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LIN28B promotes cell invasion and colorectal cancer metastasis via CLDN1 and NOTCH3

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The RNA-binding protein LIN28B is overexpressed in over 30% of patients with colorectal cancer (CRC) and is associated with poor prognosis. In the present study, we unravel a novel mechanism by which LIN28B regulates colonic epithelial cell-cell junctions and CRC metastasis. Using human CRC cells (DLD-1, Caco-2 and LoVo) with either knockdown or overexpression of LIN28B, we identified Claudin 1 (CLDN1) tight junction protein as a direct downstream target and effector of LIN28B. RNA immunoprecipitation revealed that LIN28B directly binds to and post-transcriptionally regulates CLDN1 mRNA. Furthermore, using in vitro assays and a novel murine model of metastatic CRC, we show that LIN28B-mediated CLDN1 expression enhances collective invasion, cell migration, and metastatic liver tumor formation. Bulk RNA-sequencing of the metastatic liver tumors identified NOTCH3 as a downstream effector of the LIN28B-CLDN1 axis. Additionally, genetic and pharmacologic manipulation of NOTCH3 signaling revealed that NOTCH3 was necessary for invasion and metastatic liver tumor formation. In summary, our results suggest that LIN28B promotes invasion and liver metastasis of CRC by post-transcriptionally regulating CLDN1 and activating NOTCH3 signaling. This discovery offers a promising new
therapeutic option for metastatic CRC to the liver, an area where therapeutic advancements have been relatively scarce.
INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second most common cause of cancer death in the world (1). The 5-year survival rate in patients with localized tumors is 90%, but the survival rate drastically decreases to 14% in patients with distant metastasis despite various interventions such as chemotherapy, immunotherapy, and surgery (2, 3). Therefore, it is imperative to elucidate the molecular mechanisms underlying CRC metastasis, especially to the liver, a common site for colonization and outgrowth.

LIN28 is an evolutionarily conserved RNA-binding protein (RBP), initially identified in Caenorhabditis elegans as having developmental functions (4). In mammals, there are two paralogs of LIN28: LIN28A and LIN28B, both sharing similar domain structure and function. LIN28A plays a key role in maintaining embryonic stem cell pluripotency and enhances epigenetic reprogramming by OCT4/SOX2/KLF4 (5). LIN28B has also been suggested to play a role in pluripotency (6); however, its role in cancer has been more extensively investigated due to increasing evidence demonstrating that LIN28B serves as an oncogene. For example, LIN28B is associated with advanced stage and early recurrence of...
hepatocellular carcinoma (7). In esophageal cancer, LIN28B overexpression promotes cell invasion in vitro and correlates with poor overall and disease-free survival (8). In CRC, LIN28B is overexpressed in 30-60% of patients and associated with poor prognosis (9-11). Additionally, we previously reported that LIN28B acts as an oncogene in a genetic mouse model of CRC and promotes liver metastasis in a subcutaneous xenograft model (9, 12). Thus, LIN28B is associated with poor prognosis and tumor metastasis in diverse cancers. However, the underlying molecular mechanisms of how LIN28B functions in tumor metastasis, especially in CRC, are unclear and require elucidation for potential translational therapeutics.

Let-7 microRNA (miRNA) is downregulated in various human cancers. LIN28 directly targets the let-7 family, inhibiting its biogenesis. Similar to that of LIN28B, let-7 dysregulation is associated with poor prognosis and increased metastatic potential in several cancer types, including breast, lung, colorectal, and ovarian cancers (13). While many studies have investigated the role of the LIN28B and let-7 loop in promoting cancer hallmarks, such as cell proliferation, metabolism, evasion of immune destruction, and metastasis (13), other potential targets of LIN28B and their contribution to cancer progression have received less attention.

We previously performed cross-linking immunoprecipitation sequencing (3-seq;
CLIP-seq) analysis of the target mRNAs of LIN28B and revealed that LIN28B binds to various adherens and tight junction mRNAs, including Claudin-1 (CLDN1) (12). Of the 27 claudin family members discovered to date, CLDN1 is strongly implicated in cancer progression (14-16). For example, CLDN1 promotes invasiveness in CRC and hepatocellular carcinoma (17, 18). Additionally, CLDN1 regulates growth and metastasis in mouse models of CRC (19). The invasive function of CLDN1 may be a consequence of it being a tight junction protein and its integral role in maintaining cell-cell contacts. Cell-cell interaction is required for an important step in cancer metastasis called collective cell invasion, a type of cell migration that retains cell-cell contacts to form a structural and functional unit (20-23).

Therefore, we hypothesized that LIN28B binds to and promotes translation of CLDN1, resulting in collective cell invasion and subsequent metastasis of CRC.

The Notch signaling pathway regulates cell proliferation, differentiation, and maturation in several tissues and cell types. Notch signals are activated and transduced when NOTCH1-4 transmembrane receptors bind to transmembrane ligands expressed on adjacent cells. Activation of the Notch receptors initiates a proteolytic cascade via gamma-secretase, leading to the transcription of downstream target genes, including HES and HEY transcription factors. The targets of these transcription factors regulate cell differentiation, maturation, and
proliferation (24). As such, dysregulated Notch signaling has also been implicated in several human cancers. For example, NOTCH3 overexpression is associated with poorly differentiated colorectal tumors (25), increased tumor growth rate (26), higher rates of venous invasion, and shorter recurrence-free survival (25).

In the present study, we investigate the role of LIN28B in regulating cell-cell junctions and the functional consequences of modulating LIN28B expression on CRC progression. We show that LIN28B promotes collective cell invasion through post-transcriptional upregulation of CLDN1 gene expression. Furthermore, we demonstrate that LIN28B-mediated upregulation of CLDN1 induces NOTCH3 signaling to promote liver metastasis of CRC, and genetic modulation as well as pharmacologic inhibition of NOTCH signaling reduces metastasis. Our results suggest that a newly delineated LIN28B-CLDN1-NOTCH3 axis regulates CRC metastasis to the liver, thereby providing new perspectives on therapy of this deadly manifestation of CRC.
RESULTS

LIN28B promotes cell migration and collective cell invasion of CRC cells

To study the functional role of LIN28B in CRC, we generated CRC cells with genetic modification of LIN28B expression. Endogenous LIN28B expression is high in human Caco-2 CRC cells, whereas it is low in human DLD-1 cells and LoVo CRC cells (27, 28). Thus, we generated Caco-2 cells with LIN28B knockdown by using short hairpin RNA (shRNA) and DLD-1 and LoVo cells with LIN28B overexpression (LIN28B\textsuperscript{high}). The downregulation or upregulation of LIN28B protein levels were confirmed by immunoblotting (Supplemental Figure 1, A and B).

