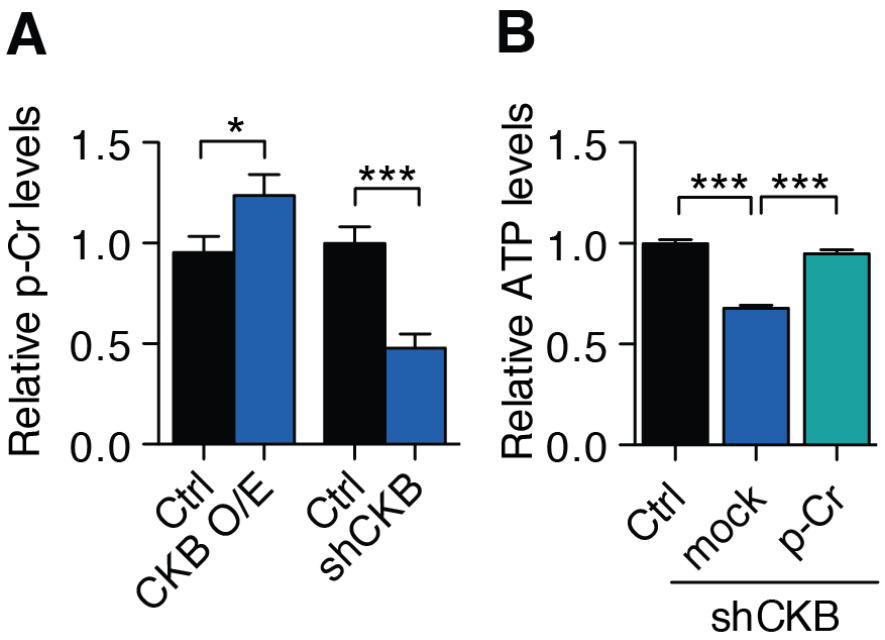


**Figure 3.1 | CKB regulates intracellular levels of high-energy phosphate metabolites.** A, Relative intracellular phosphocreatine levels in colorectal cancer cells over-expressing CKB or depleted for CKB using a bioluminescent assay (n=5). Bioluminescent signals from experimental groups are directly proportional to intracellular phosphocreatine levels and normalized to signal from control cells. B, Relative intracellular ATP levels in colorectal cancer cells depleted for CKB, with and without exogenous 10mM phosphocreatine supplementation (n=5). Error bars, s.e.m; P values are based on one-sided Student's t-tests. *$p<0.05$; ***$p<0.001$.

As miR-483-5p and miR-551a are regulators of CKB expression, modulation of either microRNAs also modulated intracellular phosphocreatine (Fig. 3.2A, B) and ATP levels (Fig. 3.2C, D). These findings demonstrated regulation of high-energy metabolites by CKB as a potential avenue through which CKB can promote liver metastasis.

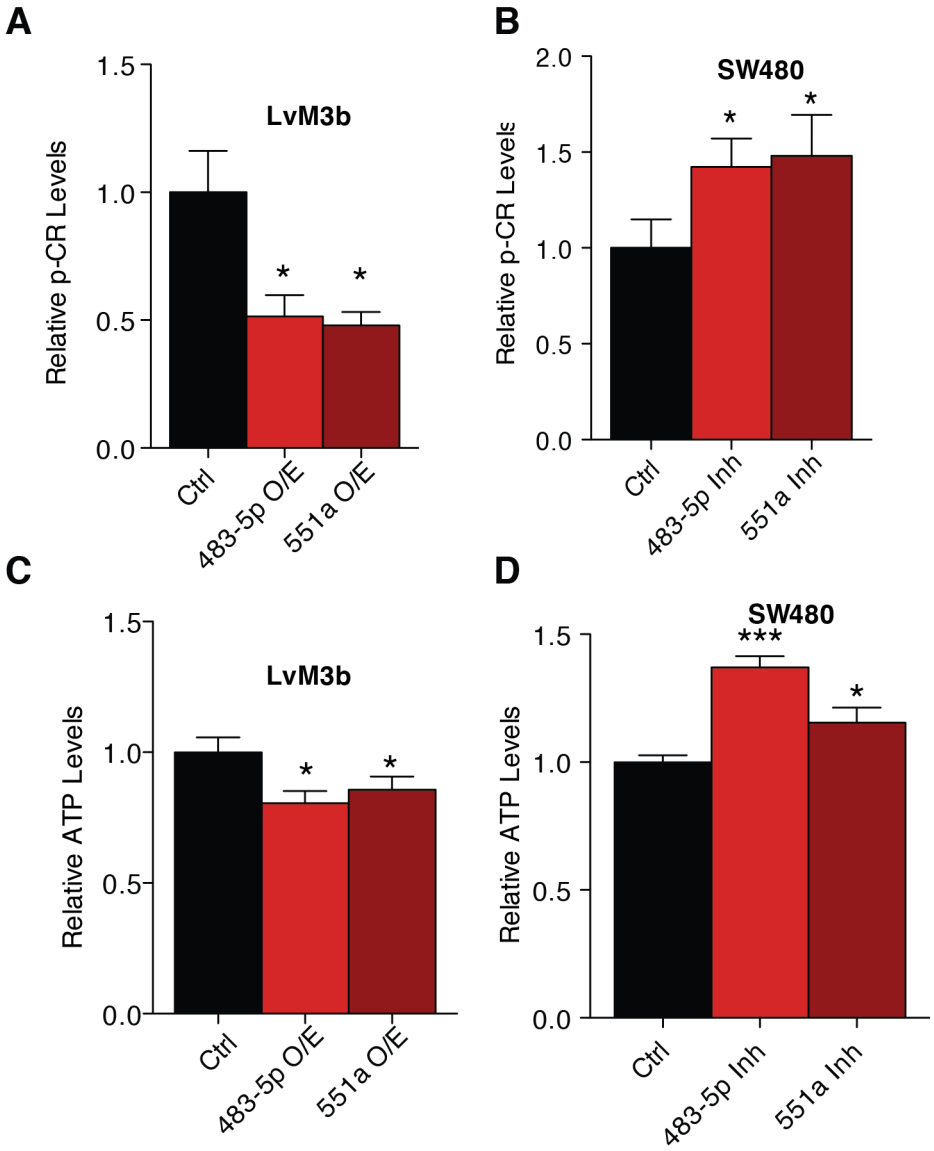


**Figure 3.2 | Mir-483-5p and miR-551a regulate intracellular levels of high-energy phosphate metabolites.** A, Relative intracellular phosphocreatine levels in LvM3b cells over-expressing miR-483-5p and miR-551a (n=3). B, Relative intracellular phosphocreatine levels in SW480 cells in which endogenous miR-483-5p and miR-551a was inhibited (n=3). C, Relative intracellular ATP levels in LvM3b cells over-expressing miR-483-5p and miR-551a (n=3). D, Relative intracellular ATP levels in SW480 cells in which endogenous miR-483-5p and miR-551a was inhibited (n=3). Error bars, s.e.m; all P values are based on one-sided Student's t-tests. *$p<0.05$; ***$p<0.001$.

**CKB mediates colon cancer cell survival in the hypoxic liver microenvironment**

In the previous chapter, it was showed that CKB promotes colon cancer cell persistence and survival at an early phase after dissemination to the liver microenvironment. Newly disseminated colon cancer cells that arrive in the liver could thus encounter a physiological context that can result in low ATP levels unless buffered by a higher intracellular phosphocreatine reservoir as a result of high CKB expression.

The liver microenvironment is known to contain hypoxic regions (Arteel et al., 1995), with metabolically active hepatocytes at the periportal region displaying high rates of oxygen consumption and hepatocytes at the perivenous region actively undergoing glycolysis (Jungermann and Kietzmann, 1996, 2000). Additionally, colon cancer cells metastasize to the liver via the portal circulation, which is relatively hypoxemic (Weiss et al., 1986). It is possible that colon cancer cells experience acute hypoxia and intense competition for glycolytic substrates during initial dissemination to the liver and could be poorly adapted to the liver microenvironment prior to HIF activated responses (Semenza, 2011). To determine if colon cancer cells are disseminated to hypoxic regions of the mouse liver, a HIF-1 alpha transcriptional luciferase reporter (HRE-Luc) was used as an *in vivo* sensor and reporter of cellular hypoxia (Fig. 3.3A). Subsequent to colon cancer cells inoculation into the liver, it was observed that colon cancer cells indeed experience hypoxia early after hepatic dissemination (Fig. 3.3B). As an independent *in vivo* reporter for hypoxia that is not dependent on HIF1A activation, a luciferase reporter fused to an oxygen degradative domain (ODD-

Luc) was used (Fig.3.3C) (Safran et al., 2006). Colon cancer cells that disseminated to the liver expressed ODD-Luc (Fig. 3.3D), independently indicating that low oxygen levels were indeed a physiological stress present in the liver microenvironment.

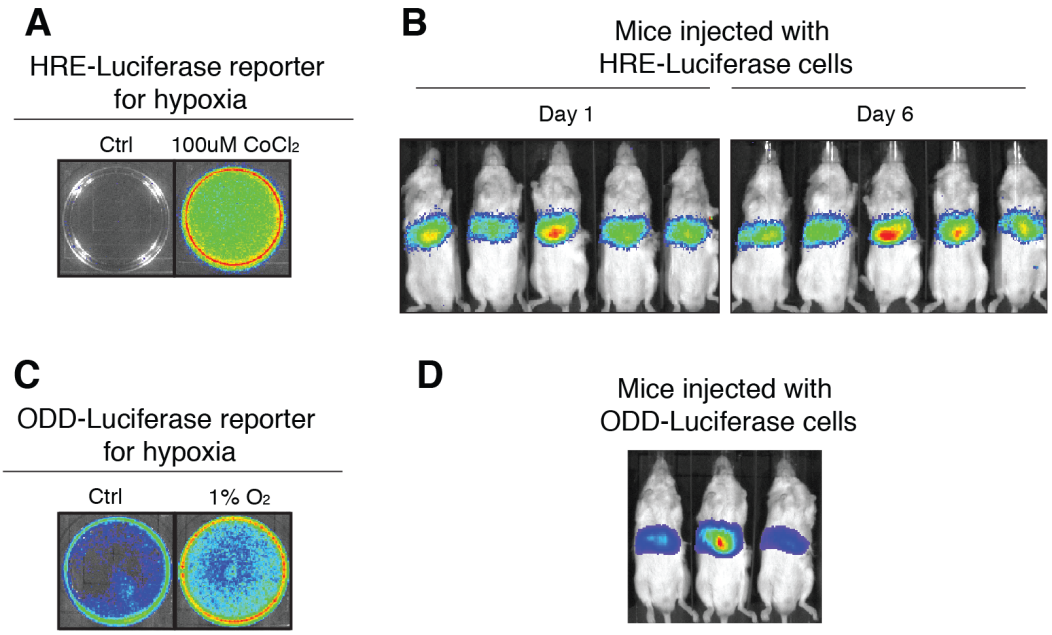


**Figure 3.3 | Incipient colorectal cancer cells experience hypoxia within the liver microenvironment during metastasis.** A, Bioluminescent imaging of SW480 cells expressing an HRE-luciferase reporter with and without 24hr $CoCl_2$ treatment. B, Bioluminescence imaging of mice (n=5) injected with 5 X $10^5$ SW480 cells expressing the HRE-luciferase reporter. C, Bioluminescent imaging of SW480 cells expressing the ODD-luciferase reporter cultured in atmospheric conditions or 1% $O_2$. D, Bioluminescent imaging of mice injected with 5 X $10^5$ SW480 cells expressing ODD-luciferase reporter.

Hypoxia can result in cellular energetic stress, ATP depletion and subsequently apoptotic cell death (Steinbach et al., 2003). Using the *in vivo* HRE-Luc reporter

for hypoxia and the caspase-activated luciferin substrate as system for investigating colon cancer cell death under hypoxia *in vivo*, it was observed that CKB depletion increased caspase-mediated cell death in HRE-Luc expressing cells experiencing hypoxia *in vivo* (Fig. 3.4A). Conversely, inhibition of either miR-483-5p or miR-551a protected colon cancer cells experiencing hypoxia *in vivo* (Fig. 3.4B). These results indicated CKB to be a pro-survival metabolic kinase in colon cancer cells during hypoxia.

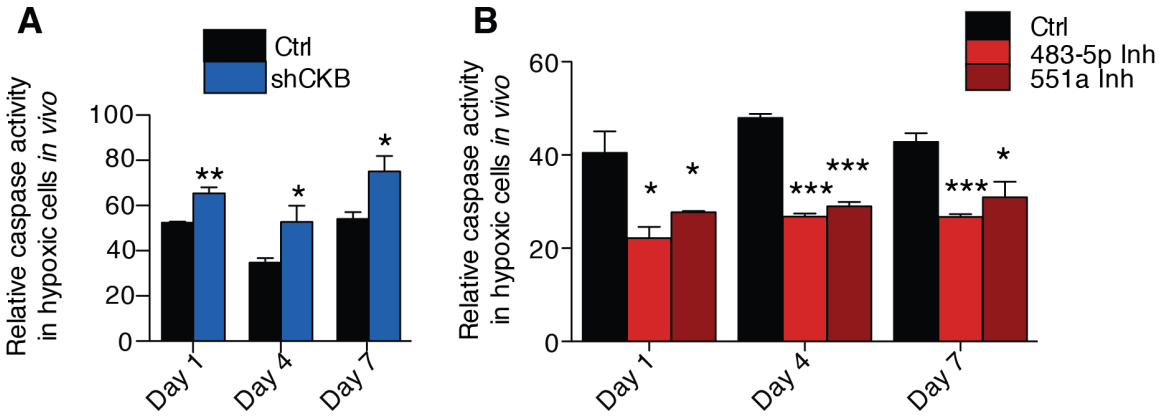


**Figure 3.4 | CKB promotes colon cancer cells survival within the hypoxic liver microenvironment during incipient metastasis.** A, *In vivo* caspase activity of control SW480 cells and CKB knockdown SW480 cells experiencing hypoxia within the livers of mice (n=3). Cells expressed an HRE-driven luciferase reporter and caspase-3 activity was measured using DEVD-luciferin and normalized to using regular luciferin. B, *In vivo* caspase activity of hypoxic SW480 cells with inhibition of miR-483-5p or miR-551a in the livers of mice (n=3). Imaging was performed on day 1, 4, and 7 after injection. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. *p<0.05; **p<0.01, ***p<0.001.

Consistent with a role for CKB and its enzymatic product, phosphocreatine, in promoting tissue integrity and cell survival during hypoxia or ischemia (Miller et

al., 1993; Prabhakar et al., 2003; Sharov et al., 1987), cells depleted of CKB through RNAi displayed reduced survival while experiencing hypoxia *in vitro*—an effect that was abrogated upon phosphocreatine supplementation (Fig. 3.5).

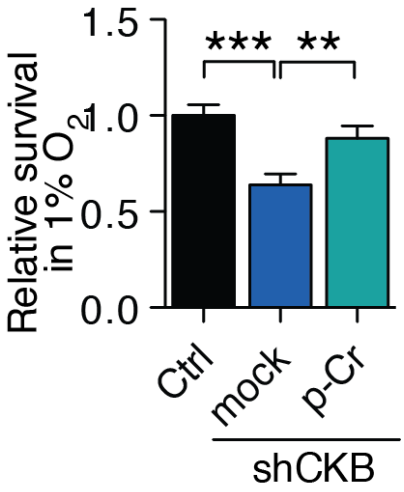


**Figure 3.5 | Phosphocreatine is protective of colon cancer cells experiencing hypoxia.** Survival of colorectal cancer cells in hypoxia *in vitro* with and without CKB knockdown, and 10mM phosphocreatine supplementation (n=3). Cells were culture in 1% $O_2$ for 96hrs. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. **p<0.01; ***p<0.001.

In agreement with the *in vitro* findings, pre-incubation of colon cancer cells depleted of CKB with phosphocreatine enhanced their ability to metastasize to the liver (Fig. 3.6A) while depletion of intracellular phosphocreatine by pre-incubating colon cancer cells with cyclocreatine, an inhibitor of CKB, suppressed liver metastasis (Lillie et al., 1993) (Fig. 3.6B). The above findings suggested that hepatic hypoxia poses a survival barrier for colon cancer cells during early metastatic colonization and cells endure this phase through the generation of ATP from phosphocreatine reserves. The ability of phosphocreatine pre-loading to enhance metastasis *in vivo* supports the importance of the acute initial hypoxic

barrier and energetic demands in shaping metastatic colonization by cancer cells as they enter the liver microenvironment. The findings that exogenous phosphocreatine can be protective for colorectal cancer cells during hypoxia were in agreement with earlier studies demonstrating that exogenous phosphocreatine can be protective against hypoxic, ischemic and other energetic insults in neurons and myocardium, with increased phosphocreatine uptake observed in ischemic myocardium (Brustovetsky et al., 2001; Li et al., 2012; Sharov et al., 1987).

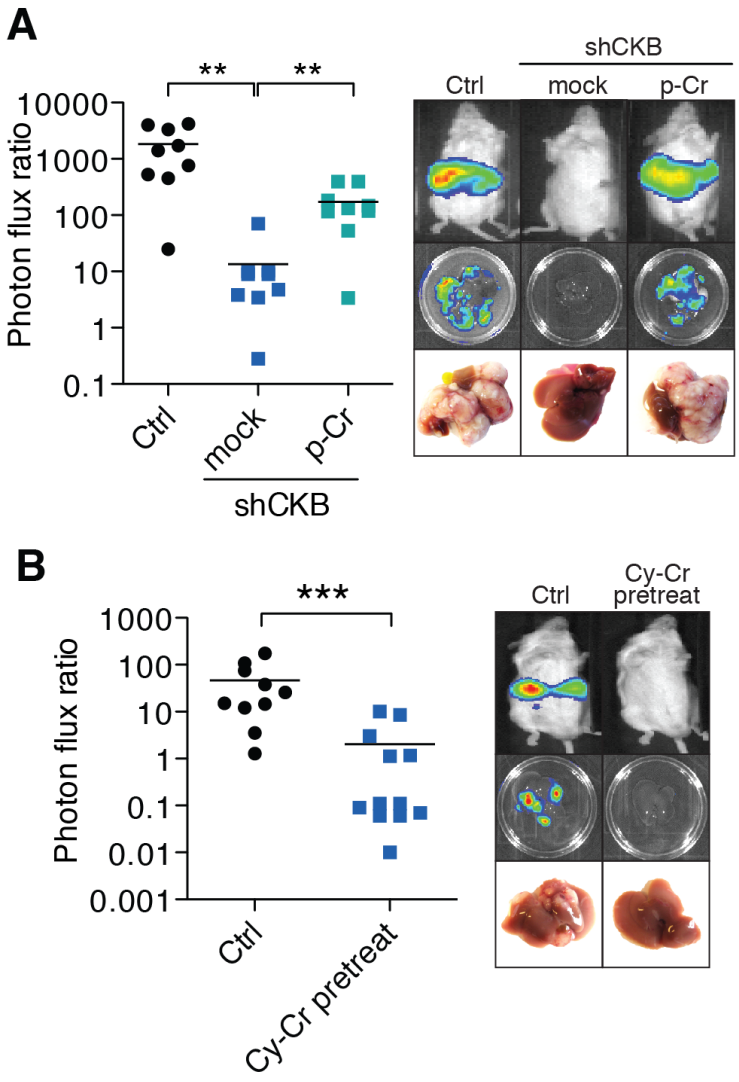


**Figure 3.6 | Intracellular phosphocreatine contributes to colon cancer cell metastasis to the liver.** A, Liver metastasis by CKB depleted LvM3b cells pre-incubated overnight with 10mM phosphocreatine. 5 X $10^5$ cells were then inoculated into the liver of mice through intrasplenic injection. B, Liver metastasis in mice injected with 5 X $10^5$ LvM3b cells with and without pre-treatment with 10mM cyclocreatine for 48hrs prior to injection. P values are based on Mann-Whitney test for non-Gaussian distribution. **p<0.01; ***p<0.001.

