Gankyrin activates IL-8 to promote hepatic metastasis of colorectal cancer

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Running title: Gankyrin promotes colorectal cancer metastasis

Word count (excluding references): 5,482
Total number of figures: 6
Total number of supplementary tables: 3
Total number of supplementary figures: 5

Conflict of interest: The authors have no conflicts of interest to disclose.

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Abstract

Hepatic metastasis is responsible for the majority of colorectal cancer (CRC)-related mortalities. Although Gankyrin (PSMD10) has been implicated in cancer metastasis, its exact role and underlying mechanisms of CRC hepatic metastasis remain largely unknown. Herein, we demonstrated that the expression of Gankyrin was higher in primary CRC with hepatic metastasis compared to CRC without metastasis. RNAi-mediated silencing of Gankyrin expression in highly metastatic human CRC cells impaired their migratory and metastatic capacity in vivo. Genome-wide transcriptome profiling revealed activation of the IL-8 signaling pathway by Gankyrin. Protein levels of IL-8 and cyclin D1 (CCND1), the two important molecules in the IL-8 pathway, were positively correlated with Gankyrin expression in human CRC specimens. Furthermore, genetic deletion of cyclin D1 demonstrated its requirement in Gankyrin-mediated cell migration. Lastly, administration of recombinant IL-8 rescued the migratory defect of CRC cells where Gankyrin expression was silenced. Together, our findings highlight the importance of Gankyrin in hepatic metastasis of CRC and point out its candidacy as a potential prognostic marker and therapeutic target to improve the clinical management of metastatic CRC.

Key words: Colorectal cancer, Metastasis, Gankyrin, Interleukin 8, Cyclin D1.
Introduction

Gankyrin (also known as PSMD10 or p28gank or nas6) was initially identified as the p28 component, a regulatory subunit of the 26S proteasome complex (1). Gankyrin consists of seven ankyrin repeats, which are required for protein-protein interactions (2-4). The expression of Gankyrin has been investigated in a variety of cancers with nearly universal findings that showed elevated protein and/or mRNA levels in cancers comparing to normal counterparts (4-5). Gankyrin has been shown to be involved in carcinogenesis and oncogene-induced tumor onset and progression (4, 6-10). Overexpression of Gankyrin promotes cellular proliferation by targeting retinoblastoma protein (Rb), leading to subsequent Rb degradation, activation of E2F transcription factor, and DNA synthesis of genes required for G1 to S phase transition (11-13). Overexpression of Gankyrin promotes oncogenic transformation in NIH3T3 cells (4).

Multiple mechanisms by which Gankyrin acts as an oncoprotein have been proposed: i) Gankyrin enhances Rb phosphorylation and inactivation, and dis-engages E2Fs from Rb repression complex (4). E2F, as an oncoprotein, initiates the expression of genes which encode proteins required for cell cycle progression. ii) Gankyrin targets p53 for degradation through ubiquitination-proteasome pathways (14). iii) Gankyrin participates in C/EBP protein degradation. C/EBP is a tumor suppressor in liver, and reduced protein expression of C/EBP is required for carcinogen-induced tumor initiation and progression (15). Thus, Gankyrin acts as oncogene by negatively regulating tumor suppressors through post-translational modification and subsequent degradation of these proteins. iv) Overexpression of Gankyrin prevents nuclear translocation of NF-κB subunit and inhibits the transcriptional activity of NF-κB (2). v)
Gankyrin mediates Ras-induced tumorigenesis by suppressing ROCK activity (16), and vi) Gankyrin enhances PI3K/AKT/HIFalpha signaling pathway in HCC (17-19).