Next, to investigate whether LIN28B mediates cell invasion and migration, we compared the wound healing capacities of CRC cells with LIN28B-high vs. -low expression. DLD-1 LIN28B\textsuperscript{high}, Caco-2 control (Ctrl) (with endogenous high LIN28B level) and LoVo LIN28B\textsuperscript{high} cells had significantly higher wound-healing rates at 48 hours after the initial scratch (Figure 1A and B and Supplemental Figure 1B). Transwell 2D migration and invasion assays also revealed that DLD-1 LIN28B\textsuperscript{high} and LoVo LIN28B\textsuperscript{high} were significantly more migratory and invasive (Supplemental Figure 1 C and D). Of note, the transwell assays did
not work with the Caco-2 cells, perhaps reflecting their intrinsic well-differentiated status (data not shown). The 3D extracellular matrix (ECM)-based assay is used commonly to assess collective cell invasion in vitro (29, 30). Therefore, we performed spheroid 3D invasion assay using ultra-low attachment plates (Figure 1C). By day 7, DLD-1 LIN28B\textsuperscript{high}, Caco-2 Ctrl and LoVo LIN28B\textsuperscript{high} cells invaded to a greater area when compared to LIN28B\textsuperscript{low}-expressing cells (Figure 1D and E, Supplemental Figure 1E). These results suggest that LIN28B enhances migration and collective cell invasion of CRC cells.

LIN28B enhances CLDN1 expression and cell aggregation of CRC cells

We have previously performed cross-linking immunoprecipitation and sequencing (CLIP-seq) which revealed various adherens and tight junction RNA transcripts as targets of LIN28B (12). To follow up with these findings, we measured the protein expression levels of the following CLIP-seq target transcripts: E-CADHERIN and p120-catenin as representatives of the adherens junctions, and CLDN1, CLDN4 and OCLN as representatives of the tight junctions. DLD-1 cells and LoVo cells with LIN28B overexpression (LIN28B\textsuperscript{high}) had significantly higher CLDN1 protein levels than the corresponding empty vector (EV) controls (Figure 2A and B, Supplemental Figure 2A and B). Similarly, Caco-2 cells with
LIN28B knockdown had significantly lower CLDN1 protein levels than the control cells (Caco-2 Ctrl) (Figure 2A and B, Supplemental Figure 2A and B). By contrast, E-CADHERIN, p120-catenin, CLDN4 or OCLN protein levels were not significantly changed between LIN28B<sup>low</sup> and LIN28B<sup>high</sup> CRC cells (Figure 2A and B, Supplemental Figure 2A and B).

Immunofluorescence of CLDN1 protein expression further confirmed higher CLDN1 expression in DLD-1 LIN28B<sup>high</sup>, Caco-2 Ctrl and LoVo LIN28B<sup>high</sup> cells when compared to their respective control groups (Figure 2C, Supplemental Figure 2C).

Due to the pivotal role of CLDN1 as a tight junction protein and the newly revealed association between LIN28B and CLDN1 transcript, we hypothesized that LIN28B expression enhances cellular aggregation. To evaluate this, we measured cellular aggregation of LIN28B expression-modified CRC cells in ultra-low attachment plates. This revealed that DLD-1 LIN28B<sup>high</sup>, Caco-2 Ctrl and LoVo LIN28B<sup>high</sup> cells promoted greater cell aggregation when compared to DLD-1 Ctrl, Caco-2 LIN28B<sup>low</sup>, and LoVo Ctrl cells, respectively (Supplemental Figure 3A and B). These findings support the notion that LIN28B enhances CLDN1 expression and cell aggregation of CRC cells.

**LIN28B directly binds to and stabilizes CLDN1 mRNA**
We next focused on identifying the mechanism(s) by which LIN28B regulates CLDN1 expression in CRC cells. As an RBP, LIN28B is responsible for post-transcriptional regulation, such as RNA splicing, transport, stability and localization (31-33). Therefore, we hypothesized that LIN28B binds to CLDN1 mRNA and regulates it in a post-transcriptional fashion. We first used an anti-LIN28B antibody to perform RNA immunoprecipitation (RIP) in DLD-1 LIN28B<sup>high</sup>, LoVo LIN28B<sup>high</sup>, and Caco-2 cells. Western blot of the precipitated samples confirmed the efficiency of the anti-LIN28B antibody (Figure 2D). The amount of CLDN1 mRNA precipitate was significantly greater than the amount of CLDN1 mRNA pulled down by the IgG negative control antibody in LIN28B<sup>high</sup> cells (Figure 2E).

To further clarify how LIN28B regulates CLDN1 protein expression, CLDN1 mRNA stability was evaluated by using actinomycin D, a transcription inhibitor which intercalates into DNA to prevent RNA polymerase activity (34-36). Upon treatment of CRC cells with actinomycin D, CLDN1 mRNA decay was analyzed every two hours by qRT-PCR. Our quantification revealed delayed decay of CLDN1 mRNA expression in LIN28B<sup>high</sup> cells after actinomycin D treatment (Figure 2F), supporting our hypothesis that LIN28B upregulates CLDN1 protein expression by binding to and stabilizing CLDN1 mRNA.
LIN28B-induced cell aggregation and collective cell invasion is dependent on CLDN1

To determine whether CLDN1 upregulation plays a pivotal role in collective cell invasion induced by LIN28B, we suppressed CLDN1 expression in DLD-1 LIN28B\textsuperscript{high}, LoVo LIN28B\textsuperscript{high}, and Caco-2 cells by two different shRNAs. We confirmed first that CLDN1 mRNA and protein expression was significantly suppressed in shCLDN1 cells of all three cell lines (Supplemental Figure 4A and B). Next, to verify whether CLDN1 knockdown inhibits LIN28B-induced cell aggregation, we compared the ability of shCLDN1 and control cells to aggregate. The number of cell aggregates was significantly lower in shCLDN1 cells (Figure 3A and B, Supplemental Figure 4C). We performed a wound healing assay to examine the role of CLDN1 in LIN28B-induced cell invasion and migration. shCLDN1 cells displayed a significantly lower wound healing rate, suggesting for reduced migratory abilities in CLDN1 knockdown cells (Figure 3C and D, Supplemental Figure 4D). Moreover, the 3D tumor spheroid invasion assay confirmed that shCLDN1 cells invaded to a smaller area than control cells (Figure 3E and F, Supplemental Figure 4E). Taken together, these results indicate that CLDN1 contributes to LIN28B-induced cell aggregation and collective cell invasion.
LIN28B-induced CLDN1 upregulation promotes metastatic liver tumor formation