**Secreted CKB catalyzes an extracellular reaction that promotes colon cancer progression**

CKB was shown to regulate the cellular energetics of colon cancer cells and promoted cancer cell survival during *in vivo* and *in vitro* hypoxia through phosphocreatine generation. While further considering CKB's role in the setting of the hypoxia hepatic microenvironment, a conundrum arose: how can colon cancer cells arriving and residing in a hypoxic hepatic microenvironment generate and replenish phosphocreatine if they are depleted of ATP, especially during the acute phase, prior to any hypoxia-response (Bertout et al., 2008; Semenza, 2013; Wheaton and Chandel, 2011)? An exogenous source of ATP would therefore be required for supplemental phosphocreatine generation.

Earlier clinical studies have described the detection of CKB proteins and CKB activity in the sera of patients with various forms of malignancies and physiological insults (Huddleston et al., 2005; Rubery et al., 1982; Wyss and Kaddurah-Daouk, 2000). The presence of extracellular ATP in the microenvironment of macro-metastases was also reported (Pellegatti et al., 2008; Stagg and Smyth, 2010). Interestingly, the liver is the main synthetic organ for creatine synthesis in the body, responsible for releasing creatine into the circulation for distribution to other tissues. Given that the substrates and enzyme required for phosphocreatine generation were reported in earlier literature, a hypothesis was developed that colon cancer cells may release CKB into the extracellular space, which can then convert extracellular ATP and liver-produced creatine into phosphocreatine. This exogenous phosphocreatine can then taken

up by cancer cells, thereby exerting a protective effect on hypoxic colon cancer cells prior to their adaption to the hypoxic liver microenvironment.

To investigate this hypothesis, the presence of extracellular ATP and CKB has to be confirmed. Cell culture supernatant from metastatic LvM3b cells and LvM3b cells depleted of CKB through RNAi were collected and examine for CKB protein levels. Extracellular CKB were released from LvM3b cells, but not CKB-depleted LvM3b cells (Fig. 3.7).

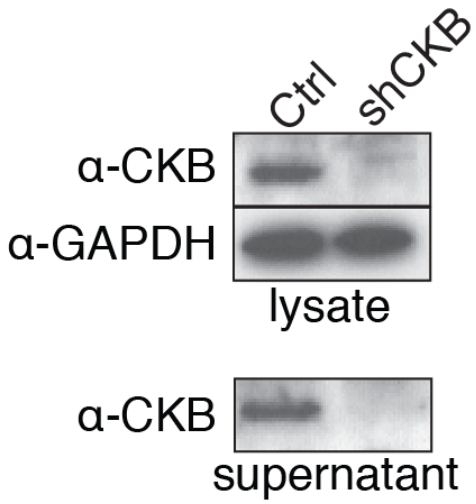


**Figure 3.7 | Colon cancer cells release CKB extracellularly.** Extracellular and intracellular CKB protein levels in control and LvM3b cells depleted of CKB through RNAi. Supernatant was harvested by culturing LvM3b cells overnight in serum-free media. Cells viability was confirmed to be above 99% using trypan blue exclusion assay. Supernatant was concentrated 20-fold using column centrifugation and 40µL was used for western-blot.

In order to determine if extracellular CKB was released from live or dying cells, a FLAG-tagged CKB expression construct was generated. The FLAG-epitope is

attached to CKB through a peptide linker containing a caspase-3/7 recognition DEVD motif. Caspase activation in apoptotic cells would result in caspase recognition and cleavage of the DEVD motif between the FLAG-epitope tag and CKB, causing loss of the FLAG-epitope from the expression CKB (Fig. 3.8A). Supernatant from colon cancer cells expressing this FLAG-tagged CKB indicated that the FLAG-epitope was not lost, demonstrating that extracellular CKB to be released by primarily live cells (Fig. 3.8B). Interestingly, release of extracellular CKB was not inhibited by brefeldin A or dimethyl amiloride (DMA) treatment (Fig. 3.8C) raising the possibility that CKB release was not mediated by golgi-related secretory pathways or exosomes respectively.

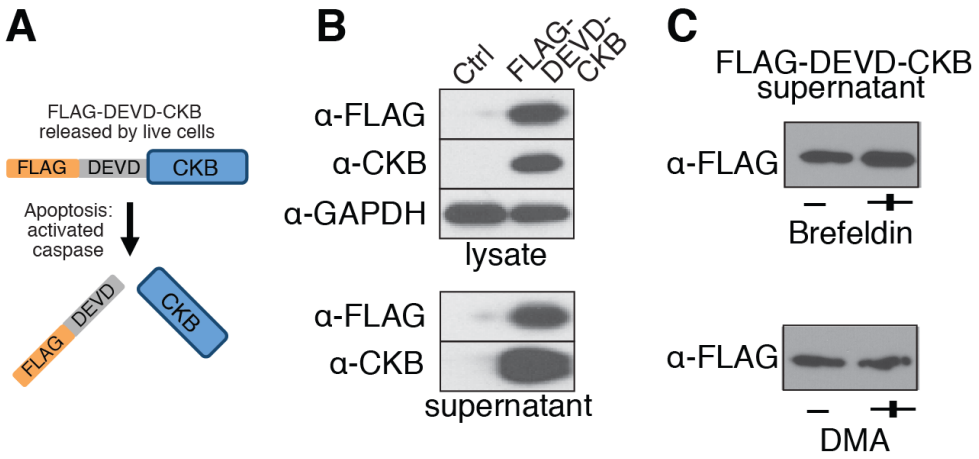


**Figure 3.8 | CKB is released by live cells.** A, FLAG-tagged CKB with a caspase 3/7 recognition site linker. The FLAG-DEVD-CKB has a FLAG-tag linked to the N-terminal of CKB by a linker containing a caspase 3/7 recognition motif (DEVD-amino sequence). Caspase activation by apoptotic cells will result in cleavage of linker and release of FLAG-tag. B, Western-blot of FLAG-DEVD-CKB over-expressing cells demonstrates release of CKB by live cells into the extracellular space. FLAG-tag was not cleaved upon CKB release into the extracellular space. C, Western-blot of FLAG-tagged CKB in supernatant of cells treated with Brefeldin A or Dimethyl-amiloride.

As generation of extracellular phosphocreatine requires exogenous ATP, the presence of extracellular ATP in the microenvironment of newly disseminated colon cancer cells or incipient micro-metastases was investigated using a plasma membrane-anchored luciferase reporter for detecting extracellular ATP (pME-Luciferase)(Pellegatti et al., 2005). Extracellular ATP was confirmed to be present in the microenvironment of disseminated colon cancer cells (Fig. 3.9).

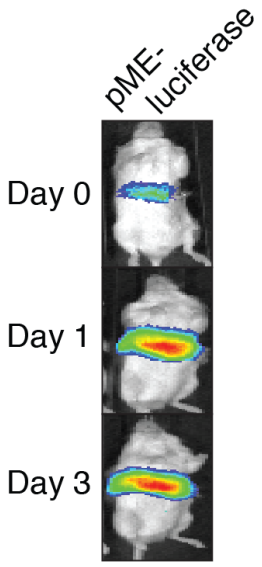


**Figure 3.9 | Extracellular ATP is present in the extracellular microenvironment of disseminated colon cancer cells within the liver.** 5 x $10^5$ SW480 cells expressing pME-Luc were inoculated into the liver of mice through intrasplenic injection. The presence of extracellular ATP was detected by bioluminescent imaging.

Given the presence of extracellular ATP and CKB, if the pro-metastatic effects of CKB resulted from utilization of extracellular ATP as a substrate for phosphocreatine generation by CKB, then depleting extracellular ATP should suppress the pro-metastatic activity of CKB. Indeed, expressing CD39, a plasma membrane anchored ATP hydrolase in SW480 cells (Kaczmarek et al., 1996), significantly precluded the ability of CKB over-expression to promote metastasis without affecting CKB levels (Fig. 3.10).

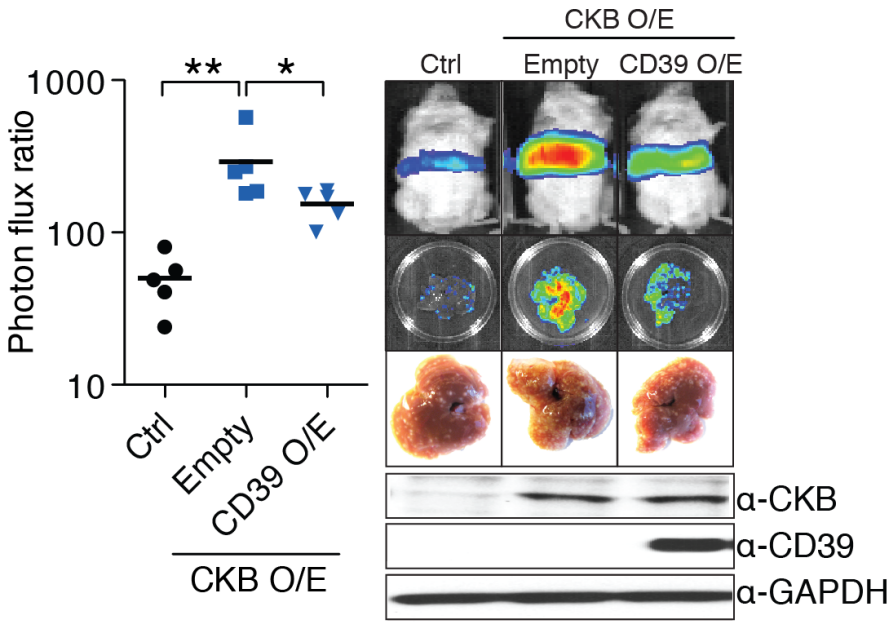


**Figure 3.10 | Depletion of extracellular ATP attenuates CKB mediated liver metastasis by colon cancer cells.** 5 x $10^5$ SW480 control cells, or cells over-expressing CKB alone, or with concomitant over-expression of CD39 was introduced into livers of mice through intrasplenic injection (n=5). Metastatic burden was monitored by bioluminescent imaging and mice euthanized at day 28. Liver was excised for *ex vivo* bioluminescent imaging and gross examination. Western-blot was performed to determine CD39 over-expression. P values are based on one-sided Mann-Whitney test for non-Gaussian distribution. *$p<0.05$; **$p<0.01$.

Consistent with CKB consumption of extracellular ATP, cells over-expressing CKB or cells whose endogenous miR-483-5p or miR-551a were inhibited displayed significantly lower extracellular ATP levels *in vivo* relative to the control cells (Fig. 3.11A, C). Conversely, the microenvironment surrounding CKB knockdown cells displayed higher extracellular ATP levels (Fig. 3.11B).

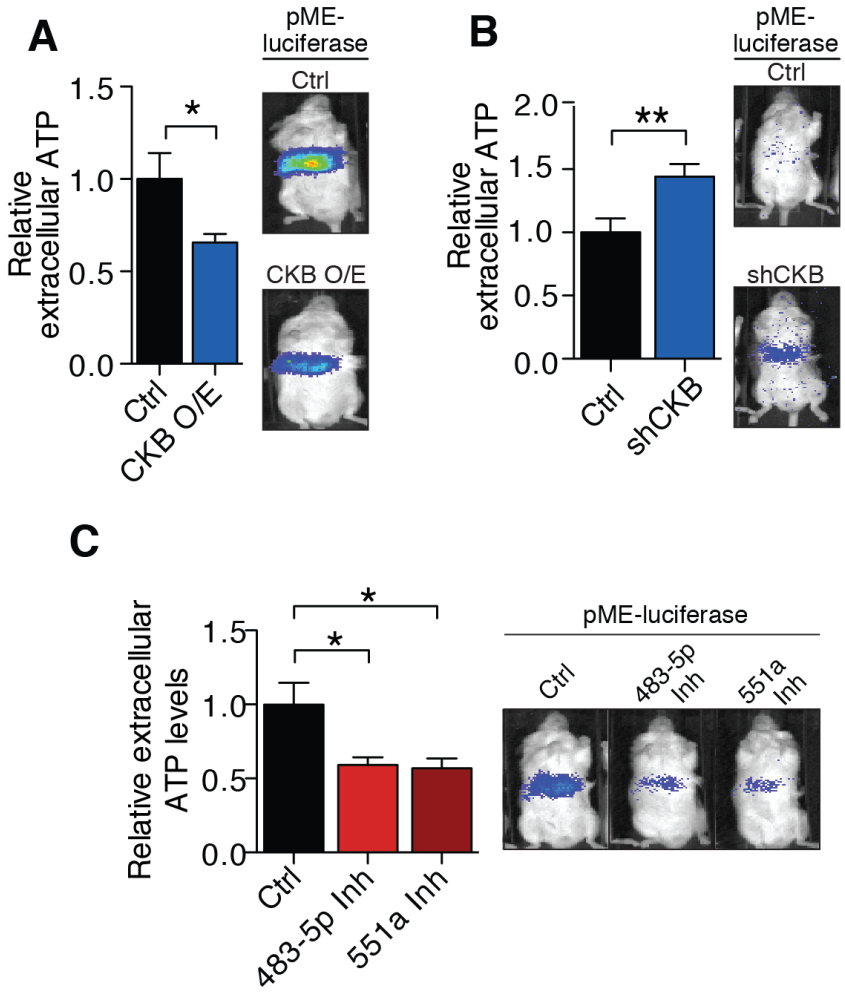


**Figure 3.11 | Extracellular ATP levels of incipient metastases is modulated by CKB expression.** A, Relative extracellular ATP levels in CKB over-expressing cells. Control and CKB over-expressing pME-Luc SW480 cells were injected into mice. Mice were imaged one hour after injection to detect the presence of extracellular ATP (n=5). Bioluminescent signal is directly proportional to levels of extracellular ATP. B, Control and CKB depleted pME-Luc SW480 cells were injected into mice (n=5). C, Relative extracellular ATP levels in microenvironment of SW480 cells whose endogenous miR-483-5p and miR-551a were inhibited. 5 X $10^5$ SW480 cells expressing pME-Luc and whose endogenous miRNAs were inhibited were introduced into the portal circulation through intrasplenic injection and bioluminescent imaging performed (n=5). Error bars, s.e.m; all P values are based on one-sided Student's t-tests. *p<0.05; **p<0.01.

If extracellular CKB catalysis can enhance metastasis, presence of the product of CKB-mediated catalysis, phosphocreatine, in the extracellular space should partially rescue the effect of CKB loss-of-function. In order to investigate this possibility, mini osmotic pumps that continuously released phosphocreatine were implanted into the peritoneal cavities of immunodeficient mice. Exogenous phosphocreatine released from the pump and draining into the portal circulation was able to significantly enhance metastasis (>10 fold) by CKB-depleted cells *in vivo* (Fig. 3.12).

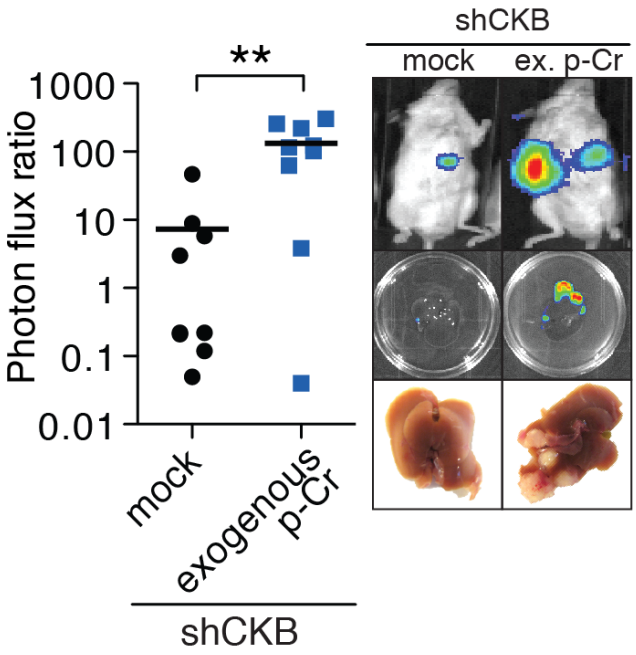


**Figure 3.12 | Exogenous phosphocreatine contributes to colon cancer metastasis to the liver.** Liver metastasis by CKB-depleted LvM3b cells in mice implanted with an osmotic pump releasing phosphocreatine into the portal circulation (n=8). P values are based Mann-Whitney test for non-Gaussian distribution. **p<0.01.

To further confirm that extracellular CKB can promote the survival of colon cancer cells depleted of CKB during hypoxia, a boyden chamber co-culture system was used (Fig. 3.13A). CKB over-expressing cells were able to compensate for the survival of CKB depleted cells across the trans-well, while addition of a CKB-activity neutralizing antibody abrogated this effect (Fig. 3.13B).

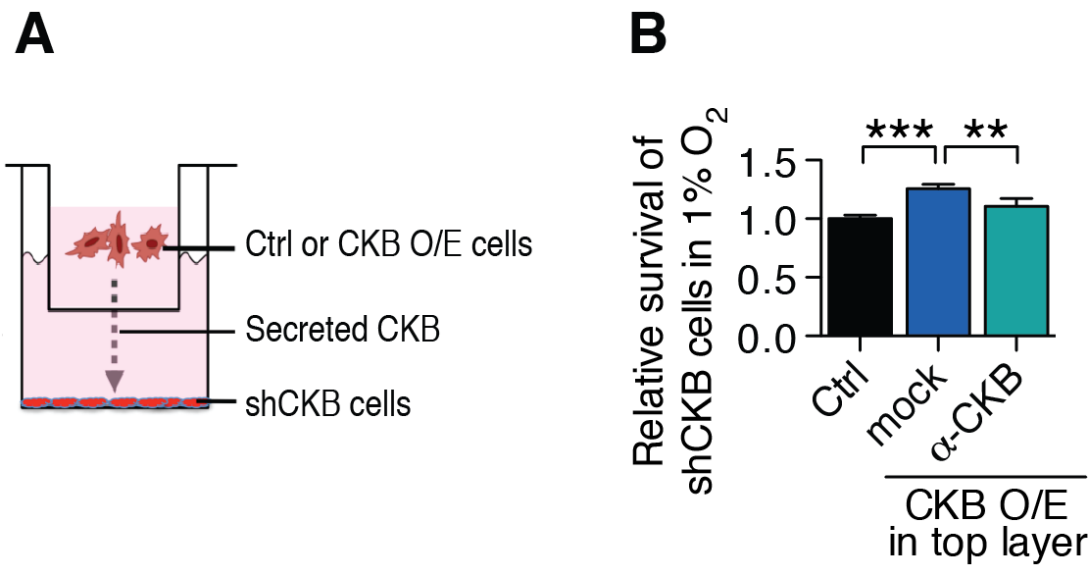


**Figure 3.13 | Extracellular CKB enhances colon cancer cell survival in hypoxia *in vitro*.** A, 5 X $10^4$ CKB-knockdown cells were cultured on the bottom of 24-well plates, while control or CKB-over-expressing cells were plated onto boyden chambers above CKB-knockdown cells with pores for exchange of metabolites and proteins. Cells at the bottom of the well were counted after 4 days in hypoxia. B, Relative survival of CKB-knockdown cells in 1% oxygen when co-cultured with control, CKB-over-expressing cells or with CKB-over-expressing cells in the presence of a neutralizing antibody (n=4). Error bars, s.e.m; all P values are based on one-sided Student's t-tests. **p<0.01; ***p<0.001.

To further extend these findings to an *in vivo* system, colon cancer cells depleted of intracellular CKB but expressing a secreted form of CKB wherein CKB is fused

to the IgK secretory signal sequence was generated. Over-expression of secreted CKB was sufficient to enhance colon cancer metastasis in CKB depleted colon cancer cells (Fig. 3.14).