Gankyrin-mediated regulation of cancer metastasis has been proposed, however the mechanisms through which Gankyrin regulates cell migration/invasion and cancer metastasis remain largely unknown. Colorectal cancer (CRC) remains to be one of the most lethal forms of cancers worldwide. In Unite States, the predicted new cases and CRC-related deaths in 2012 is 143,460 and 51,690, respectively. Synchronous hepatic metastases are detected in 25% of all CRC patients and additional 25% CRC patients without distant metastasis at initial diagnosis subsequently develop hepatic metastasis (20). Attempted chemotherapy for hepatic metastatic CRC has been showing a disappointing patient responsiveness rate at only 10% to 40% (21). The efforts for screening for cellular molecules that are responsible for CRC progression in recent years have added several proteins that could be targeted pharmaceutically. Chemokines and the corresponding receptors have been implicated in cancer metastasis. The involvement of chemokines/receptors has been summarized in a comprehensive review article (22). Wnt signaling pathway has a well-established role in CRC progression and metastasis (23). The IL-8 levels in the CRC tissue showed a significant correlation with the micro-vessel density of primary tumor and hepatic metastasis (24). Cyclin D1, a component of both IL-8 and Wnt signaling pathways (25-26), is increased in CRC, and its overexpression predicts poor clinical outcome for metastatic CRC (27). The identification of these signaling molecules has changed the clinical management of CRC patients with hepatic metastasis; however, only modest effectiveness was achieved with these advances. Therefore, there is still an urgent need to develop bio-markers that predict CRC metastasis and to discover novel therapeutic targets for treating hepatic metastasis.
Herein, we demonstrated that Gankyrin could serve as a biomarker for differentiating CRC with hepatic metastasis potential from non-metastatic CRC. Gankyrin was found to be highly expressed in colorectal cancers and nuclear translocation of Gankyrin in the primary tumor correlates with hepatic metastasis. We further showed that Gankyrin is required for cell proliferation, migration and invasion in both *in vitro* and *in vivo* models. Targeting Gankyrin expression impaired tumor growth and metastasis. Lastly, transcriptome regulated by Gankyrin populated signaling pathways responsible for cellular proliferation and migration. Together, these results established Gankyrin as a biomarker that can be used to evaluate hepatic metastasis potential in primary CRC. This study also provided mechanistic insights how Gankyrin functions as an oncoprotein to promote tumor metastasis through targeting IL-8 and Cyclin D1.
Materials and Methods

Clinical Specimens, immunohistochemistry (IHC) staining, scoring and statistical analysis

Formalin-fixed and paraffin-embedded samples recruited in this study were from tissue bank maintained in the Department of Pathology at the 90th Hospital of Jinan, China. All tumors were primary and untreated before surgery. All of the specimens were anonymous and tissues collected in compliance with institutional review board regulations from the hospital. The characteristics of patients with colorectal cancer recruited to this study were summarized in Supplementary Table 1.

IHC staining and scoring for Gankyrin, cyclin D1 and IL-8 was performed as previously described (28). A minimum of 100 cells were evaluated in calculating the H-score (29). Means of continuous variables for Gankyrin staining intensity between colorectal cancers, matched normal tissue and colorectal adenoma samples were compared by One-way analysis of variance (multiple comparisons). The comparison of Gankyrin staining intensity between colorectal cancer with and without hepatic metastasis was evaluated with the Mann-Whitney U test. The correlation between Gankyrin expression and that of IL-8, Cyclin D1 was determined using Spearman Bivariate correlation test. All statistical tests were two-sided, and \( p \) values less than 0.05 were considered as statistically significant. The statistical analyses were performed using SPSS 13.0 software (SPSS Inc, Chicago, IL).

Plasmids, antibodies, and reagents

pLKO-TRC shRNA clones targeting Gankyrin were purchased from Open Biosystems. shRNA targeting human Gankyrin sequence are A, 5’-GCTCAAGTGAATGCTGTCAAT-3’. 
sequence; B, 5’-CAAGGGTAACTTGAGATGAT-3’, or the scrambled derivative 5’-
AAGCCAGAGACGTTACGTA-3’. The shRNA targeting mouse Gankyrin sequence is: 5’-
GCCATACAGAAATTGTTGAAT-3’.

Western blotting and IHC assays were conducted using following antibodies as indicated:
anti-FLAG (M2 clone, Sigma), anti-C/EBPα, anti-Gankyrin and anti-Cyclin D1 (Santa Cruz
Biotechnology), anti-IL-8 (Abgent), anti-α-tubulin or anti-GAPDH (BD Bioscience).

Cell culture

The human embryonic kidney 293T cells (HEK293T) and 3T3 cells were maintained in
DMEM culture solution with 10% fetal bovine serum (FBS). 3T3 cells derived from Ccnd1−/−
mice were previously described (30). Human colon cancer cell line SW480 was purchased from
the American Type Culture Collection (ATCC). Mouse colon cancer cell line MC38 was kindly
provided by Dr. Schlom (31). CT26 cell line was previously described (32). The basal culture
mediums are: L15 (SW480) and DMEM (MC38 and CT26). For cell maintenance, the basal
medium was supplemented with 10% fetal bovine serum (FBS).

Cell transfection and transduction

For transient transfection, Superfect transfection reagent was used following
manufacturer’s protocol (Qiagen, Valencia, CA). For cell transduction, retroviruses were
prepared by transient co-transfection with helper viral vector into HEK 293T cells using calcium
phosphate precipitation. HEK 293T cells were transfected with plasmid DNA and cultured at
37°C for 6 hrs, the medium was replaced and after 36 hrs the supernatant was collected and
filtered through a 0.45 μm filter. Cells were infected at approximately 70% confluence in
DMEM supplemented with 8 μg/ml of polybrene. The following day, the medium was changed to basal medium supplemented with 10% FBS and cultured for further assay.