Portal vein injection of LIN28B$^{\text{high}}$ CRC cells result in liver metastases formation by undergoing extravasation, colonization, and outgrowth (37). Given this, we next explored the potential role of CLDN1 in LIN28B-induced liver metastasis. LIN28B$^{\text{high}}$ CRC cells with GFP fluorescence were injected into the portal vein of 6-8 weeks old Taconic NCr nude mice (CrTac:NCr-Foxn1nu), and the liver tissues were harvested four weeks after injection (Figure 4A). Experiments using Caco-2 cells were excluded because previous studies showed that the cell line does not form liver metastases in mice (37-39), potentially due to higher expression of let-7 miRNA in Caco-2 cells (Supplemental Figure 4F). As expected, injection of parental DLD-1 cells (empty vector; EV) with non-target control shRNA (shNTC) did not result in liver tumors (0/9, 0%), and injection of DLD-1 LIN28B$^{\text{high}}$ cells with shNTC induced liver metastases (6/9, 66.7%) (Figure 4B). Similarly, LoVo LIN28B$^{\text{high}}$ cells with shNTC formed more liver metastases (6/13, 46.2%) than LoVo EV with shNTC (1/15, 6.7%) (Figure 4B). Intriguingly, knockdown of CLDN1 completely inhibited liver tumor formation in mice injected with DLD-1 LIN28B$^{\text{high}}$ cells (0/6 and 0/7, 0%) (Figure 4B). Similarly, the number of liver tumors in mice injected with LoVo LIN28B$^{\text{high}}$ was decreased upon CLDN1 knockdown (Figure 4B). The presence of metastatic liver tumors was confirmed by GFP.
staining (Figure 4C). Histological analysis showed that tumors derived from LoVo LIN28B\textsuperscript{high} cells have higher expression of CLDN1 than tumors with EV, consistent with our \textit{in vitro} experiments (Figure 4C). We have previously published that overexpression of LIN28B in intestinal epithelial cells (\textit{Vil\textsuperscript{Cre};Lin28b\textsuperscript{high}}) induces spontaneous tumorigenesis (without liver metastasis) in a transgenic mouse model (12). Analysis of intestinal tumors from \textit{Vil\textsuperscript{Cre};Lin28b\textsuperscript{high}} mice showed that the tumors with higher LIN28B expression have higher CLDN1 expression when compared to tumors from \textit{Vil\textsuperscript{Cre};Lin28b\textsuperscript{low}} mice (Figure 4D).

\textbf{NOTCH3 regulates collective invasion and liver metastasis formation downstream of LIN28B-CLDN1 axis}

To identify downstream targets of LIN28B-CLDN1 axis, we performed bulk RNA sequencing of the liver tumors generated from portal vein injection of DLD-1 LIN28B\textsuperscript{high} and LoVo LIN28B\textsuperscript{high} cells with or without CLDN1 depletion. Amongst commonly downregulated genes upon CLDN1 depletion, we selected NOTCH3 as a potential downstream regulator of CLDN1 (Supplemental Figure 5) (please see the deposited supplementary document for the full list of genes measured with RNA-seq). We reasoned that cell-cell junctions (e.g., CLDN1 as a component of tight junctions) are required for the
ligand-receptor interaction to induce NOTCH-mediated signaling. Additionally, there is evidence that CLDN1 regulates intestinal homeostasis through NOTCH signaling (40). Interestingly, NOTCH3 oncogenic signaling has been implicated in invasiveness and metastasis of various cancer types (41, 42). Therefore, we hypothesized that NOTCH3 acts downstream of LIN28B-CLDN1 axis to regulate collective invasion and subsequent liver metastasis formation.

To determine whether NOTCH3 regulates LIN28B-mediated effects in vitro, we suppressed NOTCH3 expression in LoVo and DLD-1 LIN28B<sup>high</sup> cells. We first confirmed downregulation of NOTCH3 in LIN28B<sup>high</sup> shNOTCH3 and LIN28B<sup>high</sup> shCLDN1 cells by qRT-PCR (Figure 5A and B, Supplemental Figure 6A and B). Of note, we also measured CLDN1 mRNA expression in DLD-1 and LoVo LIN28B<sup>high</sup> shNOTCH3 cells and observed no significant difference between the groups, confirming that NOTCH3 acts downstream of CLDN1 (Supplemental Figure 6C). We next performed wound healing and cell aggregation assays to assess the invasion, migration, and aggregation abilities of LIN28B<sup>high</sup> shNOTCH3 CRC cells. When analyzed 48 hours after the scratch, DLD-1 LIN28B<sup>high</sup> and LoVo LIN28B<sup>high</sup> cells had significantly higher wound-healing rates compared to LIN28B<sup>high</sup> NOTCH3 knockdown cells (Figure 5C, Supplemental Figure 6D). Similarly, quantification
of cell aggregation between LIN28B\textsuperscript{high} shNOTCH3 and LIN28B\textsuperscript{high} shRNA control cells showed that the number of cell aggregates was significantly lower in NOTCH3 knockdown cells (Figure 5D, Supplemental Figure 6E). These results underscore that NOTCH3 contributes to LIN28B-induced cell aggregation and collective cell invasion.

To validate whether NOTCH3 mediates liver metastasis formation downstream of LIN28B \textit{in vivo}, LIN28B\textsuperscript{high} shNOTCH3 cells labeled with RFP were injected into the portal vein of immunocompromised mice, and the liver tissues were harvested four weeks after injection (Figure 5E). As expected, injection of LIN28B\textsuperscript{high} cells with shNTC readily formed liver metastases in both DLD-1 (10/12, 83.3%) and LoVo (7/9, 77.8%) cells (Figure 5F, Supplemental Figure 6F). Importantly, injection of LIN28B\textsuperscript{high} shNOTCH3 cells significantly reduced the formation of liver metastases in both DLD-1 cells (1/6, 16.7% and 3/10, 30%) and LoVo cells (1/6, 16.7% and 2/7, 28.6%) (Figure 5F, Supplemental Figure 6F).

\textbf{Pharmacologic inhibition of Notch signaling pathway reduces LIN28B-induced liver metastasis}

To evaluate the potential of Notch signaling as a therapeutic target, we used a $\gamma$-secretase inhibitor DAPT to study the effects of Notch signaling inhibition \textit{in vivo} and \textit{in
vitro. qRT-PCR analysis showed no significant difference in \textit{NOTCH3} mRNA expression between DAPT-treated and DMSO vehicle-treated LIN28B\textsuperscript{high} CRC cells (Figure 6A, Supplemental Figure 7A). However, DAPT treatment resulted in a significant decrease in the expression of hairy and enhancer of split homolog-1 (\textit{HES1}), a downstream effector target for mammalian Notch signaling pathway (21) (Figure 6B, Supplemental Figure 7B).

We next compared the effects of DAPT on the invasiveness of LIN28B\textsuperscript{high} CRC cells. DAPT treatment significantly reduced wound healing rates at 48 hours after the initial scratch (Figure 6C, Supplemental Figure 7C), suggesting that pharmacologic inhibition of Notch signaling reduces migration and invasion of CRC cells. Similarly, comparison of cell aggregation between DAPT treatment group and the vehicle-treated control group revealed significantly reduced number of cell aggregates upon DAPT treatment (Figure 6D, Supplemental Figure 7D).