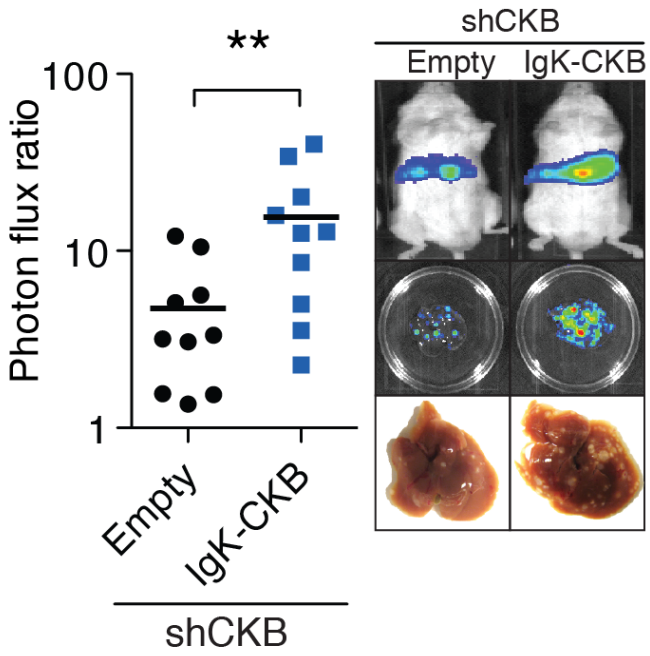


**Figure 3.14 | Extracellular CKB promotes liver metastasis by colon cancer cells.** Liver metastasis by SW480 CKB-knockdown cells over-expressing a secreted form of CKB. 5 x $10^5$ cells were introduced into mice through intrasplenic injection. Liver metastasis were monitored by bioluminescent imaging and mice were euthanized after 28 days, liver excised for *ex vivo* bioluminescent imaging and gross morphology. P values are based on one-sided Mann-Whitney test for non-Gaussian distribution. **p<0.01.

Further evidence for the role of extracellular CKB during metastatic progression was observed from serum of mice injected with CKB knockdown cells—mice with tumors invariably exhibited increased serum CKB levels (Fig. 3.15), in agreement with earlier clinical observations of patients with advanced

malignancies having higher serum CKB activity or protein levels (Huddleston et al., 2005; Rubery et al., 1982).

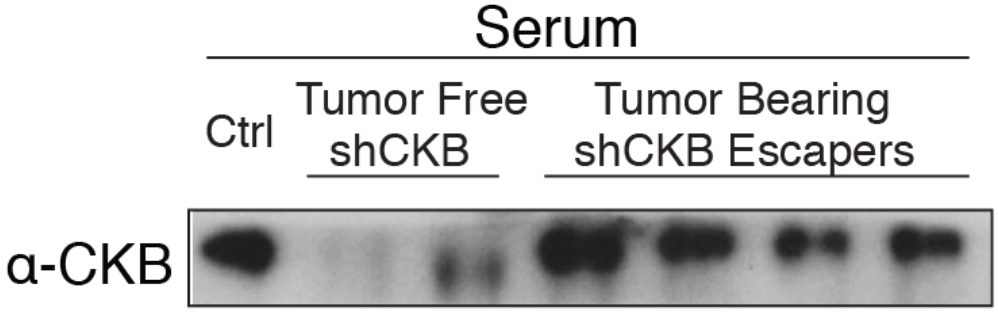


**Figure 3.15 | Extracellular CKB is present in the circulation of mice with metastatic burden.** CKB levels in serum of mice injected with CKB-knockdown LvM3b cells with and without metastatic burden as a result of escaped tumors. Blood was obtained from mice via cardiac puncture after anesthesia. After incubation at room temperature for 30min, serum was obtained after centrifugation at 200g for 10min. Serum was diluted 1:10 and 10ul used for western-blot.

**The SLC6a8 membrane channel is an effector in the CKB axis**

The present findings implicated CKB and exogenous creatine/phosphocreatine metabolism in colon cancer metastasis. It is possible that other components of creatine/phosphocreatine metabolism could also mediate colon cancer metastatic progression. Depletion of guanidinoacetate methyltransferase (GAMT), the enzyme for the final step of creatine synthesis (da Silva et al., 2009), in colon cancer cells did not affect metastasis (Fig. 3.16), consistent with a model wherein an extracellular source (the liver is the primary site of creatine biosynthesis) of, rather than intracellular creatine drives colon cancer metastasis.

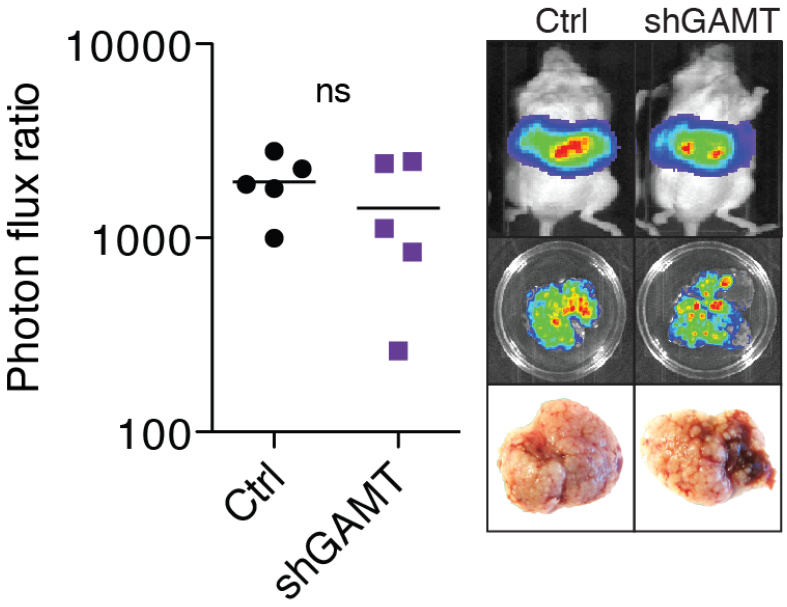


**Figure 3.16 | Colon cancer cells-derived creatine does not contribute to liver metastasis.** 5 X $10^5$ LvM3b control cells or cells depleted of GAMT by RNAi were introduced into immunodeficient mice and liver metastases were monitored by bioluminescence (n=5). Mice were euthanized at day 21 and liver excised out for *ex vivo* bioluminescent imaging and gross morphology examination. P values are based on one-sided Mann-Whitney test for non-Gaussian distribution.

In agreement with the above observations, SLC6a8, a transporter of creatine compounds (Salomons et al., 2001), was found to modulate phosphocreatine levels in colon cancer cells; SLC6a8 knockdown reduced intracellular phosphocreatine and ATP levels (Fig. 3.17A, B).

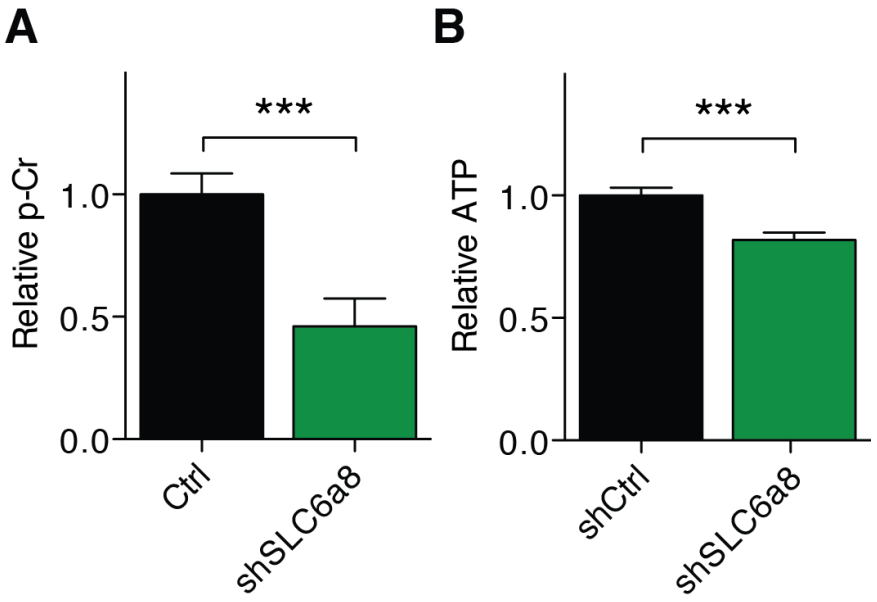


**Figure 3.17 | SLC6a8 regulates intracellular levels of phosphocreatine and ATP.** A, Relative intracellular phosphocreatine levels in LvM3b cells expressing a shRNA targeting SLC6a8 (n=4). B, Relative intracellular ATP levels in LvM3b cells expressing a shRNA targeting SLC6a8 (n=4). Error bars, s.e.m; all P values are based on one-sided Student's t-tests. ***$p<0.001$.

As depletion of phosphocreatine and ATP in colon cancer cells from CKB knockdown or inhibition was able to suppress metastasis, depletion of SLC6a8 should also suppress colon cancer metastasis. Indeed, LvM3b and SW480 colon cancer cells depleted of SLC6a8 displayed substantially reduced (10 to 100-fold) metastatic activity (Fig. 3.18A, B). Metastatic tumors that eventually grew out from SLC6a8 knockdown cells were 'escapers' and displayed restored SLC6a8 expression (Fig. 3.18C).

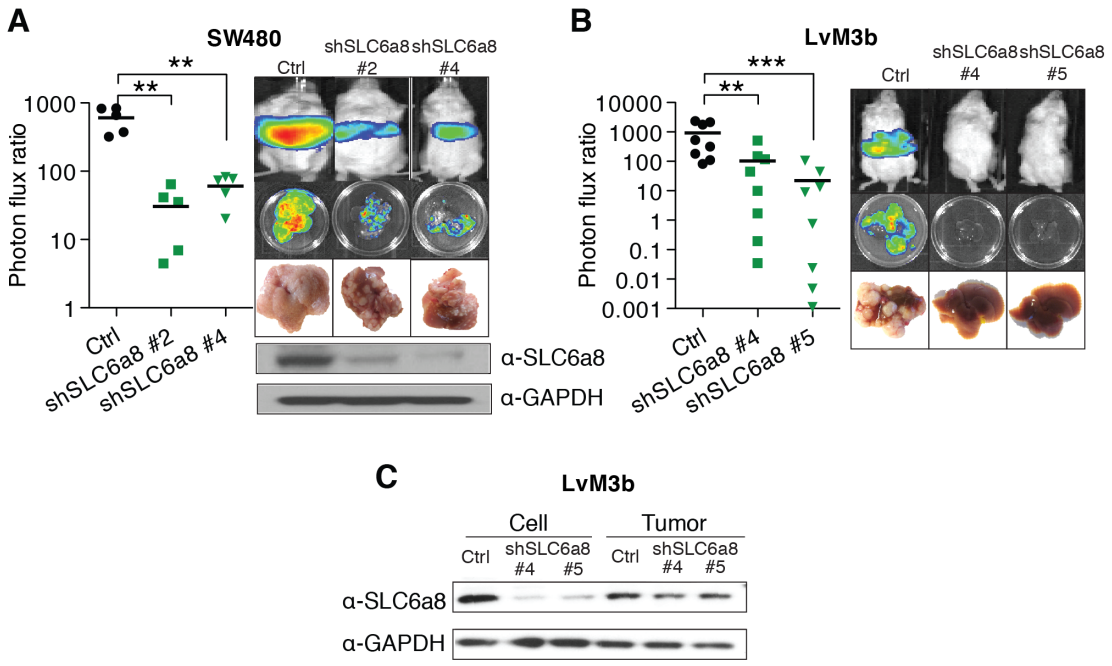


**Figure 3.18 | SLC6a8 is a regulator of colon cancer metastasis to the liver.** A, Liver metastasis by SW480 cells expressing two independent short hairpins targeting SLC6a8 (n=5). 5 X $10^5$ cells were injected into immunodeficient mice. Metastatic progression was monitored using bioluminescence imaging. Mice were euthanized at day 28 and livers excised. B, Liver metastasis by LvM3b cells expressing two independent short hairpins targeting SLC6a8 (n=8). 5 X $10^5$ cells were injected into immunodeficient mice. Metastatic progression was monitored using bioluminescence imaging. Mice were euthanized at day 21 and livers excised. C, CKB levels in SLC6a8-knockdown LvM3b cells and from escaped tumors growing out from SLC6a8-knockdown cells injected intro mice. P values are based on one-sided Mann-Whitney test for non-Gaussian distribution. **p<0.01; ***p<0.001.

Importantly, SLC6a8 knockdown, which depleted cellular uptake of extracellular phosphocreatine, also abrogated the effect of CKB over-expression on colorectal cancer metastasis (Fig. 3.19), revealing extracellular phosphocreatine uptake to be downstream of CKB catalysis.

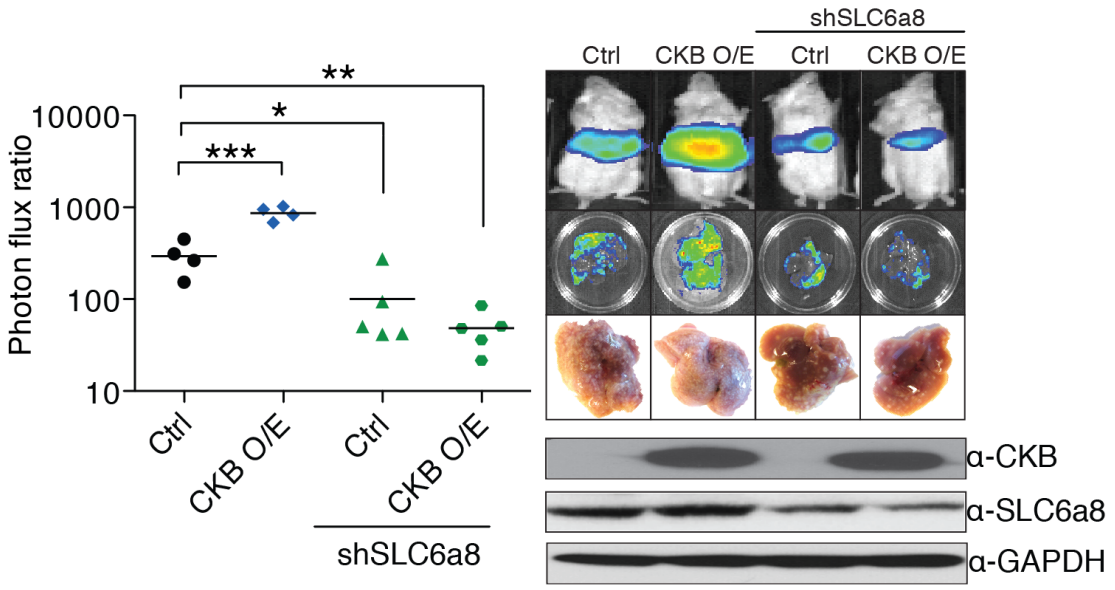


**Figure 3.19 | SLC6a8 is required for CKB mediated colon cancer metastasis to the liver.** Liver metastasis by SW480 cells over-expressing CKB with and without SLC6a8 depletion. 5 X $10^5$ cells were injected into immunodeficient mice (n>4) and metastatic burden monitored by bioluminescent imaging. Mice were euthanized at day 28 and liver excised for gross morphological examination.

Additionally, depleting SLC6a8 in CKB knockdown cells abrogated the protective effect of phosphocreatine during hypoxic stress *in vitro* (Fig. 3.20A), while exogenously added phosphocreatine was not able to promote liver metastasis by SLC6a8 knockdown cells (Fig. 3.20B). These findings revealed SLC6a8 to be downstream of CKB and phosphocreatine in mediating their metastasis-promoting effects.

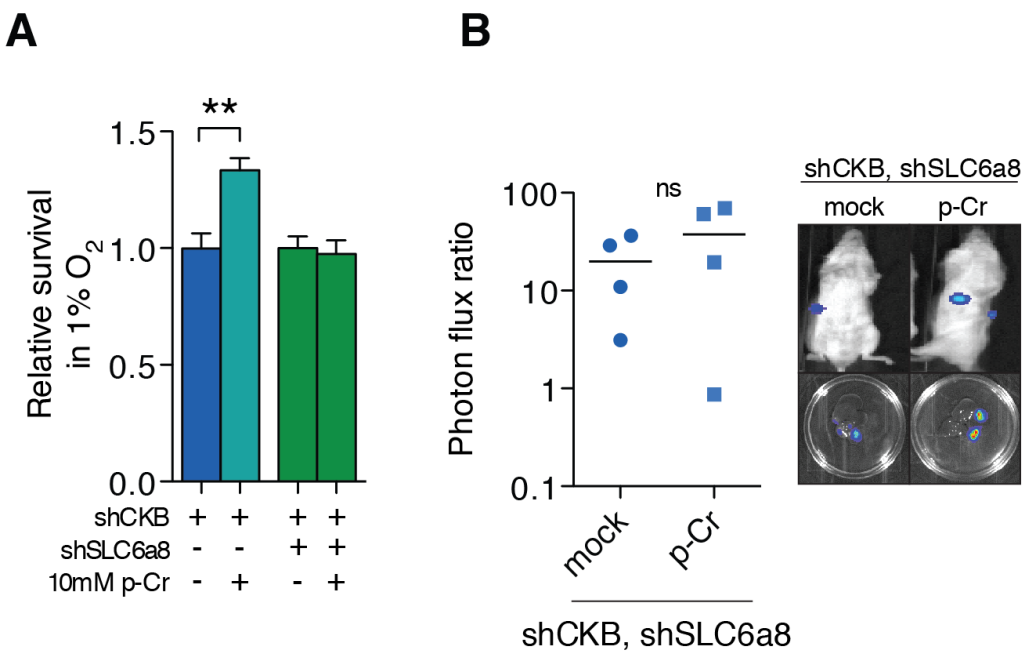


**Figure 3.20 | Depletion of SLC6a8 abrogated the effects of exogenously added phosphocreatine.** A, *In vitro* survival of LvM3b cells depleted of CKB, SLC6a8 with and without phosphocreatine supplementation in hypoxia (n=3). B, Liver metastasis in mice injected with 5 X $10^5$ LvM3b cells transduced with shRNA targeting SLC6a8 in mice implanted with osmotic pumps delivering phosphocreatine into the portal circulation. Metastatic progression was monitored by bioluminescent imaging and mice were euthanized 28 days after injection. Error bars, s.e.m; all P values are based on one-sided Student's t-tests or when appropriate, one-sided Mann-Whitney test for non-Gaussian distribution. **p<0.01.

**Summary of Chapter III**

In Chapter III, efforts to delineate the mechanisms mediated by CKB in promoting colon cancer cell survival in the hepatic microenvironment were described. It was observed that disseminated colon cancer cells experience hypoxia upon arrival into the liver. CKB enhanced survival of colon cancer cells under hypoxia *in vivo* and *in vitro* through regulation of intra- and extracellular

phosphocreatine and ATP levels. Maintenance of intracellular phosphocreatine and ATP levels was shown to be necessary for cancer cell survival under hypoxic stress during liver metastasis as depletion of these high-energy metabolites prevented liver metastasis by colon cancer cells. In addition to CKB's role in modulating intracellular ATP and phosphocreatine levels, CKB can be secreted by colon cancer into the extracellular milieu where it converts exogenous ATP and creatine into phosphocreatine that can be imported into cancer cells as an additional source of ATP to fuel metastatic survival and progression.

Additional components of the creatine/phosphocreatine pathway were also examined for their role in colon cancer metastasis. Exogenous, liver-derived creatine was found to be important for cancer metastasis as endogenous GAMT was not required for successful liver colonization by colon cancer cells. SLC6a8, a membrane transporter that is involved in the transport of creatine compounds was required for colon cancer metastasis downstream of CKB, further indicating the contribution of exogenous phosphocreatine to successful metastatic colonization in coordination with extracellular CKB. In light of the critical roles played by various components of the CKB metabolic axis, Chapter IV will examine the translational viability of these scientific observations. The therapeutic potential of the above pathway will also be investigated through proof-of-principle therapeutic intervention.

**Clinical Relevance and Therapeutic Targeting of the Creatine Kinase Axis**

With the identification of a miR-483-5p and miR-551a regulated, CKB-mediated pathway (Chapter II and Chapter III) that is critical for colon cancer metastasis to the liver, it is important to examine the relevance of this pathway to human patients. Should this pathway be pertinent to colorectal cancer patients, it would be of clinical interest to see if therapeutic perturbation of this pathway could prevent colon cancer metastasis to the liver. This chapter describes the interrogation of multiple archival patient samples for expression levels of the microRNAs as well as CKB and SLC6a8 and their relation to metastatic progression. The potential for therapeutic intervention in this pathway in preventing metastasis is also investigated in this chapter. Finally the relevance of this pathway to liver metastasis by another gastrointestinal cancer, pancreatic cancer was investigated.