The siRNAs of Gankyrin and control Photinus pyralis luciferase gene were from Invitrogen, and the target sequences were described previously (10). The Lipofectamine Reagent (Invitrogen) was used for transfections, and the final concentration of siRNA was 50 nM. MC38-luc/shGankyrin and CT26-luc/shGankyrin stable cell lines used for in vivo experiments were generated by the sequential induction of luciferase 2 (Luc2) and shGankyrin expression in MC38 and CT26 cells.

RNA isolation, quantitative real-time PCR

Total RNA was prepared using TriZol reagent (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. 1μg of total RNA was subjected to reverse transcription to synthesize cDNA using the SuperScript™ II Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). A 25 μl volume reaction consisted of 1 μl reverse transcription product and 100 nM of each primer. The primers used for qRT-PCR are listed in Supplementary Table 3.

Cell migration and 3-Dimensional invasion assays

Cell migration assays were carried out as previously described (10). Each determination represents the average of three individual experiments. For 3-Dimensional invasion assay, 100 μl of 1.67 mg/ml rat tail collagen Type I (BD biosciences) was pipetted in the top chamber of a 24-well 8 μM-pore Transwell (Corning). The Transwell was incubated at 37°C overnight to allow the collagen to solidify. 3×10^4 cells were then seeded on the bottom of the Transwell membrane and allowed to attach for 4 hr. Serum free growth medium was placed in the bottom chamber
while 5% serum was used as a chemo-attractant in the growth medium of the upper chamber. The cells were then chemo-attracted across the filter through the collagen above for 3 days. Cells were fixed in 4% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS then stained with 40 μg/ml propidium iodide (PI) for 2 hr. Fluorescence was analyzed by confocal z-sections (4 μm interval) at 10× magnification from the bottom of the filter. 3-dimensional reconstructions of the PI stained cells were done using Carl Zeiss Zen software (2007 Light Edition).

**Mouse models for in vivo tumor growth and metastasis**

MC38 is a murine colon adenocarcinoma derived from immune competent C57BL/6 mice. MC38 cells (2×10⁶) were injected subcutaneously behind the anterior forelimb of C57BL/6 mice. Each animal group was consisted of 8 animals. Tumor volumes were calculated and the growth curves for tumors were plotted as mean ± SD of tumors from eight mice.

For intrasplenic transplantation, 4×10⁶ SW480-Luc/shControl and SW480-Luc/shGankyrin cells were transplanted in the spleen of 8 weeks old NMRI nu/nu mice at day 0 (4 mice/group). Furthermore, 2×10⁶ MC38-luc/shControl and MC38-luc/shGankyrin cells were transplanted in the spleen of 6 weeks old male C57BL/6 mice at day 0 (6 mice/group). 2×10⁶ CT26-luc/shControland CT26-luc/shGankyrin cells were transplanted in the spleen of 6 weeks old male BALB/c mice at day 0 (6 mice/group). The animals were staged for 70 days (SW480) or 28 days (MC38 and CT26 cells) to allow for the development of distant metastases. After animals were sacrificed, tumors and metastasis target organs were removed. All experiments were performed in accordance with the relevant guidelines and approved by our institute (National Center of Biomedical Analysis).

**Genome-wide gene expression analysis**

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Gene expression profiling was conducted in cells with knockdown of Gankyrin using Affymetrix Human Genome U133 Plus 2.0 Array. Microarray data have been deposited in GEO (Accession No. GSE44029; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44029). The core set of probe set clusters was used with annotation version na30, dated December 2009. The dataset was imported into Matlab version R2010b (The Mathworks), and One-way ANOVA was used to evaluate the significance of differential expression between biological conditions. Genes with a differential expression p-value of 0.05 or less and an absolute fold change of 1.5 or more were clustered and visualized using a clustergram heatmap. The differentially expressed gene list was loaded into Ingenuity Pathway Analysis (IPA) 8.0 software (http://www.ingenuity.com) to perform biological network and functional analyses.

**Bioluminescence imaging**

Animals were injected with 10 mg/kg D-luciferin (Xenogen) in PBS intraperitoneally and anesthetized by isoflurane using the XGI-8 gas anesthesia system (Xenogen). Bioluminescence images were acquired by using the IVIS Imaging System (Xenogen) 10-15 min after injection.