To evaluate the effects of DAPT treatment on the formation of liver metastasis \textit{in vivo}, LIN28B\textsuperscript{high} CRC cells labeled with mCherry fluorescence were injected into the portal vein of immunocompromised mice. Two weeks after portal vein injection, the mice received intraperitoneal injection of DMSO or DAPT 3x/week for 4 weeks. At the experimental endpoint, liver tissues were harvested for analyses (Figure 6E). One hundred percent of mice
injected with DLD-1 LIN28B<sup>high</sup> cells and treated with DMSO developed liver metastases as visualized by RFP fluorescence (8/8, 100%), whereas mice treated with DAPT developed significantly fewer liver tumors (2/9, 22.2%) (Figure 6F). Similarly, mice injected with LoVo LIN28B<sup>high</sup> cells and treated with DMSO formed more liver metastasis (4/7, 57.1%) than DAPT-treated experimental group (0/8, 0%) (Supplemental Figure 7E). These results support the novel finding that LIN28B-CLDN1-NOTCH3 axis contributes to cell aggregation and collective cell invasion, and Notch signaling pathway might be a promising pharmacologic target for the treatment of metastatic colon cancer.

Pharmacologic inhibition of NOTCH3 reduces LIN28B-induced liver metastasis

To evaluate the potential of NOTCH3 as a therapeutic target, we used an anti-NOTCH3 antibody MOR20350 (43) to study the effects of NOTCH3 inhibition <i>in vivo</i> and <i>in vitro</i>. qRT-PCR analysis showed no significant difference in NOTCH3 mRNA expression between MOR20350 and vehicle-treated LIN28B<sup>high</sup> DLD-1 and LoVo cells. However, MOR20350 treatment resulted in a significant decrease in the expression of HES1 (Figure 7A, Supplemental Figure 8A). Cell migration (Figure 7B and C, Supplemental Figure 8B and C), aggregation (Figure 7D, Supplemental Figure 8D), and the propensity of the cells to
invade through the ECM (Figure 7E and F, Supplemental Figure 8E and F) were significantly
decreased in MOR20350-treated LIN28B\textsuperscript{high} cells when compared to the vehicle-treated
control group. Intraperitoneal injection of the vehicle or MOR20350 after the portal vein
injection of LIN28B\textsuperscript{high} CRC cells resulted in the inhibition of liver metastases formation
(0/8, 0%) when compared to the vehicle-treated mice (4/7, 57.1%) (Figure 7G, Supplemental
Figure 8G). These results further support the contribution of the LIN28B-CLDN1-NOTCH3
axis in cell aggregation, migration, and collective cell invasion and identify NOTCH3 as a
promising pharmacologic target for the treatment of metastatic colon cancer.

LIN28B-CLDN1-NOTCH3 axis positively correlates with metastatic progression of
human colorectal tumors

To translate our findings into human CRC cases, we quantified the expression of
LIN28B in tumor tissues obtained from CRC patients. Of 126 CRC cases observed, 60 cases
did not develop liver metastases whereas 66 cases developed liver metastases and required
partial hepatectomy (part of standard of care). Tumor samples were divided into (1) primary
colorectal tumors from patients who did not develop liver metastases, (2) primary colorectal
tumors from patients who developed liver metastases, and (3) corresponding liver metastases
from the same patients as group 2. Intriguingly, 42% of primary tumors from patients who developed liver metastases and 58% of the corresponding liver mets had high expression of LIN28B, whereas only 19% of primary tumors from patients who did not have liver mets had high LIN28B expression (Figure 8A and B). These data suggest that LIN28B expression may be upregulated in the CRC metastatic cascade.

We next stained for CLDN1 and NOTCH3 in the primary tumors and matched liver metastases collected from the CRC patients. 74% of LIN28B\textsuperscript{high} primary tumors also had high expression of CLDN1, providing evidence for positive correlation between LIN28B and CLDN1 in primary tumors (Figure 8 C and Supplemental Table 1). 48% of LIN28B\textsuperscript{high} primary tumors also had high expression of NOTCH3 (Figure 8 C and Supplemental Table 1). More relevant to the investigation of the metastatic cascade, however, 79% and 63% of LIN28B\textsuperscript{high} liver metastases had increased expression of CLDN1 and NOTCH3, respectively (Figure 8 D and Supplemental Table 1). These data suggest a positive correlation between LIN28B, CLDN1 and NOTCH3 in metastatic human CRC tissues.

To correlate LIN28B expression in primary colorectal tumors with the likelihood of developing liver metastases, we quantified the proportion of CRC patients who developed liver metastases over five years. LIN28B expression in primary tumors was associated with
patients with lower relapse-free survival, suggesting that patients with LIN28B\textsuperscript{high} primary tumors are more likely to develop liver metastases in five years when compared to patients with LIN28B\textsuperscript{low} primary tumors (Figure 8E). We also measured the expression of LIN28B, CLDN1, and NOTCH3 in primary tumors collected from metastatic CRC patients post hepatectomy. The same patients were followed over five years to track their recurrence of liver metastases. Interestingly, a greater proportion of patients with tumors that expressed high levels of LIN28B, CLDN1, and NOTCH3 developed more liver metastases following a hepatectomy procedure when compared to all other patients (Figure 8F). Together, our data indicate a positive correlation between LIN28B-CLDN1-NOTCH3 and CRC metastasis to the liver.
DISCUSSION

The RBP LIN28B is associated with tumor development, invasion, and poor prognosis in various types of cancers such as esophageal, colon, ovarian, prostate, and breast cancers (7-9, 12, 44, 45). We and others have shown that LIN28B expression is associated with metastatic behavior of cancer cells in murine xenograft models (9, 46, 47). More specifically to CRC, LIN28B expression is associated with colorectal tumorigenesis, tumor growth, cell migration, and tumor recurrence (48, 49). LIN28 functions as a negative regulator of let-7, a family of miRNA known to regulate the expression of a number of genes involved in development and cellular proliferation. Due to the role of let-7 as a tumor suppressor, let-7-dependent effects of LIN28B in cancer is currently an area of active research (13). For example, a recent study published by Qi et al. revealed a mechanism by which LIN28B promotes lung metastasis of breast cancer by building an immune-suppressive pre-metastatic niche, and this process may be dependent on the release of exosomes that express low levels of let-7 (50). Interestingly, recent studies have suggested that LIN28B may also regulate cancer progression in a manner independent of let-7 (9, 27, 51). In the present study, we investigated the mechanism by which LIN28B promotes
collective cell invasion, migration, and metastasis of CRC cells. We provide the first evidence for the direct binding of LIN28B with CLDN1 mRNA. Upon stabilization by LIN28B, CLDN1 induces expression of NOTCH3 to promote cell aggregation and collective cell invasion. Activation of this novel LIN28B-CLDN1-NOTCH3 axis mediates the metastatic cascade of CRC. In line with the established importance of cell cluster formation in cancer metastasis to secondary sites (52, 53), our data suggest that LIN28B promotes cancer cell invasion by maintaining tight junctions of colorectal epithelial cells, allowing for the formation of cellular aggregates that likely circulate as part of the metastatic cascade.