**Mir-483-5p and miR-551a expression are decreased in liver metastases relative to primary tumors**

To determine if miR-483-5p and miR-551a expression levels were suppressed in liver metastases of colon cancer patients, archival RNA samples from a set of 66 surgically resected primary colon cancer tumors and liver metastases were obtained from Memorial Sloan-Kettering Cancer Center (MSKCC). Quantitative real-time PCR was performed to examine the relative levels of these microRNAs in these patient samples. Consistent with a metastasis-suppressive role for these microRNAs during cancer progression, miR-483-5p and miR-551a both independently displayed significantly reduced expression levels in human liver metastases relative to primary colon cancers (Fig. 4.1A, B).

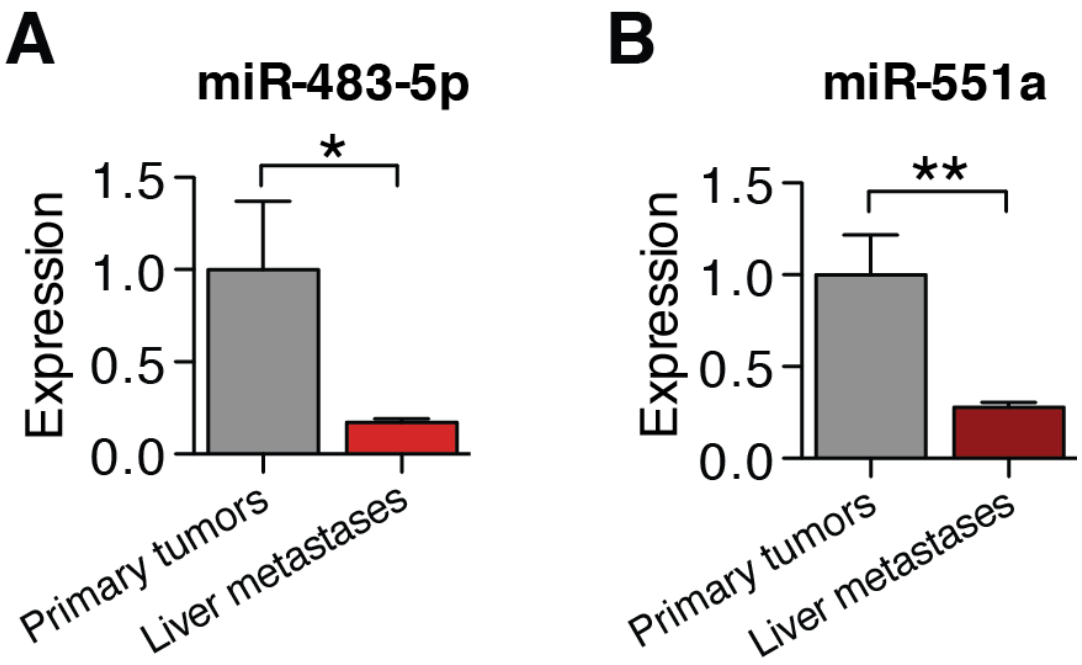


**Figure 4.1 | Expression levels of mir-483-5p and miR-551a are decreased in liver metastases compared to primary tumors from patients.** A, MiR-483-5p and B, miR-551a levels in 36 primary colorectal adenocarcinomas and 30 liver metastases were quantified by quantitative real-time PCR. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. *p<0.05; **p<0.01.

**CKB and SLC6a8 display increased expression in liver metastases relative to primary tumors**

With the experimental observations that CKB and SLC6a8 were important effectors for liver metastasis and the strong selective pressure for CKB and SLC6a8 in escaped liver nodules, the expression levels of CKB and SLC6a8 were examined in multiple sets of archival samples to determine if CKB and SLC6a8 expression were selected for in liver metastases of patients. CKB and SLC6a8 transcript levels in the above-described archival RNA samples from MSKCC were examined by quantitative real-time PCR. Both CKB and SLC6a8 transcripts were significantly increased in liver metastases relative to primary tumors (Fig.

4.2A, B), supporting a model in which CKB and SLC6a8 are selected for during colon cancer progression.

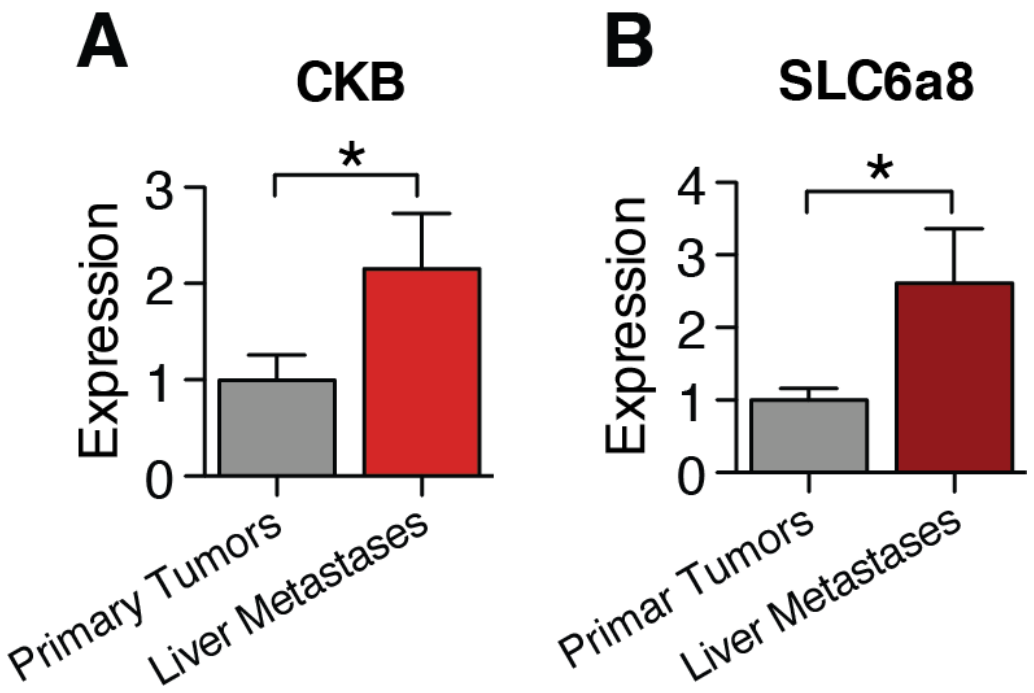


**Figure 4.2 | Expression levels of CKB and SLC6a8 are increased in liver metastases compared to primary tumors from patients.** A, CKB and B, SLC6a8 levels in 36 primary colorectal adenocarcinomas and 30 liver metastases were quantified by quantitative real-time PCR. Error bars, s.e.m; all P values are based on one-sided Student's t-tests. *p<0.05.

Given that both miR-483-5p and miR-551a were negative regulators of CKB, the correlation between the expression levels of miR-483-5p and miR-551a and CKB in patients that had low expression of these microRNAs was examined. Indeed, there was significant negative correlation between CKB expression levels and that of the microRNAs (Fig. 4.3A). Additionally, in nine patient-derived colon

cancer cell-lines, the expression levels of the microRNAs and CKB were significantly negatively correlated as well (Fig. 4.3B).

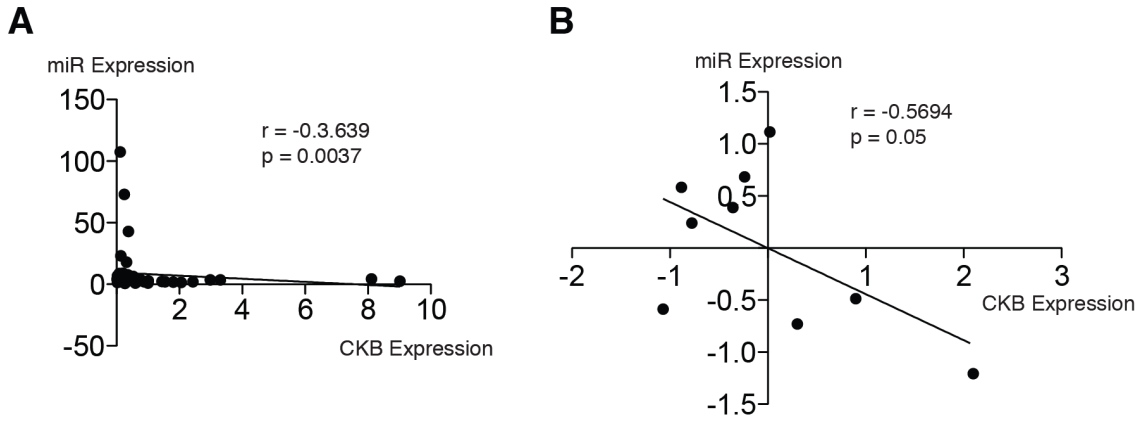


**Figure 4.3 | Expression levels of CKB and the microRNAs are negatively correlated in patient samples.** A, MicroRNA and CKB expression levels in archival RNA samples from MSKCC. B, Expression levels of microRNAs and CKB in patient-derived primary colon cancer cell-lines.

As an independent validation set, a tissue microarray was constructed from a collection of primary colorectal cancer tumors and liver metastases that were surgically resected from patients at Weill-Cornell Medical Center (WCMC) and New York Presbyterian Hospital (NYPH). The tissue microarray was immunohistochemically stained for CKB and SLC6a8 expression. Both CKB and SLC6a8 protein expression levels were found to be elevated in liver metastases relative to primary tumors of patients (Fig. 4.4A, B).

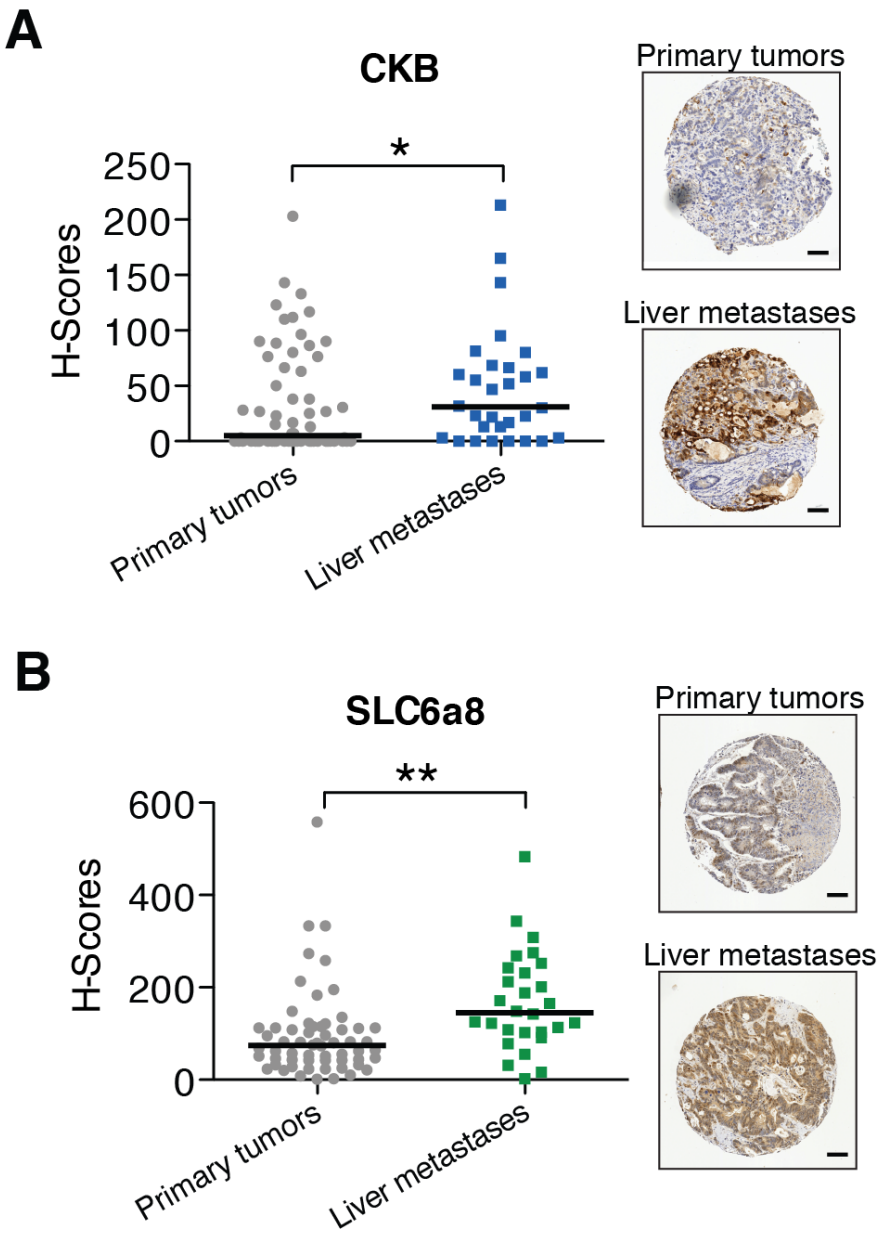


**Figure 4.4 | Protein expression of CKB and SLC6a8 are increased in liver metastases of an independent cohort of patients compared to primary tumors.** A, Immunohistochemical staining of CKB on a tissue microarray constructed from liver metastases and primary tumors of patients from New York Presbyterian Hospital. B, Immunohisochemical staining of SLC6a8 in liver metastases and primary tumors of patients. P values are based on one-sided Mann-Whitney test for non-Gaussian distribution. *p<0.05; **p<0.01.

In addition to the clinical samples obtained from MSKCC, WCMC and NYPH, a publicly available gene expression dataset was also analyzed for expression levels of CKB and SLC6a8 (Sheffer et al., 2009). This additional dataset demonstrated higher levels of CKB and SLC6a8 in liver metastases of patients compared to primary tumors as well (Fig. 4.5A, B).

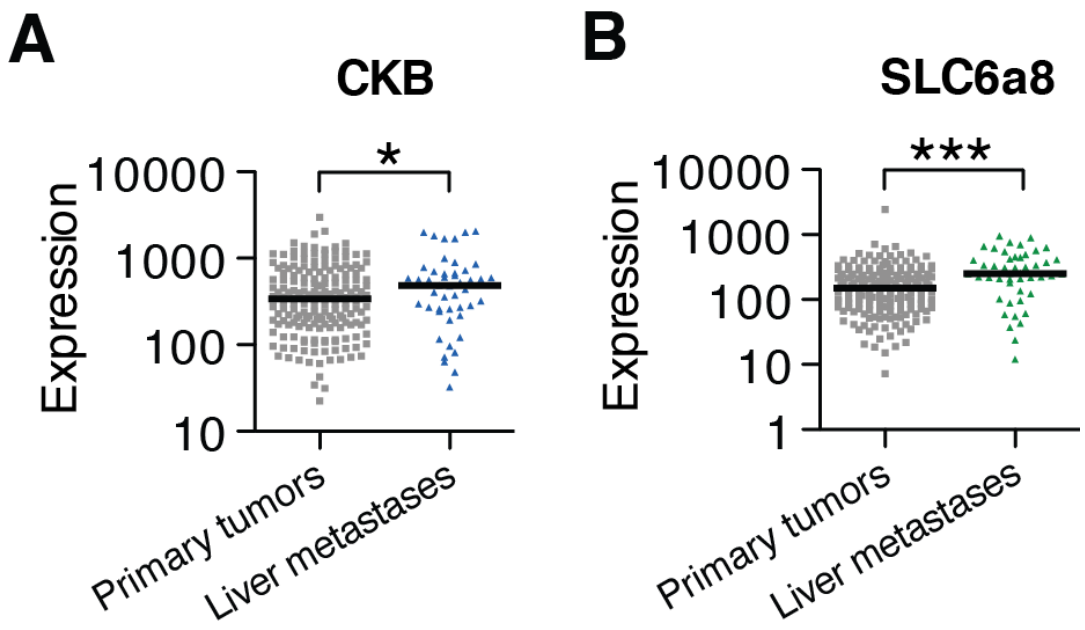


**Figure 4.5 | Expression levels of CKB and SLC6a8 in primary tumors and liver metastases obtained from a publicly available microarray dataset.** A, B, CKB and SLC6a8 expression from a public microarray dataset (GSE41258) comparing primary tumors and liver metastases (N=233). P values are based on one-sided Mann-Whitney's test. *p<0.05; ***p<0.001.

The above findings are consistent with the present experimental findings, and suggest the pathophysiological basis for, previous studies describing elevated expression levels of CKB in advanced stage cancers (Wallimann and Hemmer,

1994) and reveal significant association between the components of miR-483-5p and miR-551a regulated multi-microRNA network and colon cancer progression.

**Therapeutic delivery of miR-483-5p and miR-551a suppress colon cancer metastasis**

With the findings that deregulation of the expression levels of microRNAs, CKB and SLC6a8 is relevant to colon cancer patients; the potential for therapeutic intervention targeting this multi-microRNA regulatory network was investigated. Adeno-associated virus delivery of microRNAs was previously described to suppress hepatic carcinogenesis in a mouse model of hepatocellular carcinoma (Kota et al., 2009). It may be possible that adeno-associated virus could infect colon cancer cells and suppress colon cancer metastasis to the liver *in vivo*. As a first step towards determining if adeno-associated viruses are viable vectors for delivery of microRNAs into colon cancer cells, the ability of adeno-associated virus to transduce colon cancer cells *in vitro* was examined. Adeno-associated viruses were able to transduce colon cancer cells even at low multiplicity of infection *in vitro* (Fig. 4.6A). Injection of mice bearing macroscopic hepatic metastases with adeno-associated virus revealed that adeno-associated virus was able to infect colon cancer metastases *in vivo* as well and could therefore be a suitable microRNA delivery vector (Fig. 4.6B).

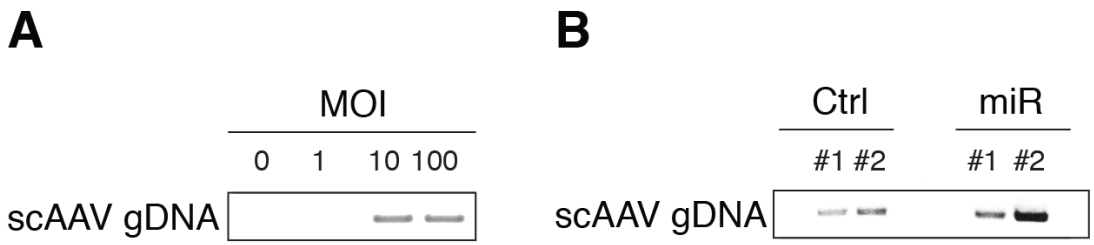


**Figure 4.6 | Adeno-associated viral vectors are able to infect colon cancer cells *in vitro* and *in vivo*.** A, PCR amplification of adeno-associated viral DNA extracted from SW480 cells directly transduced with AAV *in vitro* at indicated multiplicity of infection. DNA was extracted 48hrs after infection and primers specific for adeno-associated viral sequences were used. B, PCR amplification of adeno-associated viral DNA extracted from hepatic metastases of mice injected with adeno-associated viruses. Tumors were extracted 48hrs after injection, genomic DNA extracted and PCR amplification of viral-specific DNA was performed.

A proof-of-principle experiment was performed in which mice were injected with highly metastatic LvM3b cells and a single dose of adeno-associated virus encoding miR-483-5p and miR-551a from a single transcript was injected intravenously 24hrs after colon cancer cells inoculation. A single therapeutic dose of AAV delivering both microRNAs were able to significantly reduced metastatic colonization (Fig. 4.7A). Therapeutic efficacy was also seen in mice injected with SW480 cells (Fig. 4.7B) and a primary patient-derived cell-line (Fig. 4.7C) In these experiments, there were no adverse phenotypic outcomes or pathological abnormalities in the treated mice.