**ELISA assay**

Cells were plated at 60% confluence at day 1 and changed to serum-free medium at day 2. After 24 hrs, cell culture medium was harvested and IL-8 concentration was determined by ELISA using a kit purchased from R&D Systems.
Results

Elevated Gankyrin expression in primary colorectal cancers with hepatic metastasis

We previously reported that Gankyrin is overexpressed in human lung and breast cancers and plays an essential role in breast cancer metastasis (10, 16). To determine the role of Gankyrin in tumor metastasis, we extended our observation to colorectal cancer given that Gankyrin promoted cell proliferation and tumor growth in a colon cancer cell line HCT116 as recently reported (33). Immunohistochemical staining (IHC) was deployed to determine the expression and subcellular distribution of Gankyrin in a large cohort of colorectal cancer specimens. The expression levels of nuclear and cytoplasmic Gankyrin were scored separately, showing that Gankyrin expressed in both normal and cancer tissues. The expression levels of Gankyrin were significantly increased in cancer cells compared to adjacent normal epithelium \( (p = 0.0000, \text{Supplementary Fig. S1}) \) or colorectal adenoma (CRA) \( (p = 8.659E-14, \text{Supplementary Fig. S1}) \). In normal epithelial cells, the majority of Gankyrin was found in the cytoplasm, while in cancerous tissue, Gankyrin was detected in both cytoplasm and nucleus. These observations demonstrated that Gankyrin is highly expressed in colorectal cancers and the nuclear distribution marks the difference between cancer and normal cells.

Patients with hepatic metastases from primary colorectal cancer were considered to be one of the clinical challenges. To explore the correlation between Gankyrin expression and subcellular localization with metastasis in colorectal cancer, we investigated an independent cohort of patient samples. This cohort was consisted of 48 cases with hepatic metastasis and 33 cases randomly selected from colorectal cancer patients without developing hepatic metastasis in a minimal 5-year follow-up (Supplementary Table S1). The hepatic metastasis lesions were
surgically resected at the time of diagnosis. IHC was performed to determine the Gankyrin expression in these specimens. There was a significant increase of Gankyrin in the primary tumors from 48 cases with hepatic metastasis comparing to cancer tissues from 33 cases without hepatic metastasis ($p = 0.009$, Fig. 1). The expression levels of Gankyrin were significantly upregulated in the nucleus of CRCs with hepatic metastasis compared to those without metastasis ($p = 0.002$, Fig. 1B). No observable difference in Gankyrin expression was found between the metastatic cancer tissues comparing to tissues from the primary tumors. These results suggest that the highly expressed Gankyrin and its nuclear translocation could define a subtype of CRCs that are potentially metastatic.

**Gankyrin knockdown impairs CRC cell migration, invasion and tumor metastasis in vivo.**

Our observation that Gankyrin expression correlates with hepatic metastasis promoted us to further investigate the role of Gankyrin in cellular migration and invasion. To do so, we performed Transwell migration assay using SW480 cells stably expressing Gankyrin shRNA (shGankyrin) or control shRNA (shControl). We found that Gankyrin knockdown reduced the number of migrating cells by 2-fold (Supplementary Fig. S2). We examined the effects of Gankyrin on colorectal cancer cell movement using wound healing assays. Representative examples of cell moving toward the wound were shown (Fig. 2A). Gankyrin knockdown significantly reduced the velocity of cell movements ($p = 1.337\times10^{-8}$, $n = 24$), suggesting that Gankyrin is required for cell movement in cancer cells, a key aspect of cancer metastasis. As shown in Fig. 2B, the shRNA of Gankyrin achieved nearly 100% knockdown efficiency at protein levels.
To further test whether the endogenous Gankyrin contributes to the invasiveness of SW480 cells, we carried out a modified Transwell assay by coating the device with ECM matrix. The cells were seeded in the upper chamber in serum-free medium. The medium supplemented with 10% fetal bovine serum was provided in the lower chamber, serving as chemo-attractant. Compared to control group, the transmigration distance through the ECM of SW480 Gankyrin-knockdown cells was significantly reduced (Fig. 2C).

In order to evaluate the effect of Gankyrin in tumor metastasis in vivo, we adopted a well-established spleen-hepatic metastasis model. We transduced Gankyrin knockdown and control SW480 cells with a retroviral plasmid DNA encoding luciferase in order to visualize the metastasis. In each group of the experiment, 4×10⁶ cells were inoculated into the distal tip of the spleen in immune-deficient nu/nu mice. At day 70, mice were sacrificed and the spleen and liver organs were removed. Luciferin substrates were injected into mice 10 minutes prior to organ removal. Luciferase activity was examined for the presence of “primary” splenic tumor and hepatic metastasis lesions. We found that the “primary” tumors were detected in spleen of both Gankyrin knockdown and control groups and hepatic metastasis was only present in mice implanted with SW480 control group. The Gankyrin knockdown completely abolished the hepatic metastasis (Fig. 2D). Together, these results suggest that Gankyrin is required for cell migration and invasion and hepatic metastasis of CRC in vivo.