The tight junction complex is one of several clusters of proteins that reside between epithelial cells to bind the cells together and form a physiological barrier. The transmembrane protein CLDN1 is integral to the structure and function of tight junctions expressed by colonic epithelial cells (14-16). Despite being the most studied claudin in cancers to date, the role of CLDN1 in cancer progression is controversial and has been a subject of discussion in cancer research. The role of CLDN1 as a tumor suppressor has been demonstrated in prostate cancer (54), lung adenocarcinoma (55), and ER+ subtypes of breast cancers (56). Loss of CLDN1 has also been shown to be a strong predictor of disease recurrence and poor patient survival in CRC (57). By contrast, a tumor-promoting role of CLDN1 has also been
highlighted. High CLDN1 expression is found in thyroid carcinomas (58, 59) and correlate with shorter overall survival of patients with gastric (60) and ovarian (61) cancers. Additionally, TNF-α-mediated CLDN1 expression is associated with increased proliferation of pancreatic cancer cells (62). In CRC, CLDN1 overexpression has been associated with invasion and metastasis through the regulation of E-CADHERIN, MMP-9, β-catenin signaling and the PI3K/Akt pathway (63), but these studies have been descriptive and non-mechanistic.

In our study, we demonstrate that suppression of CLDN1 inhibited LIN28B-induced invasion and migration of CRC cells. Additionally, we found that suppression of CLDN1 inhibited cell aggregation. With the establishment that tumors cells circulate in clusters (64), maintenance of this clustered state by CLDN1-mediated cell adhesion may be key to acquiring the metastatic properties of CRC cells. In line with this, our findings support the notion that CLDN1 upregulation by LIN28B contributes to cell aggregation and collective cell invasion of CRC cells. Another proposed mechanism by which CLDN1 regulates cancer metastasis is by its functional role in cellular transformation. CLDN1 has been suggested to promote epithelial-mesenchymal transition (EMT), allowing for migration and metastasis in CRC (19, 65). CLDN1 may be the link between LIN28B and EMT and provide an
explanation as to how LIN28B induces EMT during CRC metastasis, a phenomenon that we
have described previously (37).

Our investigation into the downstream effectors of CLDN1 revealed NOTCH3 as
an important regulator of LIN28B-CLDN1-mediated CRC metastasis. Both down-regulation
of NOTCH3 and pharmacologic inhibition of the Notch signaling pathway led to decreased
cellular aggregation, invasion, and metastasis of CRC cells. The Notch signaling pathway
has emerged as a critical regulator of various types of cancer, leading to the generation of
Notch pathway inhibitors such as $\gamma$-secretase inhibitors (66) and monoclonal antibodies (67,
68). Despite having promising indications in hematological and solid tumors, one of the
limitations of Notch signaling inhibition is intestinal toxicity, leading to the reliance on
intermittent dosing of the inhibitors (69, 70). Targeting individual Notch1, Notch2, and
Notch3 paralogs may bypass the human toxicities of pan-Notch inhibition. Our work has
identified NOTCH3 as an important regulator of cellular aggregation, invasion, migration,
and liver metastases formation. Consistent with the findings by Varga et al. (71), inhibition
of NOTCH3 led to reduced CRC metastasis in mice, offering a rationale for specific targeting
of NOTCH3 to manage CRC metastasis.

Our analyses of tumor samples collected from CRC patients revealed a positive
correlation between LIN28B, CLDN1, and NOTCH3 expression and CRC metastasis to the liver. Furthermore, the likelihood of CLDN1 and NOTCH3 expression seems to be dependent upon LIN28B expression, further supporting our discovery that CLDN1 and NOTCH3 function downstream of LIN28B. Our clinical data suggest that LIN28B, CLDN1, and NOTCH3 may be used as potential prognostic markers of CRC metastasis to the liver. Additionally, LIN28B expression negatively correlated with disease-free survival status of the patients, implicating a greater likelihood of patients with high LIN28B-expressing tumors developing metastases.

Taken together, our results indicate that LIN28B promotes cell aggregation, invasion, and liver metastasis in CRC through post-transcriptional induction of CLDN1 and upregulation of NOTCH3. Development of new therapies that target LIN28B-CLDN1-NOTCH3 axis may provide an effective strategy for inhibiting the CRC metastasis to the liver.
METHODS

Cell culture

DLD-1 (RRID: CVCL_0248), Caco-2 (RRID: CVCL_0025) and LoVo (RRID: CVCL_0399) cells were purchased from the American Type Culture Collection (ATCC). All cell lines were authenticated by short tandem repeat profiling analysis. The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences), penicillin-streptomycin (100 IU/ml, Thermo Fisher Scientific) at 37°C in a humidified incubator with 5% carbon dioxide (CO₂). Experiments were performed within at most 15 passages after thawing. The absence of mycoplasma was confirmed every two months using MycoAlert Mycoplasma Detection Kit (Lonza, #LT07-118).

Establishment of LIN28B overexpression cell lines

LoVo and DLD-1 cells with stable LIN28B expression were established using MSCV-PIG vector plasmids provided by Dr. Joshua Mendell as described previously (9). Viral particles were generated in Phoenix A cells. The transduced CRC cells were selected by 2 µg/mL
puromycin for DLD-1 and LoVo cells, and 5 µg/mL for Caco-2 cells.

Knockdown of LIN28B, CLDN1, or NOTCH3 expression in colon cancer cells

BII-mirT3G-Puro vector was used for LIN28B knockdown in Caco-2 cells as described previously (12). For knockdown of CLDN1 and NOTCH3 expression, short hairpin RNAs (shRNAs) designed by Genecopoeia were used. After making lentiviral particles in 293T, CRC cells with LIN28B-high expression (LoVo LIN28B\textsuperscript{high}, Caco-2 Ctrl and DLD-1 LIN28B\textsuperscript{high}) were transduced using 8 µg/ml of polybrene and subjected to 30 min spinning at 1,200G. The transduced cells were selected by hygromycin. shRNA particles used are shown in Supplemental Table 2.