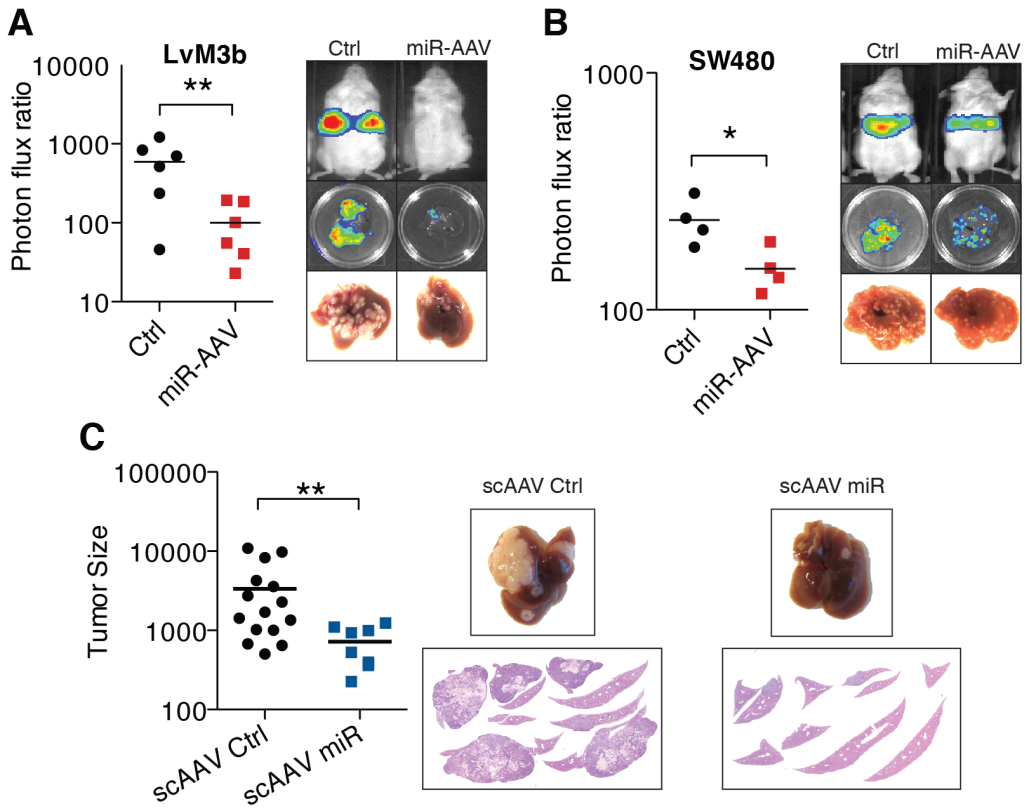


**Figure 4.7 | Adeno-associated viral delivery of miR-483-5p and miR-551a suppress liver metastasis by colon cancer cells.** A, Liver metastasis in mice injected with LvM3b cells and treated with a single dose of 1 x $10^{12}$ AAV doubly expressing miR-483-5p and miR-551a one day after injection cells (n=6). B, Liver metastasis in mice injected with SW480 cells and treated with a single dose of AAV doubly expressing miR-483-5p and miR-551a one day after injection cells (n=4). Metastatic burden was monitored by bioluminescent imaging and mice euthanized at day 21 for LvM3b and day 28 for SW480. C, Metastatic burden in mice inoculated with a patient-derived primary colon cancer cell-line and treated with control AAV or AAV-miR. Representative H&E stained liver sections with liver metastases were shown. P values are based on one-sided Mann-Whitney test for non-Gaussian distribution. *p<0.05; **p<0.01.

In mice injected with BEAS-2B immortalized lung epithelial cells that are prone to oncogenic transformation (Amstad et al., 1988; Pacurari et al., 2013), treatment with microRNA delivering AAV also did not result in spontaneous tumors, demonstrating the relatively safety of this single-dose AAV treatment in the context of immunodeficient mice (Fig. 4.8). However, given possible unforeseen pleiotropic effects of continued ectopic delivery of microRNAs (Grimm et al., 2006), further extensive characterization with regards to adeno-associated viral delivery of microRNAs has to be performed prior to clinical evaluation in human patients (Garzon et al., 2010).

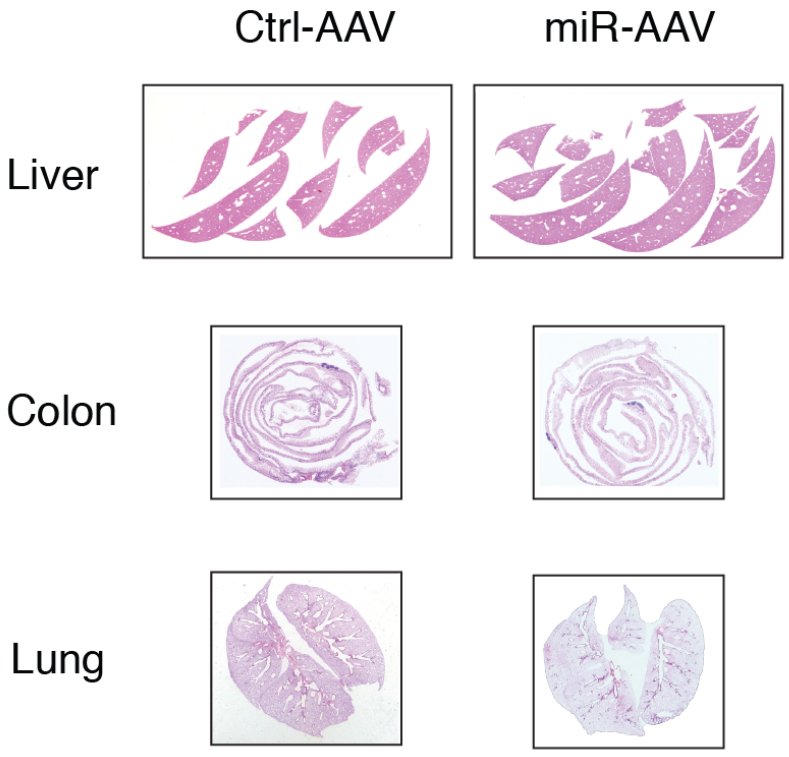


**Figure 4.8 | Adeno-associated viral delivery of microRNA does not result in oncogenic transformation in various tissues.** H&E sections of indicated organs from mice treated with control or microRNA encoding AAV.

**Small molecule inhibition of CKB and SLC6a8 suppress colon cancer metastasis**

As an alternative to adeno-associated viral delivery of miR-483-5p and miR-551a, which might have unforeseen pleiotropic effects, targeting of the downstream effector, CKB, using a small molecule inhibitor was performed. Cyclocreatine is a low-potency inhibitor of CKB. Therapeutic treatment of mice with cyclocreatine after colorectal cancer cell inoculation significantly reduced metastatic colonization, demonstrating proof-of-principle for targeting this kinase as a means of metastasis suppression (Fig. 4.9A). The modest but significant effect of cyclocreatine on metastasis suppression could be due to cyclocreatine being a relatively poor inhibitor of CKB (Lillie et al., 1993). A more potent inhibitor could potentially demonstrate a higher therapeutic efficacy. Treatment of mice inoculated with colon cancer cells with guanidinopropionic acid (GPA), a small molecule inhibitor of SLC6a8 was also able to significantly inhibit the formation of liver metastasis (Fig. 4.9B).

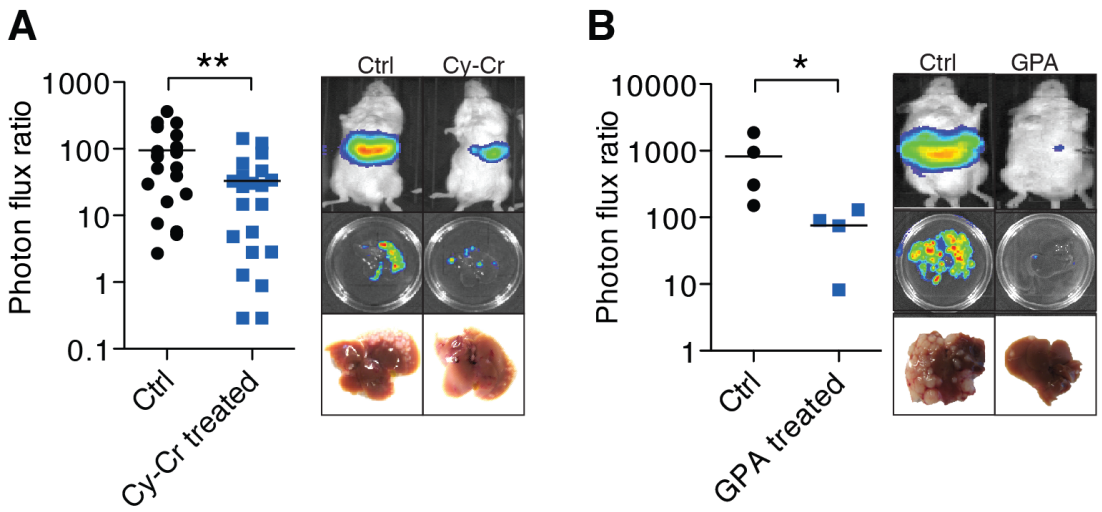


**Figure 4.9 | Small molecule inhibition of CKB or SLC6a8 suppress metastasis by colon cancer cells.** Liver metastasis in mice injected with 5 X $10^5$ LvM3b cells and treated with cyclocreatine daily for two weeks (n>15). Liver metastasis in mice injected with 5 x $10^5$ LvM3b cells and treated with GPA daily for three weeks (n=4). P values are based on one-sided Mann-Whitney test for non-Gaussian distribution. *p<0.05; **p<0.01.

**Depletion of CKB and SLC6a8 in pancreatic cancer cells suppressed liver metastasis**

Liver metastasis is a common occurrence during the progression of pancreatic cancer. As pancreatic cancer cells will also experience hypoxia within the liver microenvironment, depletion of CKB and SLC6a8 in pancreatic cancer cells could possibly suppress liver metastasis by pancreatic cancer cells. Indeed knockdown of CKB and SLC6a8 in PANC1 cells (a K-RAS mutant human pancreatic cell-line) with multiple shRNAs, strongly suppressed their ability to metastasize to the liver (Fig. 4.10A, B). This preliminary finding suggested that CKB and SLC6a8, and their associated metabolic pathways, might broadly govern liver metastasis by other gastrointestinal cancers. Inhibition of this pathway in the clinic might

therefore provide therapeutic benefit to patients with pancreatic cancer as well as other gastrointestinal cancers.

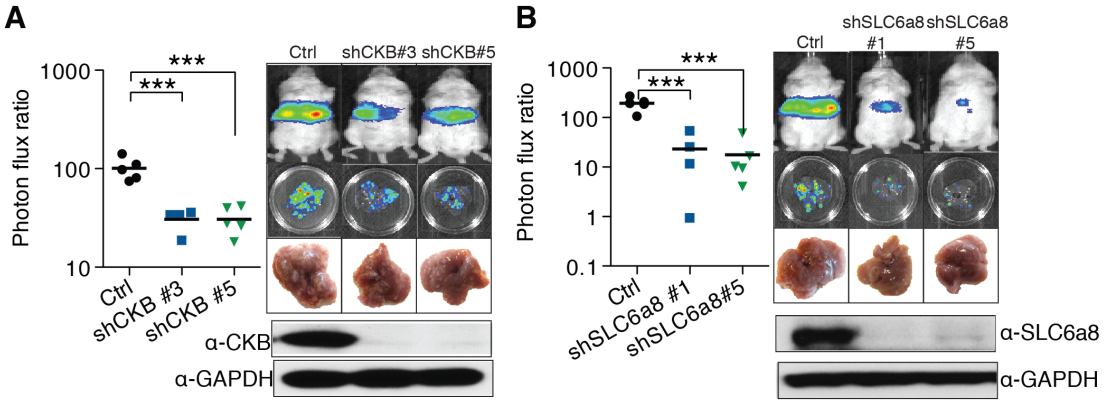


**Figure 4.10 | Depletion of CKB and SLC6a8 suppress pancreatic cancer metastasis to the liver**. A, Liver metastasis by pancreatic cancer cells, PANC1, with knockdown of CKB with two independent shRNA hairpins (n=5). B, Liver metastasis in mice injected with 5 X $10^5$ PANC1 cells transduced with two independent SLC6a8 shRNAs (n=5). Metastatic progression was monitored by bioluminescent imaging. Mice were euthanized 35 days after injection and liver excised for bioluminescent imaging and gross morphology. P values are based on one-sided Mann-Whitney test for non-Gaussian distribution. ***p<0.001.

**Summary of Chapter IV**

This chapter presents evidence from archival clinical samples that corroborated the role of miR-483-5p and miR-551a as suppressors of colorectal cancer metastatic progression from primary tumors to liver metastases. MiR-483-5p and miR-551a expression was silenced in liver metastases relative to primary tumors. Consistent with experimental findings that CKB and SLC6a8 are promoters of

liver metastasis and are selected for during metastatic progression, liver metastases from patients had expressed higher levels of CKB and SLC6a8 across multiple clinical sets. Proof-of-principle therapeutic delivery of miR-483-5p and miR-551a using adeno-associated viral-vectors demonstrated efficacy in suppressing colon cancer metastasis, as did small molecule inhibition of CKB and SLC6a8. Depletion of CKB and SLC6a8 in a pancreatic cancer cell-line also suppressed pancreatic cancer liver metastasis, which suggested that therapeutic targeting of CKB and SLC6a8 in other gastrointestinal cancers might prevent liver metastasis.

# Chapter V: Summary and Perspectives

**Overall Summary**

Colorectal cancer is one of the most prevalent cancers in the United States and contributes to almost 10% of all cancer deaths. Majority of patients die as a result of colorectal cancer cells metastasis to the liver (Siegel et al., 2014a; Siegel et al., 2014b). Understanding the cellular and physiological basis of colorectal cancer metastasis is therefore of great interest to the medical and scientific community with regards to developing new therapies targeting this important step in colorectal cancer progression.

This thesis describes the use of two complementary approaches to identify two microRNAs, miR-483-5p and miR-551a, as suppressors of colon cancer cell survival in the liver microenvironment and suppressors of metastatic colonization. Systematic dissection of the downstream effector pathways using *in vitro* cell culture, organotypic slice culture and *in vivo* experimental metastasis systems identified CKB as a direct target and downstream effector of both microRNAs. Over-expression of CKB was sufficient to enhance metastasis, while depletion of endogenous CKB suppressed metastasis by colon cancer cells. Hypoxia was identified to be a relevant physiological stress during colorectal cancer metastasis to the liver and CKB was found to promote metastasis to the liver by enhancing cell survival in the hypoxic liver microenvironment.

CKB belongs to a group of enzymes that readily modulate high-energy phosphate metabolite levels within the cell by catalyzing the transfer of a high-energy phosphate group from phosphocreatine to ADP (Wallimann et al., 1992; Wyss and Kaddurah-Daouk, 2000). In the physiologically hypoxic environment

of the liver, maintenance of intracellular ATP levels by CKB resulted in enhanced colon cancer cell survival. This enables surviving cells to subsequently activate hypoxia response pathways that can result in successful completion of the metastatic cascade and liver colonization (Semenza, 2013). Remarkably, colon cancer cells can release CKB extracellularly to take advantage of the ATP-rich extracellular milieu and liver-synthesized creatine to generate an exogenous source of phosphocreatine that can be further taken up by colon cancer cells through the SLC6a8 membrane transporter as an additional source of ATP for metastatic survival and progression (Fig. 5.1).

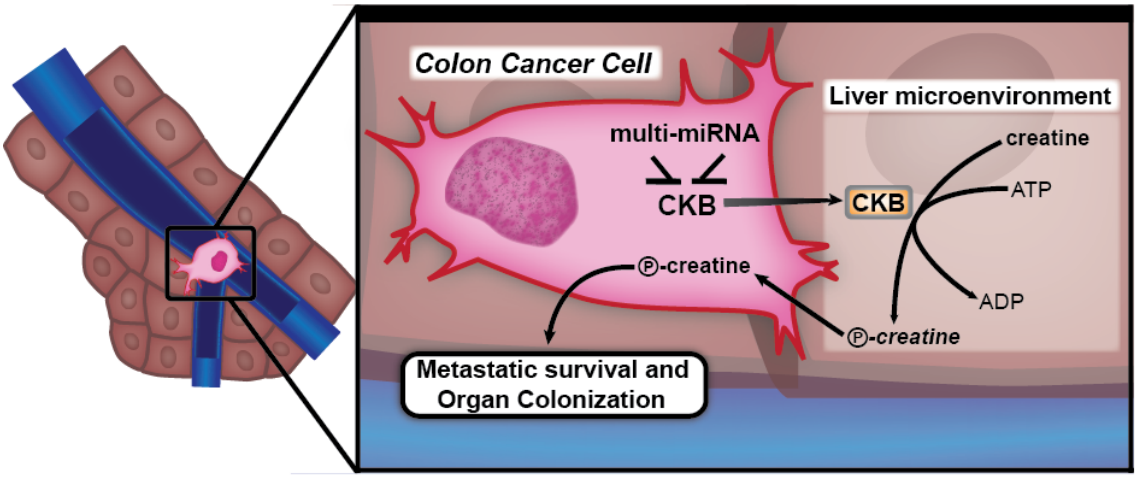


**Figure 5.1 | Model for the role of CKB during metastatic progression of colorectal cancer.** Disseminated colon cancer cells arrive in the liver microenvironment through the hypoxemic portal circulation. Within the liver microenvironment, they experience hypoxic stress and ATP depletion. Cells that up-regulate CKB through loss of miRNAs, release CKB into the extracellular matrix where it converts available creatine and ATP into phosphocreatine that is then taken up by the cell to fuel metastatic survival and subsequent organ colonization. Colon cancer cells with higher levels of CKB also build up a larger pool of intracellular phosphocreatine that acts as a buffer against energetic stress.

In the final chapter of this thesis, clinical evidence was presented that supported the roles of miR-483-5p and miR-551a in suppressing colorectal cancer progression to liver metastases. A role for CKB and the transport protein SLC6a8 during metastatic progression was supported by increased expression of CKB and SLC6a8 in human liver metastases compared to primary tumors. As the microRNAs and the effector proteins in the CKB network are clinically relevant, proof-of-concept therapeutic targeting of this pathway through adeno-associated viral delivery of the microRNAs and small molecule inhibition of CKB was investigated and demonstrated to be effective *in vivo* in mice.

In summary, this thesis presented the application of two unbiased approaches to identify a microRNA network that regulates colon cancer metastasis through CKB. CKB promotes colon cancer cell survival in the hypoxic liver microenvironment through modulation of high energetic metabolites intra- and extracellularly. The identified pathways were found to be clinically relevant and could potentially be therapeutically targeted, either through restoration of microRNA levels or small molecule inhibition of the effector proteins.

**Library-based identification of microRNAs that suppress colon cancer metastasis**

Recently, many investigators have used library-based screening approaches to identify molecular mediators of various biological processes during tumorigenesis and cancer progression (Eifert and Powers, 2012; Mohr et al., 2010). Molecular determinants of phenotypes such as *in vitro* cell proliferation in different contexts, *in vitro* migration and invasion (Quintavalle et al., 2011) and

drug sensitivity (Gupta et al., 2009) have been described through the use of library-based screening approaches. However, while identified mediators of the studied phenotypes can be characterized and further validated through downstream experiments, a caveat concerning some of the studies is that the initial screen was performed in an *in vitro* context that might not necessarily lend itself to the appropriate *in vivo* physiological context. It is only recently that *in vivo* library based functional screens have been reported (Beronja et al., 2013; Schramek et al., 2014). Because of the above caveat, there could be false negatives that are missed by *in vitro* screens, beyond the identified mediators that are subsequently validated experimentally. A pre-determined selective pressure *in vitro* would also occlude the identification of novel mediators involved in aspects of cancer progression that are not readily apparent *in vitro*.

As an approach to identify novel determinants of colon cancer liver colonization, an *in vivo* library screen of 661 microRNAs was performed through injection of colon cancer cells directly into the liver parenchyma. To develop into a metastatic liver nodule, colon cancer cells would have to survive the stringent selective pressure of the liver microenvironment. The use of an *in vivo* platform for the screen allowed for identification of microRNAs that when over-expressed, suppress colon cancer metastasis *in vivo* through pathways that are physiologically relevant and potentially lend themselves to *in vivo* microRNA-delivery based therapeutics. The identification of miR-483-5p and miR-551a in part through this screen, which mediated colon cancer cell survival under hypoxia, a physiological stress present in the liver microenvironment,

demonstrated the utility of this *in vivo* strategy in highlighting previously overlooked biological bottlenecks critical to colon cancer metastasis to the liver.