**Gankyrin is required for tumor metastasis in immune-competent mice.**

The immune-deficient mouse models are ideal for evaluating tumor growth using human cancer cells, however, these models do not reflect the nature of colon cancer in hosts developing antitumor immune response. Two murine colon carcinoma cells were tested in immune-
competent allograft models. The murine carcinoma-38 (MC38) colon cancer cell line was derived from a high-grade murine colon tumor, induced by carcinogen and able to grow in C57BL/6 mice. MC38 cells were transfected with shRNA vector targeting mouse Gankyrin, and stable clone was established by antibiotic selection. qRT-PCR showed a successful knockdown of Gankyrin expression (Supplementary Fig. S3A). MC38/shGankyrin and MC38/shControl cell were implanted subcutaneously. As shown in Fig. 3A, knockdown of Gankyrin significantly reduced tumor growth rate reflected by tumor size at each measuring time point (left panel) and tumor weight at day 28 (middle and right panel).

Next, in assays using spleen-hepatic metastasis model, 2×10^6 MC38/shGankyrin and MC38/shControl cells were injected into the spleen of C57BL/6 mice, respectively. Liver was removed at day 28 and the metastasis was visualized by bioluminescence imaging, showing dramatic decrease of hepatic metastasis in mice with injection of MC38/shGankyrin cells (p = 0.017, Fig. 3B). The observation was further confirmed by the second immune-competent mouse model. CT26 cell line was derived from a carcinogen induced colon cancer in BALB/c mice. Stable transfection of shRNA reduced the gene expression, as evidenced by qRT-PCR (Supplementary Fig. S3B). Both CT26/shGankyrin and CT26/shControl cells were inoculated into the spleen of BALB/c mice. Similar to the results above, knockdown of Gankyrinin CT26 cells also significantly reduced hepatic metastasis (Fig. 3C). To clearly display the hepatic metastasis lesions, both liver and spleen organs were removed and the number of liver surface metastatic foci was counted, showing a significant difference between the two groups (p = 0.003, Fig. 3D). These results suggest that Gankyrin expression is required for hepatic metastasis of CRC in vivo.

IL-8 Signaling Pathway is regulated by Gankyrin in CRC cells.

14
The nuclear translocation of Gankyrin observed in clinical specimens raises the question whether Gankyrin functions as a regulator of gene transcription. It has been previously reported that Gankyrin regulates transcription indirectly through NF-κB and Wnt signaling pathways (2, 17). To identify the transcriptional targets of Gankyrin, we performed genome-wide screening using SW480 cells with Gankyrin knockdown on an Affymetrix gene expression array, which identified 937 genes that were differentially regulated by Gankyrin knockdown (1.5-fold, p < 0.05). 663 were decreased and 274 increased in SW480/shGankyrin cells comparing to vector control cells (Fig. 4A and Supplementary Table S2). Ingenuity Pathway analysis revealed that the Molecular and Cellular Functions were affected by Gankyrin knockdown. These pathways include Cell Death, Cellular Movement, Cell Cycle, and Cell-To-Cell Signaling and Interaction (Fig. 4B). The genes regulated by Gankyrin also populated canonical pathways including IL-8 Signaling, AMPK Signaling, Molecular Mechanisms of Cancer, Xenobiotic Metabolism Signaling, and PI3K/AKT Signaling (Fig. 4C, D). It is worth noting that IL-8 Signaling, including IL-8 and Cyclin D1, has been linked to colorectal cancer metastasis. Some of key genes involved IL-8 pathways were validated by qRT-PCR (Supplementary Fig. S4).

**Gankyrin promotes colorectal cell migration through targeting IL-8 and Cyclin D1.**

To investigate the functional interaction between Gankyrin and IL-8 signaling, IHC staining was performed on CRC samples to determine the correlation between Gankyrin and IL-8 expression, showing that Gankyrin has a significantly positive correlation with IL-8 expression ($r = 0.312$, $p = 0.021$, Fig. 5A, B). Consistently, Gankyrin overexpression in SW480 cells increased the IL-8 production (Fig. 5C, left panel), and Gankyrin knockdown reduced IL-8 levels (Fig. 5C, right panel). To further determine whether IL-8 induced by Gankyrin contributes to cell migration, recombinant IL-8 protein was added to the medium in Transwell
migration assays. As shown in Fig. 5D, IL-8 partly rescued the migratory defect of SW480/shGankyrin cells. Together, these results demonstrate that IL-8 plays an important role in Gankyrin-mediated CRC cell migration.

Cyclin D1, encoded by CCND1 gene, has been reported to be either amplified or overexpressed in CRC, which correlates to enhanced cellular proliferation, migration and metastasis (34-37). Overexpression of Gankyrin induces Cyclin D1 protein expression in hepatic carcinoma (17). To test whether Gankyrin requires Cyclin D1 in promoting cell migration, we overexpressed Gankyrin in 3T3 fibroblast derived from Ccnd+/+ and Ccnd-/- mice (30). Consistent with the role of Gankyrin in CRC cells, overexpression of Gankyrin enhanced cell migration in Ccnd+/+ 3T3 fibroblast, and the enhanced cell migration by Gankyrin was decreased in Ccnd-/- cells (Fig. 6A). These results suggest that Cyclin D1 is also involved in Gankyrin-mediated CRC cell migration.