Quantitative real-time PCR

Total RNA was isolated using the GeneJET RNA Purification Kit (Thermo Scientific, #K0731) according to the manufacturer’s protocol. To synthesize cDNA, 1 µg of total RNA was reverse transcribed using 25 units of MultiScribe Reverse Transcriptase (Invitrogen, #4311235) and 0.5 µg of Oligo(dT) primer (Invitrogen, #18418012). Real-time PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems, #4376600)
using 1 µl of cDNA, 0.5 µM of primers and 5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, #4367659) per 10 µl reaction. Primer sequences are listed in Supplemental Table 3.

**Western blot**

Protein samples were extracted using Cell Lysis Buffer (Cell signaling Technology, #9803) containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Sigma-Aldrich, P8340). Protein concentration was quantified by the Bradford method using the Bio-Rad Protein Assay Dye (Bio-Rad, #5000006). Electrophoresis was performed with NUPAGE 4-12% Bis-Tris Gels and MOPS SDS Running Buffer (Invitrogen, #NP000102). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-FL, Millipore, #IPFL00010) using NuPAGE Transfer Buffer (Invitrogen, #NP0006). Primary antibodies used for immunoblotting are listed in Supplemental Table 4. IRDye Secondary Antibodies (LI-COR, 926-68070, 926-32213) were used before measurement. All protein measurements were normalized to GAPDH used as an endogenous control.

**mRNA stability assay**
A total $5.0 \times 10^5$ cells were seeded in 6-well culture plate. After 24 hours, culture medium was changed to medium containing 5-15 µg/ml actinomycin D (Sigma-Aldrich, A1410) to inhibit transcription. After incubation for up to 4 hours, total RNA was extracted. Quantitative real-time PCR was performed as described above.

**Cell aggregation assay**

The ability of cell aggregation was assessed by measuring the number of colonies after seeding single cells. For LoVo and DLD-1 cells, $1.0 \times 10^4$ cells were seeded into 96-well flat bottom ultra-low attachment plates (Corning, #3474). For Caco-2 cells, $5.0 \times 10^3$ cells were seeded. After incubation for 24 hours, the number of cell aggregates over 2,000 µm$^2$ was counted by Keyence BZ-X810.

**Scratch wound healing assay**

Confluent monolayer of cells in 6-well plates were scratched using a 200µl pipette tip. Images of wound closure were captured at 0, 24 and 48 hours after the scratch. The wounded area was measured using ImageJ software as previously described (72) . The wound-healing rate was normalized to the area of the initial scratch wound.
Transwell migration and invasion assay

8 μm membrane pores (#353097, Corning, NY, USA) for 24-well plates were used to measure cell migration. A total of $1.0 \times 10^5$ (Caco-2) or $5.0 \times 10^4$ (DLD-1, LoVo) cells in 100 μl serum-free medium were added to the upper chamber, and 100 μl of 10% FBS medium was added in the lower chamber. After cells were incubated at 37°C for 24 hours, non-migratory cells in the top chamber were removed by cotton swabs. To analyze invasiveness, the transwell inserts were first coated with Matrigel (Corning, #354480). Migratory or invasive cells at the bottom of the membrane were fixed in 70% ethanol and stained by 0.2% crystal violet. The number of migratory or invasive cells was counted in three different fields by microscopy (Keyence, BZ-X810).

Spheroid 3D invasion assay

$1.0 \times 10^3$ cells in 100 μl of culture medium were seeded into 96-well round bottom ultra-low attachment plates (Corning, #7007) and incubated for 3 days to form spheroid tumors. After formation of tumor spheroids, 70 μl of culture medium was carefully removed. To optimize the extracellular matrix conditions, the same amount of Matrigel (Corning, #354480) and Rat
Tail Collagen type 1 (Corning, #354236) was mixed. 70 µl of the mixed solution was added to each well and incubated at 37 °C for 30 minutes to form the gel. After 30 minutes, 100 µl of warm culture medium was added. The growth of a tumor spheroid was imaged at days 1, 2, 3 and 7 and the area of tumor cells was calculated using Image J software (72).

RNA immunoprecipitation (RIP)

The samples were extracted from CRC cells with LIN28B-high expression (LoVo LIN28B<sup>high</sup>, Caco-2 Ctrl, DLD-1 LIN28B<sup>high</sup>). LIN28B ribonucleoprotein particles were immunoprecipitated using SureBeads Protein G Magnetic Beads (Bio-Rad, 161-4023) and RIP-Assay Kit (MBL, RN1001) according to the manufacturer’s protocol. An equal amount of Normal Rabbit IgG was used as a negative control. RNA in the RIP products was analyzed by qRT-PCR. CLDN1 expression levels in the LIN28B antibody-immunoprecipitates were compared with those in the control Rabbit IgG antibody-immunoprecipitates.

Immunocytochemistry

5.0×10<sup>4</sup> cells were seeded on round coverslips in 24-well plates. After 2 days, cells were fixed for 5 minutes at -20°C in 100% methanol. Immunostaining was performed according
to Thermo Fisher Scientific’s protocol. Primary antibodies used for immunofluorescence are listed in Supplemental Table 4. Cy3-conjugated secondary antibody was obtained from Jackson Immunoresearch Laboratories (1:600, #715-165-150). Coverslips were mounted using Vectashield Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (Vector Laboratories, H-1200) and imaged by fluorescence microscopy (Keyence, BZ-X810).

Animal experiments

Female athymic nude mice (CrTac:NCr-Foxn1<sup>nu</sup>) between 6 and 8 weeks of age were obtained from Taconic Biosciences (model NCRNU). All mice were acclimated for 1 week before use. Animal experiments were conducted in accordance with a protocol approved by the Columbia University Institutional Animal Care and Use Committee.

Portal vein injection

The nude mice were anesthetized with isoflurane. 2.0×10<sup>6</sup> cells were suspended in 100 µl of PBS and were injected into the portal vein of nude mice during open laparotomy with 28G needle. After four weeks, the mice were sacrificed by CO<sub>2</sub> inhalation and the livers were removed. The resected liver tissues were fixed in 10% neutral buffered formalin for a day.
and placed in 70% ethanol prior to be embedded in paraffin. Paraffin sections of liver tissues were generated every 800 µm thickness and stained with hematoxylin and eosin (HE).

In vivo and in vitro DAPT and MOR20350 treatments

Cells were incubated with 10 µM or 20 µM DAPT dissolved in 0.1% dimethyl sulfoxide (DMSO) for 48 hours. Equal volume of DMSO was used as a negative control. 10 µg/mL of MOR20350 (Novartis) (43) was dissolved in the buffer prior to incubation with the cells for 48 hours. For in vivo experiments using DAPT, mice received 100 µl of 1 mg/kg of DAPT every other day by intraperitoneal injections for 4 weeks. For in vivo experiments using MOR20350, mice received 100 µl of 20 mg/kg of MOR20350 dissolved in dilution buffer every other day by intravenous injections for 4 weeks. Mice in the control group were given either 100 µL DMSO or IgG control antibody.