### *In vivo* selection for liver metastatic colon cancer cells

A caveat of functional over-expression screens is that the genes or microRNA that were overexpressed, might not be present at endogenously relevant levels in the studied tissues (Ashworth and Bernards, 2010; Schramek et al., 2014). *In vivo* selection thus provides a means to identify mediators that are present at endogenously relevant levels. Isaiah Fidler first pioneered *in vivo* selection when he performed *in vivo* selection for metastatic melanoma cells (Fidler, 1973). In recent years, *in vivo* selection had been used to identify molecular determinants of breast cancer and melanoma metastasis to the lungs, as well as breast cancer metastasis to the bone and brain (Kang et al., 2003; Minn et al., 2005; Pencheva et al., 2012; Png et al., 2012; Tavazoie et al., 2008). A caveat of *in vivo* selection is that observed expression changes between parental and *in vivo*-selected cell populations might be over-represented by passenger genes that may not confer a biological function. However, *in vivo* selection lends itself well as a parallel approach to a functional library screen—targets that are identified in a functional overexpression screen and whose endogenous expression levels are changed in *in vivo* selected lines will be higher confidence functional mediators that are present at physiologically relevant levels. That mir-483-5p and miR-551a were identified through the convergence of both functional screens and *in vivo* selection and had robust effects highlighted the complementarity of both approaches.

**Regulation of CKB by miR-483-5p and miR-551a**

MicroRNAs have been extensively studied for their roles in tumorigenesis and cancer progression and have been shown to modulate well-known cellular phenotypes such as migration, invasion, proliferation and cell death through regulation of effector gene targets . While it is not uncommon for studies to focus on the identification of single gene transcripts targeted by particular microRNAs, it is recognized that microRNAs, through their binding to target sequences on different gene transcripts, can exert pleotropic effects by modulating divergent gene expression programs (Pasquinelli, 2012). Based on this effect, microRNAs can regulate the expression of multiple transcripts that do not necessarily converge on the same biological pathways. However, an alternative model could also hold true in which a single microRNA can regulate multiple transcripts that eventually converge on the same biological processes and phenotypes resulting in a very robust regulation of particular biological pathways by a single microRNA. More recently, a third model of microRNA-mediated regulation of biological processes emerged in which multiple microRNAs can coordinately converge on a few genes regulating a particular biological process. The advantage would be that dysregulation of one of a few microRNAs in a pathway would not necessarily disrupt the biological processes as extensively since the other microRNAs can still provide a layer of redundancy in terms of regulation.

In the present thesis, miR-483-5p and miR-551a were identified to convergently target a functional effector, CKB. While miR-483-5p and miR-551a could regulate the level of other transcripts, *in vivo* loss-and-gain of function epistasis experiments confirmed CKB to be an important downstream effector of both

microRNAs in the context of liver colonization by colon cancer cells. Given that an increase in CKB levels is sufficient to enhance metastasis by colon cancer cells and CKB expression is necessary for successful completion of the metastatic cascade, a network of multiple microRNAs targeting CKB would provide a layer of redundancy in preventing severe dysregulation of this pathway and suppressing metastatic colonization.

The regulation of the above network is critical; in contrast to the multitude of pathways and genes that could contribute to a migratory and invasive phenotype, the specific enzymatic reaction converting creatine to phosphocreatine can only be catalyzed by a few other isoenzymes that are within the creatine kinase family, namely CKM, CKMT1 and CKMT2 (Wallimann et al., 1992). CKMT1 and CKMT2 are ubiquitous mitochondrial creatine kinases whose subcellular localization is limited to the mitochondrion, where they perform a role in transferring ATP (generated from oxidative phosphorylation) from mitochondria to the cytosol of cells. CKM is the only alternative cytosolic creatine kinase that could perform the same cellular role as CKB. However, as earlier studies had described a lack of CKM expression in colonic tissues (Trask et al., 1988; Urdal et al., 1983), CKB remains a critical enzyme in colon cancer cells that had to be tightly regulated to prevent dysregulation of creatine/phosphocreatine metabolism and metastatic progression to the liver. That extracellular CKM had not being extensively detected in the serum of patients with advanced cancer also indicates the possibility that the mechanism(s) involved in CKB release are unique to CKB and could be an alternative pathway through which CKB's functions are regulated.

**CKB and initial seeding of colon cancer cells into the liver microenvironment**

Colon cancer cells cannot easily circumvent the physiological challenges presented by the liver microenvironment. Earlier studies investigating the efficiency of colon cancer cell metastasis to the liver demonstrated that only 0.1% of all colon cancer cells that eventually arrive successfully at the liver go on to develop metastases (Sugarbaker, 1993; Weiss et al., 1986). The stresses encountered by colon cancer cells during liver colonization can only be understood by an appreciation of the physiological functions and architecture of the liver.

The liver is a complex organ that is responsible for a varied number of physiological functions required for organismal homeostasis. The enormous functional capacity of the liver is surprisingly mediated by a minimal number of cell-types comprising parenchymal cells (hepatocytes) and non-parenchymal cells consisting of sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells. The variation in cellular function is achieved through functional specialization of cells based on their location with respect to the direction of blood flow through the organ and oxygenation levels (Jungermann and Kietzmann, 1996, 2000). This division of biochemical functions is known as 'liver zonation'. Functional groups of hepatocytes can be broadly grouped into cells at the periportal region experiencing relatively higher levels of oxygenation (considering that blood reaching the liver is already hypoxemic in nature) and cells at the perivenous region which is hypoxic in nature. Oxygen pressure at the perivenous region can fall as low as 5-10mmHg, corresponding to 0.5-1% oxygen

levels due to consumption by metabolically active hepatocytes present upstream (Arteel et al., 1995). Perivenous hepatocytes actively undergo glycolysis as their main source of cellular energy. Colon cancer cells that initially arrived at the liver will eventually get arrested near the perivenous region where they experience acute hypoxia. Empirical evidence from *in vitro* co-culture and tissue-mimetic studies had also described hepatocytes to consume oxygen at a rate of 10-40 times that of colon cancer cells, resulting in an hypoxic microenvironment experienced by colon cancer cells during co-culture experiments (Jiang et al., 2013).

While hypoxia can result in depletion of ATP reserves and cell death, it is widely accepted that cancer cells utilize glucose during glycolysis as a major source of cellular ATP. In the context of the above-discussed liver microenvironment, where colon cancer cells are arrested at the hypoxic perivenous region, an understanding of hepatic glucose metabolism is important to understand the limitation of glycolysis in fueling metastatic cell survival during initial dissemination to the liver prior to any adaptive response to the hypoxic environment.

An important homeostatic role performed by the liver is the regulation of blood glucose levels through integration of glucagon and insulin signaling. Excess glucose is taken up by hepatocytes to synthesize glycogen (glycogenesis) that can be subsequently converted to glucose (gluconeogenesis) when required. Periportal and perivenous hepatocytes are responsible for very distinct roles during glycogenesis and gluconeogenesis. Particularly pertinent to colon cancer

cells at the perivenous regions are the roles of perivenous hepatocytes during these two processes. During glycogenic phase, perivenous hepatocytes will actively take up glucose to synthesize glycogen. Excess glucose is consumed by perivenous hepatocytes via glycolysis, with the release of lactate that travels through the circulation to arrive at the periportal hepatocytes where they are converted to glucose via gluconeogenesis for glycogenesis. Colon cancer cells that are newly disseminated to the liver therefore encounter intense competition for the absorption of glucose from perivenous hepatocytes undergoing glycogenesis and glycolysis; *in vivo* measurements of glucose uptake by hepatocytes had been determined to be as high as 0.61μM/min/g (Jungermann et al., 1982). In contrast, glucose uptake rates of established tumors of various cancer cell-types had been reported to vary between 0.21-0.43μM/min/g (Kallinowski et al., 1989; Kallinowski et al., 1988). Freshly disseminated cells arriving at the liver microenvironment would possibly have even lower glucose uptake rate prior to up-regulation of glucose transporters. Taking into consideration the relative abundance of hepatocytes compared to disseminated colon cancer cells, these factors contribute to a scenario in which colon cancer cells are severely out-competed for glucose by hepatocytes.

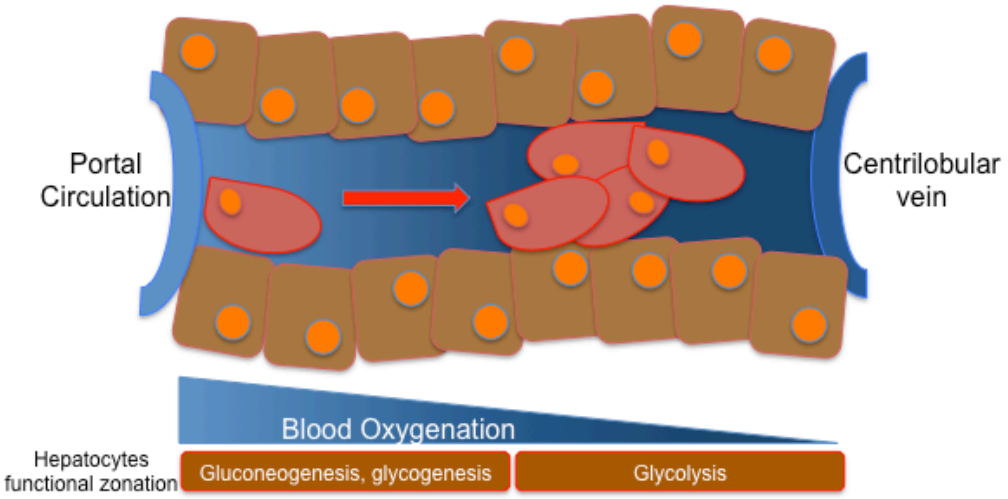


**Figure 5.2 | Liver metabolism and colorectal cancer cells metastasis.** Colon cancer cells arriving at the liver via the hypoxemic portal circulation experience progressively lower levels of oxygenation and glucose availability as they competes with hepatocytes for glucose and oxygen.

Given the above context (Fig. 5.2), CKB expression is beneficial to colon cancer cells. Increased CKB expression will allow for increased levels of phosphocreatine within colon cancer cells that will be a reservoir of ATP that can be rapidly utilized for when colon cancer cells first arrive at the liver. The release of CKB into the extracellular space will also allow for the scavenging of extracellular ATP in the form of phosphocreatine that can be an additional source of ATP that fuel cell survival and metastatic progression until further adaptation. This latter function of CKB described within this thesis requires that concentrations of extracellular ATP and creatine in the pericellular environment of colon cancer cells be sufficient for the reaction to proceed. Previous studies outside the context of cancer progression have reported resting serum creatine

levels to be approximately 50-100μM, while extracellular ATP levels are reported to be extremely low in healthy tissues, with concentrations of extracellular ATP to be in the nanomolar range. In the context of cancer progression, increased levels of extracellular ATP within the immediate microenvironment of cancer cells have been reported to be in the range of hundreds of micromolar (Pellegatti et al., 2008), while ADP levels were reported to be an order of magnitude lower, in part due to the expression of ecto-nucleotidases by cancer cells that readily converts ADP to adenosine. Earlier investigations into the kinetics of creatine kinases *in vitro* had established Michaelis constants of creatine kinases for ATP and creatine to be in the order of micromolars (Valdur A et al., 1998). While the extracellular milieu of cancer cells is more complex than the defined reaction buffers utilized in *in vitro* biochemical assays, given the relative high concentrations of extracellular ATP and creatine, the generation of phosphocreatine from ATP and creatine within the extracellular microenvironment of cancer cells is energetically favored. However, biological sensors for the detection of phosphocreatine without the need for biochemical assays or isolation of bulk biological fluids would have to be developed for more sensitive and timely detection of phosphocreatine synthesis within the tumor microenvironment.

The role CKB plays in colon cancer cell survival during the initial phase of metastatic seeding in the liver is critical as cells that survive the initial hypoxia would be able to activate additional pathways involved in energy homeostasis and generation such as activation of the AMPK (DeBerardinis et al., 2008; Hardie

et al., 2012; Inoki et al., 2012; Jeon et al., 2012). The surviving cells would also have engaged hypoxia-activated pathways that are pro-metastatic through the HIF-1A transcriptional pathway and can progress to aggressively colonize the liver (Chiang and Massagué, 2008; Kaelin and McKnight, 2013; Semenza, 2011). Cells that survive the barrier presented by the liver microenvironment through CKB expression are therefore selected for aggressive metastatic progression.

**CKB during metastatic progression**

While the role of CKB during metastatic initiation in the liver was investigated in the present thesis, CKB could also play a role during metastatic progression after macro-metastases have been established. During metastatic progression, an intracellular pool of phosphocreatine that functions as a readily accessible source of ATP through CKB can provide an energetic buffer for when ATP generated through glycolytic pathways are insufficient for the cells' energetic needs. Such stresses could occur when the growing metastases outstrip blood supply or when challenged by cytotoxic drugs that deplete intracellular ATP levels of cells. When cancer cells are challenged with such an acute stress that depletes intracellular ATP, additional ATP can be generated from phosphocreatine rapidly as a buffer until an adaptive cellular response.

Intracellular ATP levels are tightly regulated even as the energetic needs of cancer cells are tightly balanced against a requirement for biosynthetic materials through modulation of glycolysis (Locasale and Cantley, 2011). Intracellular ATP can function as an allosteric inhibitor of glycolytic process through inhibition of fructose-6-phosphokinase. CKB expression can thus allow for diversion of excess

ATP towards phosphocreatine, allowing glycolysis to continue for generation of biosynthetic substrates required during metastatic progression.

With the possibility that CKB could be beneficial during the later stages of metastatic progression, it would be interesting to investigate the effect inhibiting or depleting CKB has on metastatic progression after macro-metastases have been established. A recent study had also demonstrated increased intracellular ATP levels to be important for enhanced drug resistance in colon cancer cells (Zhou et al., 2012). The effect of CKB inhibition and decreasing intracellular ATP and phosphocreatine levels on potentiating the effects of cytotoxic drugs that stress cancer cells energetically could also be investigated as a possible therapeutic strategy against metastatic progression.

**Functional consequences of extracellular catalysis by CKB**

CKB protein levels or enzymatic activity as a serum marker for malignancies have been described in early clinical studies (Huddleston et al., 2005; Rubery et al., 1982). The presence of CKB in the serum was attributed to inconsequential release by damaged and dying cells during malignant progression. However, functional studies described in the current thesis demonstrated that extracellular CKB released by colon cancer cells within the liver microenvironment could perform its enzymatic function using substrates available extracellularly to generate phosphocreatine to enhance colon cancer metastasis. In addition to phosphocreatine, a product of CKB catalysis is ADP, which is generated from ATP.

While the functional aspects of ATP conversion to ADP by CKB was not investigated in the present thesis, numerous studies by other investigators have demonstrated the relevance of purinergic signaling to cancer progression (Di Virgilio, 2012; Stagg and Smyth, 2010). The presence of ATP in tumor microenvironment has been demonstrated to initiate innate immune responses that can be suppressive to tumor growth (Aymeric et al., 2010). However, conversion of ATP to ADP, and finally adenosine by a series of membrane-bound ectonucleotidase can result in an immune-suppressive effect that in turn could be conducive to tumor progression (Deaglio et al., 2007; Jin et al., 2010). ADP generation as a result of CKB catalysis could therefore be a source of adenosine that can mediate immune-suppressive effects. As xenograft models of colon cancer progression in immunodeficient mice were use in the current study, the effects of CKB-mediated purinergic signaling through extracellular ADP and adenosine production were not investigated. However, future studies in immunocompetent mouse models will be useful for dissecting the contribution of CKB towards immune evasion by colon cancer cells through modulation of purinergic signaling.

**An expanded functional space for intracellular enzymes**

The secretome of cancer cells have been the subject of many investigations (Paltridge et al., 2013). Unbiased proteomic studies of extracellular proteins from cancer cells have reported the presence of multiple intracellular enzymes with no known secretory routes and consequently have attributed the presence of intracellular enzymes as artifacts arising from cellular damage and death and not further investigated. Similarly, prior to the work described in this thesis, the

presence of CKB in the serum of cancer patients was considered a consequence of dying cells with no functional role during cancer progression.

The identification of CKB as an intracellular enzyme that also plays an extracellular catalytic function therefore expanded the functional space of canonical intracellular enzymes, as there could be other intracellular enzymes that may also play an extracellular role during cancer progression. Of particular interest are enzymes involved in different enzymatic reactions of glycolytic pathways, as metabolites arising from glycolytic pathways are abundant in the immediate milieu of primary nodules or metastases. Lactate dehydrogenase is a prominent intracellular enzyme responsible for the reversible conversion of pyruvate to lactate. Similar to CKB, high expression of lactate dehydrogenase has been associated with poor prognosis in cancer patients and the presence of lactate dehydrogenase have been described in literature as a marker of cell death during tumor progression. It is well established that extracellular lactate is abundant in the extracellular space of malignancies. It is therefore possible that extracellular lactate dehydrogenase could utilize exogenous lactate to generate pyruvate that could in turn be beneficial to cancer progression. Interestingly, early studies in rabbits have described physiological release and activity of lactate dehydrogenase in the oviducts (Georgiev et al., 1970) and postulated an extracellular role for lactate dehydrogenase during early embryogenesis when substrates for growth are limited, paralleling the context of early metastatic growth. It would therefore be of biological interest to investigate if extracellular lactate dehydrogenase has a functional role during metastasis.

In addition to lactate dehydrogenase, another key glycolytic enzyme, pyruvate kinase M2, has also been described to be present in the serum and plasma of cancer patients (Hugo et al., 1999) and have recently been reported to have functional effects on tumor progression through modulation of angiogenesis (Li et al., 2014). While the mechanistic pathway through which extracellular pyruvate kinase M2 promotes angiogenesis remains to be identified, the above recent study is yet another example of how intracellular enzymes can have non-canonical roles extracellularly.

With an expanded functional space for intracellular enzymes, there is a resultant expansion of the therapeutic window against intracellular enzymes. Therapeutic targeting of key intracellular enzymes using membrane-permeable inhibitors in diseases had been limited by accompanying toxicity in normal tissues. The expansion of therapeutic space as a result of extracellular roles played by intracellular enzymes will allow for the design of non-membrane permeable inhibitors that can target extracellular enzymes released by cancer cells while avoiding the deleterious effects of inhibiting their intracellular counterparts in normal tissue. The use of neutralizing antibodies against these extracellular enzymes is also a potential avenue for highly specific inhibition of these enzymes.

**Extracellular release of intracellular enzymes**

An outstanding question remains with regards to how CKB and potentially, other intracellular enzymes with no known routes of secretion are released by cancer cells. Preliminary experimental evidence presented in this thesis indicated that CKB secretion is unlikely to occur through known canonical secretory

pathways involving N-terminal signal peptides, nor through exosomes. The mechanisms by which intracellular enzymes such as CKB are released into the extracellular space by cancer cells remain to be elucidated.

In recent years, a new class of vesicles known as ectosomes has been described and is gaining interests among investigators (Cocucci and Meldolesi, 2015). Ectosomes are variable-sized vesicles that bud directly off the plasma membranes of cells and can release their contents into the extracellular space. They are also able to persist intact before fusion with the plasma membranes of other cells, releasing their contents into cells, which results in the intercellular transfer of proteins and metabolites into the recipient cell. Immunohistochemical staining of archival tissues described in this thesis revealed the presence of CKB in the vicinity of plasma membranes of cancer cells in addition to the cytosolic space. The possible release of CKB through ectosomal budding of vesicles could be investigated in further studies. Regardless of the mechanisms responsible for the extracellular release of CKB and other intracellular enzymes, identifying the cell biological pathways involved may also have potential therapeutic benefits as inhibition of CKB release could also suppress metastatic progression. The identification of additional intracellular enzymes with malignant extracellular functions will also expand the therapeutic space for metastatic cancer.