To investigate whether Gankyrin is correlated with Cyclin D1 in CRC, we determined the expression levels of Gankyrin and Cyclin D1 on consecutive slides of CRC samples. As shown in Fig. 6B, Cyclin D1 and Gankyrin were both elevated and significantly correlated, suggesting these two proteins may functionally associate to regulate cell migration. Overexpression of Gankyrin increased the Cyclin D1 abundance (Fig. 6C). Conversely, cells knockdown of Gankyrin by shRNA reduced Cyclin D1 protein. It has been reported that oncoprotein Gankyrin contributes to the ubiquitin-proteasome-dependent elimination of C/EBPα during liver cancer development (15). C/EBPα inhibits CCND1 gene expression through recruiting histone deacetylase 1 (HDAC1) (38). In order to gain mechanistic insight how Gankyrin regulates Cyclin D1 expression, we further examined the expression level of C/EBPα. Western blot showed that C/EBPα expression was significantly reduced by Gankyrin overexpression. Furthermore,
Gankyrin knockdown increased the protein abundance of C/EBPα (Fig. 6C, Supplementary Fig. S5). Together, these results suggest that Gankyrin induces cyclin D1 expression through downregulating the expression of C/EBPα, the known negative regulator of cyclin D1.
Discussion

The role of Gankyrin in tumorigenesis has been well established in a variety of human malignancies including HCC, ESCC, breast, pancreatic, oral, lung and colorectal cancers (4, 6-8, 10, 16, 19, 39-40). Our results in the current study reveal the profound impact of Gankyrin in governing the capability of colorectal cancers that metastasize to liver. This study extended our understanding of Gankyrin functioning as oncoprotein by showing that overexpression of Gankyrin promotes hepatic metastasis of CRC. Using genome-wide gene expression screening, we identified a transcriptome regulated by Gankyrin, populating IL-8 signaling pathway, which mediates the effect of Gankyrin in cell proliferation, migration and metastasis in vivo.

Mechanistic studies have been intensively focusing on the function of Gankyrin as a component of proteasome complex in explaining its oncogenic activity. Gankyrin promotes Rb phosphorylation/inactivation through sequestering p16 from CDK4 complex and activates E2F family proteins (3, 41). Through binding ubiquitin ligase MDM2, Gankyrin targets p53 degradation (14, 42). In a carcinogen-induced liver cancer model, Gankyrin eliminates the tumor suppressor C/EBPα and promotes tumor development (15, 43). Despite these significant advances in understanding the role of Gankyrin in cell proliferation, tumor onset and progression, the molecular mechanisms that underpin the function of Gankyrin in cell migration, invasion and metastasis remain largely unknown. Our results demonstrate that Gankyrin overexpression defines a patient group whose tumors have increased potential to metastasize to the liver.

Gankyrin regulation of protein stability through UPS-mediated degradation has been recognized (4, 14, 42). Several lines of evidence suggest that Gankyrin regulates gene expression indirectly through targeting transcription factors and regulators such as E2F, NFκB, C/EBP, β-
catenin and Oct-4 (2, 4, 14-15, 17, 44-45). In this study, we conducted systematic analysis of gene expression regulated by Gankyrin using a genome-wide microarray approach. 937 genes were significantly regulated by Gankyrin. Pathway analysis of these genes confirmed the previous observations that Gankyrin regulates p53, C/EBPα, and HIFα-dependent gene expression. Recent study using transgenic zebrafish revealed that Gankyrin overexpression induced liver steatosis and modulated gene and microRNA expression (46). Together, these studies suggest an important role of Gankyrin in regulating gene transcription. Future studies focusing the molecular details by which Gankyrin regulates gene transcription are warranted.

It has been previously reported that Gankyrin was mainly distributed to the cytoplasm in hepatocellular carcinoma (47-48). The IHC staining of Gankyrin showed similar subcellular distribution patterns in normal colon epithelium. A significant increase of nuclear Gankyrin distribution was observed in CRC cells comparing to adjacent normal-like epithelium (Supplementary Fig. 1). In addition, increased nuclear Gankyrin differentiated the CRC patient population with increased risk of developing liver metastasis (Fig. 1). These observations suggest that the nuclear translocation of Gankyrin could play an important role in cancer progression. It is important to investigate the mechanisms that promote Gankyrin nuclear translocation in future studies. Targeting nuclear translocation of Gankyrin could potentially serve as a novel strategy to prevent hepatic metastasis of CRC.