Immunohistochemistry

Antigen retrieval was performed by heating slides in 10 mM citric acid buffer using a pressure cooker. The non-specific staining was blocked by 3% hydrogen peroxide, 0.002% avidin, 0.002% biotin, and StartingBlock Blocking Buffer (ThermoFisher Scientific, #37539).
Primary antibodies used for immunohistochemistry are listed in Supplemental Table 4. Biotinylated secondary antibody was obtained from Vector Laboratories (1:200, #BA-1000). Signals were detected using ABC-HRP kit (Vector Laboratories, #PK-6100) and DAB substrate kit (Vector Laboratories, #SK-4100).

**Immunohistochemistry of human tissues**

Immunohistochemical staining of LIN28B/CLDN1/NOTCH3 was performed following standard protocols. Briefly, paraffin-embedded tissue blocks were cut into 4-µm thick sections. The tissue sections were incubated with anti-MTDH antibodies (Invitrogen, MilliporeSigma, Burlington, MA, USA; dilution 1:500), anti-CLDN1 (Cat #: 71-7800, Invitrogen, Carlsbad, CA, USA; dilution 1:1000), anti-NOTCH3 (Cat #: ab23426, Abcam, Cambridge, UK; 1:100) overnight at 4°C, incubated in Envision™+ Kits (Dako, Glostrup, Denmark), and visualized using 0.01% 3, 3-diaminobenzidine. The staining intensities varied from 0 (negative), 1+ (weak), 2+ (moderate) to 3+ (strong). The percentage of cells at each staining intensity level was calculated, and H-score was assigned using the following formula; H-score = 1 x (% cells 1+) + 2 x (% cells 2+) + 3 x (% cells 3+). High expression levels were designated as follows: LIN28B ≥ 120, CLDN1 ≥ 200, NOTCH3 ≥ 100. The score
of immunohistochemical staining was evaluated independently by two investigators.

Sample preparation for bulk RNA sequencing

Single metastatic CRC cells were isolated from metastatic liver tumors after portal vein injection. After mice were euthanized by CO₂, the liver tissues including CRC metastasis were minced and digested in culture medium containing 200 U/mL collagenase IV (Gibco, 17104019) at 37 °C for 30 minutes. The undigested tissues were removed using a 70 µm cell strainer (Fisherbrand, 22363547). After centrifugation, red blood cells were lysed by adding ACK Lysing Buffer (Gibco, A1049201). The cell pellet was washed by PBS containing 0.04% bovine serum albumin (BSA) and resuspended by FACS buffer containing 0.5% BSA, 1 mM EDTA and 0.1% sodium azide in PBS. The metastatic CRC cells were sorted by flow cytometer by the presence of mCherry fluorescence (BD Biosciences, BD Influx Cell Sorter).

Normal mouse liver tissues were used as a negative control for gating. Total RNAs were extracted using RNeasy plus micro kit (Qiagen, 74034). The RNA integrity numbers (RIN) were measured by RNA 6000 Pico Kit and 2100 Bioanalyzer (Agilent). Samples with RIN >7 were used for sequencing. Two biological replicates per sample were prepared.
Collection of patient samples

Human CRC and corresponding liver metastasis tissues were obtained from patients who underwent surgical resection at the Department of Frontier Surgery (for primary CRCs) or General Surgery (for liver metastases), Chiba University Hospital, Japan, from 2005 to 2014. All patients were histologically diagnosed with primary colorectal adenocarcinoma. The study protocol has been approved by the Ethics Committees of Chiba university and written informed consent was obtained from each patient before surgery.

Data Availability

The RNA-sequencing FASTQ files have been deposited in the Gene Expression Omnibus (GEO) database of the National Institutes of Health (NIH) and are accessible under accession number GSE234513.

Statistics

All analyses and experiments were repeated at least three independent times (minimum three technical replicates and three biological replicates). Statistical analyses were performed using 2-tailed Student’s t-tests when comparing two groups, or standard ANOVA analysis with
Tukey’s multiple comparison test when comparing three or more groups. For animal experiments and microarray datasets, associations between binary categories were analyzed by Pearson’s chi-squared test or Fisher’s exact test. For analyses using patient samples and data collected from patients, accumulative rates were calculated by using the Kaplan-Meier method and the significance of difference in survival rate was analyzed by the log-rank test. Fisher’s exact tests were used to compare the distribution of a categorical variable in a group with the distribution in another group with $p<0.05$ as statistically significant. Statistical analysis was performed using GraphPad Prism software version 8.0. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. All data and analyses were reviewed with our Biostatistics Shared Resource.

**Study approval**

Animal experiments were conducted in accordance with a protocol approved by the Columbia University Institutional Animal Care and Use Committee. For collection of patient samples, the study protocol has been approved by the Ethics Committees of Chiba university, and written informed consent was obtained from each patient before surgery.
This work is supported by NIH 7R01CA277795-22, NIH 5P30CA0113696 and the American Cancer Society. We thank the Biostatistics, Confocal and Specialized Microscopy Shared Resource (funded in part through NIH/NCI Cancer Center Support Grant P30CA013696), Genetically Modified Mouse Models and Molecular Pathology Shared Resources at Columbia University’s Herbert Irving Comprehensive Cancer Center, and the Children’s Hospital RNA-seq core (Dr. Renata Pellegrino). We thank Novartis for providing the MOR20350 antibody. We are grateful to Dr. Premal Shah, and members of the Rustgi, Lengner and Sims labs for advice and input. The authors declare no conflicts of interest.
AUTHOR CONTRIBUTIONS

Y.M., K.S., K.S., A.E.S., N.S., and A.K.R. contributed to conceptualization and methodology, data curation, formal analyses, and validation. Y.M., K.S., K.S., A.E.S., and A.K.R. wrote the manuscript. Y.M., K.S., K.S., A.E.S., N.S., H.M., M.O., P.A.S., C.J.L., and A.K.R. reviewed and edited the manuscript. A.K.R. acquired funding, managed project administration, and supervised the study. The basis for the designation of co-first authors was equal contribution to experimental design, generation of data, and data analysis.
DECLARATION OF INTERESTS

The authors have declared that no conflict of interest exists.
REFERENCES


FIGURE LEGENDS

Figure 1. LIN28B promotes cell migration and collective cell invasion in colon cancer cells. (A) A comparison of wound closure in DLD-1 cells with an empty vector versus LIN28B overexpression (left), and in Caco-2 cells with shControl versus shLIN28B for LIN28B knockdown. Scale bar represents 200 μm. (B) The wound area in (A) is quantified using ImageJ. The wound healing rate is defined as (initial wound area - wound area at 24 or 48 hours) / initial wound area. Data were analyzed using a Student's t-test or one-way ANOVA and are represented as mean ± SEM (n=7). (C) A schematic model of the tumor spheroid 3D invasion assay. (D) 3D invasion of DLD-1 cells with an empty vector versus LIN28B overexpression, and of Caco-2 cells with shControl versus shLIN28B is shown at day 0 and day 7. The scale bar represents 500 μm. (E) The area of the tumor cells in (D) is quantified using ImageJ. Data were analyzed using a Student's t-test or one-way ANOVA, expressed relative to the corresponding value at day 0, and are represented as mean ± SEM (n=3).
A