**Therapeutic targeting of the miR-483-5/miR-551a/CKB/SLC6a8 functional axis**

Multiple proof-of-principle therapeutic experiments presented in the current thesis demonstrated the utility of targeting the miRNAs/CKB/SLC6a8 functional axis in preventing the establishment of colon cancer metastases.

Adeno-associated viral delivery of genes has in recent years been shown to be safe and promising in the treatment of various human diseases such as hemophilia, having successfully undergone clinical trials with minimal side effects (Kotterman and Schaffer, 2014; Nathwani et al., 2011). Therapeutic delivery of microRNAs in mouse models of hepatocellular carcinoma had also proved to be efficacious (Kota et al., 2009). Adeno-associated viral vectors of different serotypes exhibit tropisms for different organs, with most serotypes showing some degree of affinity for hepatic tissue. A recently developed, recombinant serotype, AAV-DJ was shown to have increased efficiency in transducing hepatocytes compared to wild-type virus, and also displayed increased infectivity towards multiple other cell-types *in vitro* (Grimm et al., 2008). The availability of this recombinant adeno-associated virus allowed for testing of the utility of therapeutic microRNA delivery to colon cancer cells *in vivo* in the liver. In proof-of-concept experiments, a single dose of recombinant AAV-DJ viral particles was able to therapeutically deliver miR-483-5p and miR-551a to colon cancer cells in the liver and suppressed the formation of colon cancer metastases with no noticeable side effects in treated mice. While the experiments demonstrated that delivery of microRNAs early after cancer cell dissemination can result in suppression of metastases, a caveat remains that continuous dissemination of colon cancer cells from remnant primary tumors can negate the efficacy of single-dose administration of adeno-associated viruses. This concern can be addressed using xenograft orthotopic models of colon cancer and treatment of mice with multiple doses of adeno-associated viruses after surgical resection of the primary tumor. While there were no observable side

effects in mice treated with a single dose of adeno-associated virus, the effects of multiple administrations of adeno-associated viruses delivering the microRNAs should also be investigated to ensure there are no deleterious side effects from continued overexpression of either miR-483-5p or miR-551a in normal tissues.

As an alternative to delivery of microRNAs by adeno-associated viruses, therapeutic inhibition of CKB was demonstrated in the current work. The modest effect seen could be a result of the low potency of the available inhibitor, cyclocreatine. Improvement of the inhibitor through crystal structure guided design might improve the therapeutic effect. While it is possible that normal tissues with lower energetic needs are less susceptible to CKB and creatine kinases inhibition, the effects of more potent inhibitors or longer durations of inhibitor administration have to be thoroughly investigated. The extracellular role of CKB during metastatic progression also allows for the design of non-membrane permeable inhibitors that can potentially have a lower risk of undesirable side effects.

Another targetable aspect of the CKB/SLC6a8 axis is the SLC6a8 membrane transporter downstream of the extracellular catalytic reaction performed by CKB. As preliminary experiments using a small molecule inhibitor of SLC6a8 demonstrated that inhibition of SLC6a8 was also able to potently suppress liver metastases, designing of more potent small molecule inhibitors targeting SLC6a8 would provide additional avenues for targeting this pro-metastatic pathway in colorectal cancer. Recently, the use of antibodies to block activity of membrane transporters and channels have been investigated and shown to be effective in

inhibiting transporters and channels' activities by preventing conformational changes in these proteins after binding to specific epitopes on the surface of the membrane protein (Fiorio Pla et al., 2012). The development of neutralizing antibodies targeting SLC6a8 can therefore provide an alternative and highly specific therapy against metastatic colorectal cancer.

**Relevance of CKB and SLC6a8 to metastatic progression in other gastrointestinal cancers**

Liver metastasis is a common occurrence during progression of gastrointestinal cancers such as pancreatic and gastric cancers as these cancer cells can be disseminated via similar hematogenous routes involving the portal circulation. These gastrointestinal cancer cells will encounter the same hypoxic and energetic stress in the liver microenvironment as colon cancer cells. The results from this study arising from depletion of CKB and SLC6a8 in pancreatic cancer cells indicated that in addition to colon cancer, CKB and SLC6a8 might also be important for liver metastatic colonization by other gastrointestinal cancers, warranting further investigation in mouse models of pancreatic and gastric cancer. In particular, because of the availability of genetic mouse models of pancreatic cancer that could accurately mimic cancer progression in human patients, including the development of liver metastases, conditional knockout of CKB and SLC6a8 in these genetic mice models could further confirm the importance of CKB and SLC6a8 in gastrointestinal cancer progression. Therapeutic targeting of these effector proteins in pancreatic and gastrointestinal cancers through small molecule or antibody inhibition could therefore prevent metastatic progression in patients with other gastrointestinal cancers.

**Conclusion**

The present thesis describes the use of two unbiased complementary approaches to identify regulators of colon cancer liver metastasis. Mir-483-5p and miR-551a were identified as suppressors of liver metastasis. CKB, a promoter of liver metastases was identified as a direct downstream target and effector of both microRNAs. CKB promoted liver metastases through modulation of intra- and extracellular energetics. In addition to regulating intracellular levels of ATP, it can be released by cancer cells to scavenge high-energy phosphate metabolites from exogenous sources of creatine and ATP. SLC6a8, a membrane transporter protein responsible for transport of creatine compounds, was found to be a critical effector downstream of the extracellular effects of CKB. The microRNAs, CKB and SLC6a8 were found to be clinicopathologically associated with metastatic progression of colon cancer patients and proof-of-principle therapeutic targeting of the pathway identified herein demonstrated therapeutic efficacy. This thesis revealed molecular mechanisms underlying liver metastases by colon cancer, and possibly other gastrointestinal cancers and may provide insights for future development of therapeutics for the treatment of metastatic gastrointestinal cancers.

## Materials and Methods

**Animal studies**

All animal work was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at The Rockefeller University. 5-6 weeks old age-matched male NOD-SCID mice were used for organotypic slice culture, intrahepatic colonization, liver metastasis assay and primary tumor growth assays involving LS174T, SW620, WiDR, LvM3a and LvM3b cell-lines. 5-6 weeks old age-matched male NOD/SCID gamma male mice were used for liver metastasis assays for the SW480 and PANC1 cell-lines. 5-6 weeks old age-matched male athymic nude mice were used for experiments involving BEAS-2B mice. For all experiments involving anesthesia and surgery, mice were monitored after surgery to ensure recovery from anesthesia before returning to clean cages. Breeding pairs of all mice strains were originally obtained from Jackson Laboratories and bred in house to establish colonies for experiments, with supplemental purchase from Jackson Laboratories when necessary.

***In vivo* selection**

1 x $10^6$ LS174T cells expressing a luciferase reporter were suspended in a 20ul volume of 1:1 PBS/Matrigel mixture and injected directly into the livers mice (described in detail below). Colon cancer liver nodules were allowed to develop over a period of 3-4 weeks and monitored by bioluminescence imaging. Nodules formed were excised and dissociated by collagenase and hyaluronidase digestion (described in detail below) into single cell suspensions. The cells were allowed to expand *in vitro* before re-injection into mice. After three iterations of *in vivo*

selection, highly metastatic LvM3a and LvM3b derivative cell-lines were established from nodules obtained from independent mice.

**Intrahepatic injection for *in vivo* selection and liver colonization**

Each mouse was first anesthetized with injection of ketamine/xylazine solution into the peritoneal cavity. When the mouse was deeply anesthetized (confirmed by lack of reflex response after pinching of hind legs), the fur above the abdomen wall was shaved and the shaved abdomen wall scrubbed with Betadine and 70% alcohol. A 15mm incision was made through the skin and peritoneum just below the sternum of the mouse to expose the liver. The left lobe of the liver was gently pulled out and stabilized with a pair of forceps and cells in a 20μL volume of 1:1 PBS/Matrigel mixture were injected slowly using a 28-gauge needle attached to a 1/2cc insulin syringe (Becton Dickinson). Blanching of the liver at the site of injection without reflux of injected cells indicated a successful injection. The needle was retracted slowly and a Q-tip placed over the site of injection with gentle pressure for about 30 seconds to prevent bleeding and spillage of injected cells. The left lobe was then returned to its original location and the peritoneum of the mouse closed with surgical 6-0 sutures (Roboz) and the skin closed with 9mm wound clips (Roboz).

**Intrasplenic injection for liver metastasis assays and organotypic slice culture**

Each mouse was first anesthetized as described above. The left flank of the anesthetized mouse was shaved and scrubbed with Betadine and 70% alcohol. A 10mm incision was then made in the skin and peritoneum just below the ribcage

of the mouse to expose the spleen. The spleen was gently exteriorized with a pair of forceps and stabilized. 5 x $10^5$ Cells in 50μL volume of PBS were injected slowly using a 28-gauge needle attached to a 1/2cc insulin syringe. A blanching of the spleen without reflux of injected cells indicated a successful injection. The needle was retracted slowly and a Q-tip placed over the site of injection with gentle pressure to prevent bleeding and leakage of the injected cells. After 30 seconds, the spleen was removed using a cautery and the peritoneum of the mouse closed with surgical 6-0 sutures (Roboz) and the skin closed with 9mm wound clips (Roboz).

**Retro-orbital injection of luciferin and DEVD-luciferin for bioluminescent imaging**

Each mouse was anesthetized using an isoflurane anesthesia chamber. After anesthesia, the mouse was placed its left flank and restrained using the thumb and index finger of the non-dominant hand. At the same time, the index finger and thumb was used to draw back the skin below the right eye of the mouse. 100μL luciferin substrate (Perkin Elmer) was then injected using a 28-gauge insulin needle on a 1cc syringe into the retrobulber sinus of the mouse. For *in vivo* caspase activity bioluminescent imaging, 100μL of amino-DEVD-luciferin substrate (15mg/mL) (Promega) was injected for bioluminescent imaging. After imaging with DEVD-luciferin, regular luciferin substrate (15mg/mL) was injected and imaging performed to obtain a normalization signal. The needle was then retracted slowly and the anesthetized mouse can be placed into the IVIS imaging system for bioluminescent imaging. Mice are imaged with their

abdominal facing up, and 30s after injection of luciferin. Images are taken with exposure times ranging from 5s to 5min dependent on metastatic burden to avoid saturation of CCD camera sensor, leading to inaccurate measurements.

**Tail vein injection for delivery of adeno-associated viral particles**

Intravenous tail vein injection was used for delivery of adeno-associated viral particles. Mice inoculated with colon cancer cells were randomized for treatment. Each mouse was restrained using a restrainer (Braintree Scientific) designed for tail vein injection. The tail of the moue were then gently warmed in 37 degree Celsius water, and wiped with 70% alcohol. 1 x $10^{12}$ purified adeno-associated viral particles in 150μL of PBS were then injected into the lateral tail vein of the mouse using a 27 1/2 -gauge needle attached to a 1cc syringe. A paling of the vein and noticeable delivery of PBS up the tail indicated a successful injection. The needle was retracted slowly and a kim-wipe was used to exert gentle pressure on the site of injection to stop the bleeding. The mouse was released after 30 seconds.

**Subcutaneous injections for primary tumor growth assays**

Each mouse was first anesthetized as described above. 1 x $10^6$ cells were suspended in 100μl of 1:1 PBS:Matrigel mixture and injected into the subcutaneous flanks of the anesthetized mouse using a 27-gauge needle on a 1-cc syringe. Tumor growth was measured using digital calipers starting 7 days after injection. Each mouse was anesthetized using an isoflurane anesthesia chamber.

Palpable tumors were then grasped and measured. Volume of the tumors were calculated using the formula, Volume = $(\text{width})^2$ x (length)/2.

**Liver extraction and tumor nodule extraction**

Each mouse was first deeply anesthetized with a lethal dose of ketamine/xylazine solution. After confirmation of anesthesia, the fur above the abdomen wall of the mouse was shaved and the abdomen scrubbed with betadine and 70% ethanol. A 30mm incision was then made and the liver exteriorized. The liver of the mouse was then cut free of the abdominal cavity and washed gently with PBS. It could then be used for downstream experiments. If required, metastatic nodules in the liver were excised from the liver, washed with PBS and used for downstream experiments.

**Tumor nodule dissociation into cells for culture**

Each excised tumor nodule was first washed twice in PBS supplemented with penicillin-streptomycin, gentamicin and amphotericin B antibiotics. After washing, the tumor nodule was minced up as finely as possible with a pair no. 10 surgical scalpels and re-suspended in 15mL of antibiotics-supplemented PBS. The minced tumor nodule was then collected by centrifugation at 800g for 5min. The PBS was removed and the nodule re-suspended in ACK buffer (Lonza) for lysis of residual red blood cells. After 10min incubation at room temperature, the minced nodule was collected by centrifugation and re-suspended in enzymatic digestion media (300u/mL Collagenase, 1u/mL Dispase, 0.25mg/mL Hyaluronidase, 24u/mL DNAseI; Worthington Biochemicals) and incubated with gentle agitation for 2hrs at 37 degree Celsius. After enzymatic digestion,

cells were collected by centrifugation and incubated in 5mL trypsin-EDTA for 10min at 37 degree Celsius. After trypsin digestion, cells were collected again by centrifugation, re-suspended in cell culture media, and filtered successively through 70μm and 40μm cell strainers to remove undigested debris and plated onto cell culture plates. Freshly plated cells were monitored daily for contamination and tested for mycoplasma contamination before transition into routine culture.

**Tumor nodule homogenization for western-blot**

Tumor nodules up to 125mm$^3$ in size were excised and washed in PBS before immersion in 3mL of chilled RIPA buffer (Amersham) with protease inhibitors (Roche) on ice. A hand-held rotor-stator homogenizer was used to homogenize the tumor. After thorough homogenization, the mixture was incubated on ice for 30min, before centrifugation at 15,000g for 10min to clear the supernatant. The supernatant was then used for western-blot after quantification with BCA kit (Pierce).

**Serum collection from mice**

Each mouse was first deeply anesthetized with a lethal dose of ketamine/xylazine solution. After anesthesia, the chest of the mouse was shaved and scrubbed with betadine and 70% ethanol. The chest cavity of the mouse was then quickly cut open and the heart exposed. Whole blood was collected from the mouse via cardiac puncture using a 27-gauge needle attached to a 1cc syringe. Up to 500μL of blood can be collected. After collection, the blood was allowed to

clot for 30min at room temperature before centrifugation to separate the serum from the blood clot. Collected serum was then stored at -20 degree Celsius.

**Cyclocreatine treatment of mice**

One day after inoculation of colon cancer cells into mice, injected mice was randomized and treated mice were injected with 10mg of cyclocreatine in 350μL of PBS via intra-peritoneal injection. Control mice received 350μL PBS placebo injections. Treatment was performed daily for 2 weeks until the mice were euthanized.

**Guanidinoproprionic acid treatment of mice**

One day after inoculation of colon cancer cells into mice, injected mice was randomized and treated with either 200μL PBS or 0.5M GPA in 200μL PBS daily for three weeks. The treatment was delivered via intra-peritoneal injections.

**Implantation of osmotic pumps**

Each osmotic pump (Alzet) was loaded with PBS or 1M-phosphocreatine solution according to manufacturer's protocol. Briefly, for each pump, a blunt-end loading tip was attached to a 1cc syringe and solution to be loaded was slowly injected into the reservoir of each pump using the syringe. After loading, the pump was capped and primed overnight by incubation in a warm saline solution at 37 degree Celsius. On the day of surgery, osmotic pumps were inserted into the peritoneal cavity of anesthetized mice. Depleted osmotic pumps were removed 7 days after implantation to prevent discomfort to mice.

**Organotypic slice culture**

Cells were passaged such that they were 60% confluent at the start of the assay. On the day of slice culture preparation, cells were labeled by addition of either cell tracker red or green (Life Technologies) for 45min at 37°C. During incubation, tissue culture inserts (30mm, 0.4um; Millipore) was placed in 35mm tissue culture dishes and 1.1mL of liver media (Williams media E with hepatic culture supplement pack; Gibco) was added. The media from cell tracker labeled cells was then removed and replaced with fresh culture media and the cells were incubated for 30min at 37°C to ensure proper labeling. Cells were then prepared collected by trypsin digest from the cell culture plates and inoculated into mice by intrasplenic injection. After inoculation, mice were euthanized and liver excised as described above. Excised livers were washed gently with PBS supplemented with antibiotics and chopped into 150um slices using a tissue chopper (McIlwan). The slices were washed with antibiotics-supplemented PBS prior to transfer onto the tissue culture inserts using sterile transfer pipettes. Seeding of cells into within liver slices were confirmed by fluorescent light microscopy. Liver slices were cultured and media replaced daily until fixation with 4% paraformaldehyde for two-photon microscopy.

**Cell culture**

The 293T, LS174T, SW480, SW620, WiDR, PANC1 and BEAS-2B cell-lines were purchased from ATCC. 293T, LS174T, WiDR and PANC1 cells were cultured in DMEM media (Life Technologies) supplemented with 10% FBS (Sigma-Aldrich), sodium pyruvate (Life Technologies), L-glutamine (Life Technologies), amphotericin B (Lonza) and penicillin-streptomycin (Life Technologies). SW480

and SW620 cells were cultured in McCoy's 5A media (Life Technologies) supplemented with the above cell-culture supplements. BEAS-2B were cultured in BEGM media (Lonza) which was supplemented with pre-aliquoted BEGM bullet kit (Lonza) with Hydrocortisone, hEGF, Epinephrine, Transferrin, Insulin, Retinoic Acid, Triiodothyronine and Gentamicin. For phosphocreatine pre-treatment, cells were treated with 10mM phosphocreatine for 24hrs in media supplemented with 0.2% FBS prior to experiments. For cyclocreatine pre-treatment, cells were treated with 10mM cyclocreatine for 48hrs. For hypoxic cell cultures, cells were cultured in 1% oxygen within a modular hypoxia chamber (Billups-Rothenberg).

**Generation of lentivirus, retrovirus, knock-down and over-expressing cells.** For generation of lentivirus delivering shRNAs or over-expression vectors, 293T cells were seeded onto 10cm plates such that cell confluency will be approximately 70% the next day. 3μg each of pRSV-Rev, pCMV-VSVG-G and pCgpV packaging vectors (Cell Biolabs) were co-transfected with 9μg the appropriate pLKO-shRNA or pLenti-overexpression plasmids using 45μl of Lipofectamine 2000 in antibiotic-free media. After 16hrs, the media was replaced with fresh antibiotic-free media. After 24hrs, virus-containing supernatant was collected and centrifuged for 10min at 800g before being filtered through a 0.45μm filter. For generation of over-expression retrovirus, 293T cells were seeded as described above. On the day of transfection, 12μg of the appropriate over-expression vector was co-transfected with 12μg of Pol/Gag and 6μg of VSVG packaging vectors (Gift of Jiang Lab, MSKCC). Media was replaced after

16hrs. 48hrs after transfection, virus-containing supernatant was harvested and spun for 10min at 800g before being filtered through 0.45μm filter. For transduction of cells, 2mL of the appropriate virus was used to transduce 1 x $10^5$ cells in the presence of 8μg/mL polybrene. Media was replaced 24hrs later. 48hrs after transduction, antibiotic selection was performed with either blasticidin (10-15μg/mL) or puromycin (2-4μg/mL) for 2-7 days alongside a population of untransduced control cells. Selection is deemed completed after untransduced control cells were killed by antibiotic selection. After selection, cells were allowed to recover in selective antibiotic free media for 72hrs and tested for over-expression or knockdown of gene of interest by quantitative PCR and Western-blot where applicable.

**Preparation of adeno-associated virus for *in vivo* experiments**

Adeno-associated viruses was generated by transfection of 30 x 15cm plates of 70% confluent 293T cells with 10μg each of pHelper (Cell Biolabs), DJ-Packaging (Cell Biolabs), scAAV-miR or scAAV control vector. 16hrs after transfection, the media was replaced. 60hrs after transfection, cells were sloughed off by gentle pipetting and pelleted by centrifugation. To release viral particles from cells, cell pellets were subjected to three cycles of freeze thaw, alternating between a 37 degree Celsius water-bath and a dry-ice ethanol bath. After release of viral particles, adeno-associated viral particles were purified using Virabind AAV purification kit (Cell Biolabs) according to manufacturer's protocol. Non-viral nucleic acids were first digested by incubation of viral supernatant reagent A. Viral particles were then captured by affinity beads (reagent B), and loaded onto wash columns.