It has been known for years that IL-8 production is increased in patients with CRC and that IL-8 promotes tumor growth and cancer metastasis (24). Microarray analysis revealed that genes in IL-8 Signaling Pathway are down-regulated in Gankyrin knockdown cells, suggesting that Gankyrin governs this pathway in fulfilling its cellular function. Among those genes, Cyclin D1 is a rate-limiting protein controlling the G1/S transition of cell cycle progression and is
involved in cell migration and invasion (49-50). Cyclin D1 is overexpressed in CRC and required for growth and tumorigenicity of human colon cancer cells (35, 51). Loss of one allele of \textit{Ccn\textsubscript{d1}} gene resulted in decreased tumor number in the duodenum, intestines, and colons of Ap\textsuperscript{c\textsubscript{Min}} mice correlated with reduced cellular proliferation and increased differentiation (52). Our data showed that the protein levels of Cyclin D1 and Gankyrin are significantly correlated in human CRC specimens. We further demonstrated the requirement of Cyclin D1 and IL-8 in Gankyrin-mediated cell migration by showing that Cyclin D1 deficiency blocked Gankyrin-dependent cell migration phenotype and IL-8 was able to rescue the migratory defect associated Gankyrin knockdown.

In summary, our results demonstrate that Gankyrin distinguishes CRCs with hepatic metastasis from those with less metastasis potential. Knockdown of Gankyrin in CRC cells impairs cells’ ability to migrate, invade and metastasize \textit{in vivo}. Our data provide novel insights into understanding the mechanisms by which Gankyrin regulates metastasis. In addition to the known targets, Gankyrin enhanced IL-8 pathways and thus increased cell proliferation and migration/invasion. Increased Gankyrin expression and/or nuclear translocation in CRC hence represents a potentially attractive biomarker for early detection of CRC with potential to develop hepatic metastasis, as well as the therapeutic approach, which would target the tumor growth and prevents cancer progression toward hepatic metastasis. By synthesizing our findings, we propose a model by which Gankyrin overexpression enhances cellular proliferation, tumorigenesis and metastasis by regulating Cyclin D1 and IL-8-dependent pathways (\textbf{Fig. 6D}).
Acknowledgements

We would like to thank Dr. Scott Waldman for his suggestions and the valuable reagents. This work was supported by grants from the Key State Science and Technology Projects of China (2012ZX09301003-004,-001), the China National Basic Research Program (Grant 2012CB910801), the China National Natural Science Foundation (No. 81221004, No. 81172008 and No. 31270801) and in part by the Pennsylvania Department of Health grant. Work conducted at the Kimmel Cancer Center was supported by the NIH Cancer Center Core grant (P30CA56036). The Department specifically disclaims responsibility for any analyses, interpretations or conclusions.
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Figure Legends

**Figure 1. Gankyrin expression is increased in metastatic colorectal cancer.**

A, Immunohistochemistry (IHC) staining of primary colorectal cancer specimens for Gankyrin protein expression and subcellular localization. Comparison was made between cancer samples from patients with or without hepatic metastasis at the time of diagnosis. B, Boxplot of IHC staining score for Gankyrin showing increased expression in primary cancer in patient who has hepatic metastasis.

**Figure 2. Gankyrin is required for cell migration, invasion and tumor metastasis in vivo.**

A, The time-lapse video-microscopy was conducted and cell migration velocity was further analyzed in SW480/shGankyrin and SW480/shControl cells. Student t-test was used for quantitative analysis of cell migration. B, Western blot was conducted of cells with knockdown of Gankyrin in SW480 cells using a specific antibody against human Gankyrin. C, 3-dimentional invasion assays were performed with Transwell device coated with Type I Collagen (BD biosciences). Fluorescence was analyzed by confocal z-sections (1 section every 4 μm) at 10× magnification from the bottom of the filter. D, 70 days post intrasplenic transplantation, liver metastasis was determined by Bioluminescence imaging acquired using the IVIS Imaging System (Xenogen).

**Figure 3. Gankyrin is required for cancer metastasis in immune-competent mice.**

A, MC38/shGankyrin and MC38/shControl cells were implanted into C57BL/6 mice subcutaneously. Tumor growth was measured every five days by digital caliper and tumor volume was calculated (left panel). Tumor was isolated at the completion of the experiment and...
weighted (middle panel). Images of tumors isolated were shown in the right panel. B, Intrasplenic transplantation of MC38/shGankyrin and MC38/shControl expressing luciferase in C57BL/6 mice. The mice were staged for 28 days to allow the development of distant metastases. Bioluminescence images were acquired on liver immediately removed from mice. Number of metastatic foci in liver was counted. C, Intrasplenic transplantation of CT26/shGankyrin and CT26/shControl cells expressing luciferase in BALB/c mice. The mice were staged for 28 days to allow the development of distant metastases. Bioluminescence images were acquired on whole animal. D, Liver and spleen were removed from mice. Number of metastatic foci in liver was counted.