After washing, viral particles were eluted and concentrated using 100kD spin columns. Concentrated viral particles were titered using AAV quantification kit (Cell Biolabs) according to manufacturer's protocol. Viral particles were first denatured to released viral genomic DNA and viral titer was using the provided viral DNA standard and fluorometric DNA binding dye. After quantification, viral particles were aliquoted and stored at -80 degree Celsius before use. For larger preparation of adeno-associated viral particles, Vector Biolabs provided viral preparation services.

**Lenti-miR microRNA library screen**

1 x $10^6$ colon cancer cells were seeded 16hrs prior to the start of experiment. On the day of transduction, purified lenti-miR pooled-library viral particles (System Biosciences) were used to transduce the seeded colon cancer cells at a low multiplicity-of-infection (0.1-1) in the presence of polybrene (8μg/mL) such that each individual cell was likely to be transduced by only a single lentiviral particle. 48hrs after transduction, transduced cell populations were purified via flow cytometry and allowed to recover and expand *in vitro*. Once sufficient cell numbers were available (>5 x $10^6$), a portion of the cells was used for intrahepatic injection for liver colonization dropout screen. Genomic DNA was extracted from the unused population (reference population) of cells using the Qiagen DNeasy kit according to manufacturer's protocol (genomic DNA extraction from cells). 4-6 weeks after injection, mice were euthanized, liver nodules were harvested and genomic DNA extracted (using protocol for tissue samples). Lentiviral inserts, with microRNAs precursors sequences were amplified via

PCR using library-specific primers provided by System Biosciences and gel-purified using Qiagen Gel Purification kit. Genosensor Corporation provided microarray preparation and profiling services for quantification of library derived microRNA precursors inserts. The raw signal intensities for probe were median-normalized. For each cell-line used, biological replicates were performed and averaged, with independent library transductions and injection into three mice each for each independent transduction.

**LNA mediated inhibition of microRNAs**

Non-targeting control LNA and LNAs targeting miR-483-5p and miR-551a were purchased from Exiqon. 10μL of 100μM LNA were transfected into 70% confluent cells grown in 15cm cell culture plates using 60μL of Lipofectamine 2000 reagent (Life Technologies) in antibiotic free culture media. LNA and Lipofectamine 2000 reagent were pre-diluted in separate aliquots of 1.5mL of Opti-MEM media (Life Technologies) and incubated at room temperature for 5min. After incubation, both reagents were mixed gently by pipetting and incubated at room temperature for 20min. After incubation, the transfection mix was added drop-wise to cells to be transfected and the cell culture plate was swirled gently to ensure even distribution of transfection reagents. Transfection media was removed after 16hrs and replaced with fresh media. 48hrs after transfection, transfected cells were used to perform respective assays.

**Illumina expression beads hybridization and transcriptomic analysis**

To identify transcripts that were regulated by the microRNAs, total RNA was extracted from control cell populations and cells over-expressing either microRNAs using the miRVANA kit according to manufacturer's protocol (Life Technologies). The RNA was labeled and hybridized onto Illumina HT-12v3 Expression BeadChip arrays by The Rockefeller University genomics core facility. The raw signal intensities were median-normalized. Common putative targets of the microRNAs were identified if both microRNAs down-regulate transcript levels at least 1.3x fold and confirmed with quantitative real-time PCR validation. Putative target genes were further validated to be potential direct targets and effectors with dual-luciferase assay and liver metastasis assays.

**Luciferase reporter assay**

The luciferase reporter assay was performed using a Dual-Luciferase Reporter Assay Kit (Promega). The full-length 3'-UTR and CDS of CKB were cloned into the siCheck2 dual luciferase reporter vector. 2.5 x $10^4$ LS174T cells were seeded in quadruplicates for each condition onto 24-well plates and allowed to attach overnight before transfection. Cells were co-transfected with either a control LNA or LNA targeting miR-483-5p or miR-551a and 100ng of the respective siCheck2 dual luciferase reporter vector. 30hrs after transfection, luciferase activity was determined using the dual-luciferase assay kit. Cells in each well were first washed with gently with 1X PBS and lysed in 100μL of 1X Passive Lysis Buffer, with gentle rocking for 30min at room temperature. The cell lysates were then collected and spun briefly to clear the cell lysate solutions. 30μL of the

cleared solution was then transferred into white opaque 96-well plates. 50μL of LARII Reagent was then added to each well and mixed gently by pipetting. The bioluminescent signal was read using a microtiter plate reader (Perkin Elmer Envision). After the first reading, 50μL of Stop and Glo Reagent was added to each well, mixed gently and the resulting bioluminescent signal was read again.

**Cell proliferation and hypoxia survival assay**

For cell proliferation assays, 1 x $10^5$ cells were seeded onto 6-well plates (Falcon) in triplicates. Cells were kept in culture for 5 days before collection through trypsin digestion and counted using the Cellometer cell-counting machine (Nexcelom). Experiments were repeated at least three times. For *in vitro* hypoxia assays, 5 x $10^4$ cells were seeded in 24-wells plate and were cultured in 1% oxygen in DMEM with 5.6mM glucose for 4 days before counting. Cell counts were normalized to that of control conditions. For experiments with 10mM phosphocreatine supplementation, 1 x $10^5$ cells were seeded in triplicates in 6-well plates and cultured in 1% $O_2$ for 4 days before cell counting. For boyden chambers co-culture experiments, 5 x $10^4$ cells were seeded on top of transwell insert and 5 x $10^4$ cells were seeded at the bottom of the well of the 24 well-plate. Cells were cultured in 1% $O_2$ for 4 days before counting.

**Collection of cell culture supernatant**

7.5 x $10^6$ cells were seeded onto 15cm cell culture dishes. After allowing cells to attached overnight, cells were washed gently four times with PBS and routine culture media was replaced with 12mL serum free media and cell cultured for an

additional 24hrs. Supernatant was collected, and centrifuged for 800g for 10min to remove debris. Subsequently, 10mL of the supernatant was concentrated using a spin column with 10kD cut-off filter to approximately 1mL prior to downstream applications. For treatment with brefeldin A, brefeldin A (Biolegend) was added to a final concentration of 5μg/mL in the serum-free media and supernatant was collected after 6hrs. For treatment with dimethyl-amiloride (Sigma-Aldrich), the final concentration was 15nM and supernatant was collected after 16hrs.

**Phosphocreatine and ATP measurements**

Cells were cultured routinely (unless otherwise stated) and harvested at approximately 70% confluence. On day of measurement, 1 x $10^6$ cells here detached from culture dish by trypsin, washed twice in PBS and lysed in 1mL ATP assay buffer (Biovision). After vortexing to ensure homogenization, 200μL of perchloric acid (Biovision) was added to the cell homogenate. Vortexing was performed to ensure complete extraction of proteins, the homogenate was incubated on ice for 5min before centrifugation at 15,000g for 3min. 960μL of the resulting supernatant was withdrawn and 40μL of neutralization buffer (Biovision) was added to the supernatant. The solution was vortexed to ensure mixing, and incubate on ice for 5min before centrifugation at 15,000g for 3min to spin down any residual precipitate. Triplicate aliquots of 100μL of the cleared supernatant were subsequently transferred to a white flat-bottom 96 well microplate. 50μL of Vialight assay buffer (Lonza) was added and the mixture was incubated at room temperature for 10min. After 10min, 100μL of ATP

monitoring reagent (Lonza) was added and a bioluminescent reading was performed using a microplate reader (Perkin Elmer Envision). The value of the reading corresponds to the relative ATP levels. Subsequently, 5μL of 100mM ADP (Sigma-Aldrich) and 50μL of reconstituted rabbit creatine phosphokinase (500u/mL) (Sigma-Aldrich) was added and the reaction was allowed to completion (10-15min) before a bioluminescent reading was taken. The increase in bioluminescent signal corresponds to phosphocreatine levels.

**Western-Blot**

Cell lysates were prepared by lysing cells grown on 10cm plates in 1mL of RIPA buffer containing protease inhibitors. Lysate was quantitated using Bio-Rad BCA kit. 40μg protein from cell lysates were separated on a 4-12% SDS-PAGE and transferred to a PVDF membrane. Membrane was blocked for 1hr in 5% milk in PBST (except for FLAG antibody, blocking was performed in 5% BSA in PBST for 1hr). Antibodies were incubated overnight in 2% milk in PBST at 4 degrees with gentle rocking. The CKB antibody was purchased from Abcam (Cat. 38212, 1:400 dilution). CKB neutralizing antibody was purchased from Abcam (Cat. 48651, 1:10). (GAPDH antibody was purchased from Genetex (Cat. GTX627408, 1:5000 dilution). The FLAG antibody was purchased from Sigma (Cat. F3165, 1:2000 dilution). SLC6a8 antibody was purchased from Abcam (Cat. 62196, 1:1000 dilution). CD39 antibody was purchased from Abcam (Cat. 127167, 1:1000 antibody). Horseradish peroxidase-conjugated secondary antibodies were purchased from GE Health Sciences and used at a dilution of 1:1000, in 2% milk in PBST for 1hr. In between antibody incubation, membranes were washed 3X in

PBS, 15mins per wash. Chemiluminescent detection of proteins was performed using Pierce ECL plus kit.

**PCR for cloning or genomic DNA amplification**

PCR for cloning or genomic DNA amplification was performed using Phusion enzyme (NEB) according to manufacturer's protocol. Generally 200 to 400ng of starting template were used. PCR products were visualized using gel electrophoresis, excised and purified using Qiagen gel-extraction kit. Restriction digest was performed NEB restriction enzymes, at 37 degree Celsius for 6hrs and ligation into appropriate vector performed using NEB T4 ligase, at 16 degree Celsius overnight.

**Real-time PCR from archival RNA samples**

For microRNA, cDNA synthesis was performed with 50ng of total RNA using the Universal cDNA synthesis kit II (Exiqon, MA) according to protocol. qRT-PCR LNA primers for miR-551a, miR-483-5p and SNORD44 endogenous control was purchased from Exiqon and real-time PCR was performed with SYBR-green master mix (Life Technologies, CT). For mRNA, cDNA synthesis was performed with 200ng of total RNA using the Superscript III cDNA synthesis kit (Life Technologies according to protocol using oligo-dT primers.

**Analysis of publicly available microarray data**

Microarray data from GSE41258 was used to analyze mRNA expression of CKB and SLC6A8 from unmatched primary tumor samples and liver metastasis samples. Expression was compared using one-sided Mann Whitney U test.

**Analysis of tissue microarray**

Staining of tissue microarray was performed by New York Presbyterian hospital histopathology lab. Each core on the tissue microarray is given a score of 0-3 based on intensity of staining. Scores of 0 and 1 are interpreted as negative for protein expression while scores of 2 and 3 are interpreted as positive staining for each core. A tumor sample is considered positive if at least 2 cores (out of 3 replicate cores) showed positive staining, and is considered negative if cores showed 0 and 1 staining intensities, or if only 1 core (out of 3 replicate cores) showed staining intensity of 2-3. For each positive sample, the area (% tumor) corresponding to each staining intensity was recorded to allow for calculation of percentage tumor positivity and a weighted overall staining score (H-score) is calculated as (percentage area of 2+ staining x 2) + (percentage area of 3+ staining x 3). Individual cores were excluded from the analysis if no tumor was present, tumor was predominantly necrotic or falling off the slide or if tumor was of signet ring cell morphology due to rarity. A patient sample was not included in the analysis if more then one core (out of 3 replicate cores) was excluded for reasons above or if only two replicate cores were available and they showed discordant results (i.e. one core scored positive and one core score negative).

**Table M.1 | List of Primers used**

| | |
|---|---|
| **miR-551a O/E Fwd** | CGCATAGGATCCGGAGAACCTTCAGCTTCA TGTGAC |
| **miR-551a O/E Rev** | CGCATAGAATTCGGTCCCTTCCCAGGCTTGG GGGAG |
| **miR-483-5p O/E Fwd** | CGCATAGGATCCCCTGCCCCATTTGGGGGT AGGAAG |
| **miR-483-5p O/E Rev** | CGCATAGAATTCGCTGCCTCCTTCCTCAGAT GAAAAATGGGC |
| **miR-551a AAV Fwd** | CGCATAAGATCTGGAGAACCTTCAGCTTCA TGTGAC |
| **miR-551a AAV Rev** | CGCATACTCGAGGGTCCCTTCCCAGGCTTG GGGGAG |
| **miR-483-5p AAV Fwd** | CGCATACTCGAGCCTGCCCCATTTGGGGGT AGGAAG |
| **miR-483-5p AAV Rev** | CGCATAGCGGCCGCGCTGCCTCCTTCCTCAG ATGAAAAATGGGC |
| **miR Control Hairpin** | GATCCCCGGTGACTTTCTGTAACAATTTCAA GAGAATTGTTACAGAAAGTCACC |
| **AAV-Fwd** | GCTGACCCTGAAGTTCATCTG |
| **AAV-Rev** | CACCTTGATGCCGTTCTTCT |
| **CKB O/E Fwd** | CGCATAGGATCCATGACGGAGGCGGAGCA GCAGC |
| **CKB O/E Rev** | CGCATAGTCGACTCATTTCTGGGCAGGCAT GAG GTC |
| **CKB qPCR Fwd** | TGAGTTCATGTGGAACCCTCACCT |
| **CKB qPCR Rev** | AACTTCTCATGCTTGCCCAGGTTG |
| **SLC6a8 qPCR Fwd** | GGCAGCTACAACCGCTTCAACA |
| **SLC6a8 qPCR Rev** | CAGGATGGAGAAGACCACGAAG |
| **GAPDH qPCR Fwd** | AGCCACATCGCTCAGACAC |
| **GAPDH qPCR Rev** | GCCCAATACGACCAAATCC |
| **FLAG-DEVD-CKB Fwd** | CGCATAGGATCCATGGATTACAAGGATGAC GACGATAAGCTGGGCGGCACCGGCAGCGG CAGCGGCGACGAGGTGGACGGCCCCTTCTC CAACAGCCACAACGCAC |
| **FLAG-DEVD-CKB Rev** | CGCATAGTCGACTCATTTCTGGGCAGGCAT GAGGTC |
| **IgK-FLAG-CKB Fwd** | CGCATAGGATCCATGGAGACAGACACACTC CTGCTATGGGTACGCTGCTCTGGGTTCCAGG TTCCACTGGTGACGATTACAAGGATGACGA CGATAAGCCCTTCTCCAACAGCCACAACGC AC |
| **CD39 O/E Fwd** | CGCATAGGATCCATGGAAGATACAAAGGA GTCTAACGTGAAG |
| **CD39 O/E Rev** | CGCATAGTCGACTATACCATATCTTTCCAGA AATATGAAGGCTTG |
| **CKB CDS Luciferase Fwd** | CGATGCCTCGAGATGCCCTTCTCCAACAGC |

| | |
|---|---|
| | CACAACG |
| **CKB CDS Luciferase Rev** | GCGGCCGGCGGCCGCTCATTTCTGGGCAGG CATGAGGTC |
| **CKB 3′UTR Luciferase Fwd** | CGATCGCTCGAGAGCCCGGCCCACACCCGA C |
| **CKB 3′UTR Luciferase Rev** | GCGGCCGGCGGCCGCCAGACGCAGGCAGG C CAAAACC |
| **CKB 3′-UTR Mutagenesis Forward** | ATGCACCCCTGATGTTCGCAACATGGCGAG CCCTTAGCCTTG |
| **CKB 3′-UTR Mutagenesis Reverse** | CAAGGCTAAGGGCTCGCCATGTTGCGAACA TCAGGGGTGCAT |
| **CKB CDS Mutagenesis Forward** | GACAATAAGACCTTCCTGGTGTAAATCAAC GAGGAGGACCACCT |
| **CKB CDS Mutagenesis Reverse** | CCTTCTGCATGGAGATGGTTCGCAGGTGGTC CTCCTC |

**Table M.2 | List of shRNA sequences used**

| shRNA | Sequence |
|---|---|
| **shCtrl** | CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTG CTCTTCATCTTGTTGTTTTTG |
| **shCKB #3** | CCGGCCCAGATTGAAACTCTCTTCACTCGAGTGAAGAG AGTTTCAATCTGGGTTTTTG |
| **shCKB #5** | CCGGCCGCGGTATCTGGCACAATGACTCGAGTCATTGT GCCAGATACCGCGGTTTTTTG |
| **shSLC6a8 #1** | CCGGCACGGGAAAGATCGTGTACTTCTCGAGAAGTACA CGATCTTTCCCGTGTTTTTG |
| **shSLC6a8 #2** | CCGGGCTGGTCTACAACAACACCTACTCGAGTAGGTGT TGTTGTAGACCAGCTTTTTG |
| **shSLC6a8 #4** | CCGGCTTATTCCCTACGTCCTGATCCTCGAGGATCAGG ACGTAGGGAATAAGTTTTTG |
| **shSLC6a8 #5** | CCGGATTACCTGGTCAAGTCCTTTACTCGAGTAAAGGA CTTGACCAGGTAATTTTTTG |
| **shGAMT** | CCGGATGGCCATCGCAGCGTCAAAGCTCGAGCTTTGAC GCTGCGATGGCCATTTTTTG |

**Sequences of miR-551a and miR-483-5p with flanking genomic sequence in adeno-associated viral vector**

miR-551a (with flanking genomic sequence) in AAV

GGAGAACCTTCAGCTTCATGTGACCCAGAGACTCCTGTATGCCTGGCTCT
GGGAGTACAGAAGGGCCTAGAGCTGACCCCTGCCCTCCGAAGCCCCTGG
GGCACTAGATGGATGTGTGCCAGAGGGTAGTAGAGGCCTGGGGGTAGAG
CCCAGCACCCCCTTCGCGTAGAGACCTGGGGGACCAGCCAGCCCAGCAA
CCCCCTCGCGGCCGACGCCTGAGGCTGTTCCTGGCTGCTCCGGTGGCTGC
CAGAGGGGACTGCCGGGTGACCCTGGAAATCCAGAGTGGGTGGGGCCA
GTCTGACCGTTTCTAGGCGACCCACTCTTGGTTTCCAGGGTTGCCCTGGAA
ACCACAGATGGGGAGGGGTTGATGGCACCCAGCCTCCCCCAAGCCTGGG
AAGGGACCCC*GGATCC*CCAGAGCCTTTCCCTGCCTATGGAGCGTTTCTCTT
GGAGAACAGGGGGGCCTCTCAGCCCCTCAATGCAAGTTGCTGAG

miR-483-5p (with flanking genomic sequence) in AAV

CCTGCCCCATTTGGGGGTAGGAAGTGGCACTGCAGGGCCTGGTGCCAGC
CAGTCCTTGCCCAGGGAGAAGCTTCCCTGCACCAGGCTTTCCTGAGAGGA
GGGGAGGGCCAAGCCCCCACTTGGGGGACCCCCGTGATGGGGCTCCTGC
TCCCTCCTCCGGCTGATGGCACCTGCCCTTTGGCACCCCAAGGTGGAGCC
CCCAGCGACCTTCCCCTTCCAGCTGAGCATTGCTGTGGGGGAGAGGGGG
AAGACGGGAGGAAAGAAGGGAGTGGTTCCATCACGCCTCCTCACTCCTC
TCCTCCCGTCTTCTCCTCTCCTGCCCTTGTCTCCCTGTCTCAGCAGCTCCAG
GGGTGGTGTGGGCCCCTCCAGCCTCCTAGGTGGTGCCAGGCCAGAGTCC
AAGCTCAGGGACAGCAGTCCCTCCTGTGGGGGCCCCTGAACTGGGCTCA
CATCCCACACATTTTCCAAACCACTCCCATTGTGAGCCTTTGGTCCTGGTG
GTGTCCCTCTGGTTGTGGGACCAAGAGCTTGTGCCCATTTTTCATCTGAGG
AAGGAGGCAGC

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