**Figure 4. Knockdown of Gankyrin expression impairs interleukin 8 signaling.**

A, SW480 cells stably transfected with shGankyrin and control were analyzed by gene expression profiling. All genes altered by Gankyrin knockdown are shown (1.5-fold, \( p < 0.05 \)). B, Genes differentially regulated by Gankyrin were subjected to Ingenuity Pathway analysis. Top five Molecular and Cellular Functions regulated by Gankyrin are shown. C, Top five Canonical Pathways regulated by Gankyrin. D, shRNA knockdown of Gankyrin alters the expression of genes in IL-8 Signaling Pathway.

**Figure 5. IL-8 is required for Gankyrin-dependent cell migration.**

A, IHC was conducted with the anti-IL-8 antibody in CRC specimens. B, The correlation between IL-8 and Gankyrin expression levels in CRC specimens. C, SW480 cells with either Gankyrin overexpression or knockdown were culture in serum-free medium for 24 hrs. The IL-8 content in cell culture medium was measured by ELISA kit. D, SW480/shGankyrin cells were
subjected to Transwell migration assays in the presence and absence of IL-8. Addition of IL-8 rescues the migration defect of SW480/shGankyrin.

**Figure 6. Cyclin D1 is required for Gankyrin promotion of cell migration.**

A, 3T3 cells derived from *Ccnd1* wild-type (*Ccnd1*+/+) and knockout (*Ccnd1−/−*) were transduced with retroviral vectors expressing Gankyrin or control. Transwell migration assays were conducted. The number of cells migrating across the membrane was counted. Crystal violet dye staining of cells that migrated is shown. The data are shown as mean ± SEM of the number of cells migrated in three independent experiments. B, IHC was performed on colorectal cancer specimens. Representative images showed that Gankyrin and Cyclin D1 were both overexpressed in colorectal cancer. There is a significant correlation between Gankyrin and Cyclin D1 (Spearman’s *rho* = 0.372, *p* = 0.005). C, Western blot was conducted to examine the expression levels of cyclin D1 and C/EBPα in Gankyrin overexpressing and knockdown cells. D, Schematic representation of Gankyrin functions. Gankyrin upon nuclear transformation promotes cellular proliferation and tumor growth likely through upregulation of Cyclin D1. Gankyrin induction of IL-8 secretion promotes cell migration/invasion and cancer metastasis.
Figure 1

A

without hepatic metastasis (n = 33)                                    with hepatic metastasis (n= 48)

B

\[ p = 0.0129 \]

\[ p = 0.0002 \]

\[ p = 0.0009 \]
Figure 2

(A) shControl vs. shGankyrin

(B) Velocity (nm/sec.)

C

Seeding cells → Serum-free → 10% serum → Cell migration direction

D

SW480/shControl

SW480/shGankyrin

Liver

Spleen

Day 1

Day 70

Liver

Spleen

Day 1

Day 70
**Figure 3**

**A**

MC38

- shControl
- shGankyrin

Tumor size (×100 mm²) vs. Days

- p = 0.019
  - n = 8

**B**

- shControl
- shGankyrin

Number of metastatic foci per mouse

- p = 0.017
  - n = 6

**C**

CT26

- shControl
- shGankyrin

Day 1 vs. Day 14

**D**

- shControl
- shGankyrin

Liver, Spleen

Number of metastatic foci per mouse

- p = 0.003
  - n = 6
Figure 4

A: Heatmap showing the expression levels of genes in control (shControl) and Gankyrin knock-down (shGankyrin) conditions.

B: Molecular and cellular functions regulated by Gankyrin.

<table>
<thead>
<tr>
<th>Molecular &amp; Cellular Functions</th>
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<tbody>
<tr>
<td>Cell Death</td>
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<tr>
<td>Cellular Movement</td>
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<td>Cell Cycle</td>
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<tr>
<td>Cell-To-Cell Signaling and Interaction</td>
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<tr>
<td>Carbohydrate Metabolism</td>
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C: Ingenuity canonical pathways regulated by Gankyrin.

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<td>AMPK Signaling</td>
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<td>Molecular Mechanisms of Cancer</td>
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<td>Xenobiotic Metabolism Signaling</td>
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<td>PI3K/AKT Signaling</td>
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D: IL-8 Signaling Pathway.

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shControl vs shGankyrin comparison.
Figure 5

A

B

C

D
Gankyrin activates IL-8 to promote hepatic metastasis of colorectal cancer

Zhao-fang Bai, Yanhong Tai, Weihua Li, et al.

Cancer Res  Published OnlineFirst April 10, 2013.

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