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B

**A**

**B**

**C**

**D**

**E**

**F**

Time after Actinomycin D treatment
Figure 2. LIN28B directly binds to and stabilizes CLDN1 mRNA. (A) Western blot analysis of adherens and tight junctions from DLD-1 and Caco-2 cells. (B) Quantification of band densities measured by Western blot in (A), normalized to GAPDH. Data were analyzed using a Student's t-test or one-way ANOVA, expressed relative to the corresponding value in empty vector or control groups, and represented as means ± SEM (n=3). (C) Immunofluorescent staining of CLDN1 (red) in DLD-1 and Caco-2 cells. Nuclei were stained by DAPI (blue). Scale bar = 100 μm. (D) qRT-PCR analysis of claudin-1 mRNA in RNA immunoprecipitation samples. Data were analyzed using a Student’s t-test, expressed relative to the corresponding value of IgG IP samples, and represented as means ± SEM (n=3). (E) Representative images of Western blots of samples precipitated using IgG or anti-LIN28B antibodies. (F) Quantification of CLDN1 mRNA after Actinomycin D treatment. Data were analyzed using a two-way ANOVA, expressed relative to the corresponding value at 0h, and represented as means ± SEM (n=3).
Figure 3. LIN28B-induced cell aggregation and collective cell invasion is dependent on CLDN1. (A) Representative images of cell aggregates formed from DLD-1 LIN28B\textsuperscript{high} and Caco-2 cells with CLDN1 knockdown after a 24-hour incubation in ultra-low attachment plates. Scale bar = 500 μm. (B) Quantification of the number of cell aggregates counted by a Keyence BZ-X810 microscope. (C) Representative images of wound closure of DLD-1 LIN28B\textsuperscript{high} and Caco-2 cells with CLDN1 knockdown. Scale bar = 200 μm. (D) The wound healing rate in (C) was analyzed using ImageJ. Data are represented as means ± SEM (n=3). (E) 3D invasion of DLD-1 LIN28B\textsuperscript{high} and Caco-2 cells with CLDN1 knockdown at 1 and 7 days after incubation. Scale bar = 500 μm. (F) Tumor area in (E) was quantified by ImageJ. Data are expressed relative to the corresponding value at day 1 and represented as means ± SEM. All graphs were generated from data analyzed by one-way ANOVA followed by Tukey’s multiple comparison test.
A. Cell culture

DLD-1LoVo

Empty Vector (EV), shNTC
LIN28B\(^{\text{high}}\), shNTC
LIN28B\(^{\text{high}}\), shCLDN1 #1
LIN28B\(^{\text{high}}\), shCLDN1 #2

B. DLD-1

\[ \text{% of mice with liver metastases} \]

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C. LoVo

LoVo EV shNTC
LoVo LIN28B\(^{\text{high}}\) shNTC

H&E
GFP
LIN28B
CLDN1

D. 

Vil\(^{\text{Cre;Lin28b\text{low}}}\)
Vil\(^{\text{Cre;Lin28b\text{high}}}\)

H&E
LIN28B
CLDN1
Figure 4. LIN28B-induced CLDN1 upregulation promotes metastatic liver tumor formation. (A) Schematic drawing of the workflow used to study the effects of CLDN1 knockdown in liver metastasis formation in vivo. (B) Quantification of the proportion of mice with metastatic liver tumors (n ≥ 6); left graph depicts results from injection of DLD-1 cells, right graph depicts results from injection of LoVo cells. Statistical analyses were performed using Chi-square test. (C) Representative images of H&E and IHC staining for GFP, LIN28B and CLDN1 in metastatic liver tumors from LoVo with empty vector and shNTC group (left) and LoVo with LIN28B overexpression and shNTC group (right). Scale bar = 50 μm. (D) Representative images of H&E and IHC staining for LIN28B and CLDN1 in intestinal tumors from a Villin\textsuperscript{Cre};Lin28\textsuperscript{low} mouse (left, n=1) and Villin\textsuperscript{Cre};Lin28\textsuperscript{high} mice (right, n=3). Scale bar = 50 μm.
Portal vein injection of 2 million CRC cells 6-8 weeks old mice

Metastatic tumor formation

Cell culture + LIN28B high, shNTC LIN28B high, shNOTCH3 #1
LIN28B high, shNOTCH3 #2

4 weeks

shNTC shNOTCH3-1 shNOTCH3-2

% of mice with liver metastases

DLD-1 LIN28B high

Resect liver tissue

DLD-1 LIN28B high

% of wound healed

DLD-1 LIN28B high

# of cell aggregates

DLD-1 LIN28B high

Relative Notch3 mRNA

shNTC shCLDN1-1 shCLDN1-2

EV shNTC shCLDN1-1 shCLDN1-2

% of mice with liver metastases

p=0.0082
Figure 5. NOTCH3 regulates collective cell invasion and liver metastasis formation downstream of LIN28B-CLDN1 axis. (A) qRT-PCR analysis of NOTCH3 mRNA in DLD-1 LIN28B\textsuperscript{high} cells with or without shCLDN1. (B) qRT-PCR analysis of NOTCH3 mRNA in DLD-1 LIN28B\textsuperscript{high} cells with or without shNOTCH3. (C) Wound closure of DLD-1 LIN28B\textsuperscript{high} cells with or without shNOTCH3. Scale bar = 200 μm (left). Wound healing rates were quantified by ImageJ (right graph). Data represented as means ± SEM (n=3). (D) Representative images of aggregates formed from DLD-1 LIN28B\textsuperscript{high} cells transduced by shNOTCH3 (left). The number of cell aggregates were counted by Keyence BZ-X810 (right graph). Data represented as means ± SEM (n=3). (E) Schematic drawing of the workflow used to study the effects of NOTCH3 KD on portal vein injection model of metastatic CRC. (F) Representative images of RFP expressed by DLD-1 LIN28B\textsuperscript{high} tumors in the liver. Images were taken using Keyence BZ-X810. Graphs in A, B, C, and D were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. Graph in F was analyzed by Chi-square test.
Portal vein injection of 2 million CRC cells into 6-8 weeks old mice. Cell culture of DLD-1 + LIN28Bhigh LoVo. DMSO or 20µM DAPT. % of wound healed.

**F**

DLD-1 LIN28Bhigh

% of mice with liver metastases

8/8
2/9

Control
DAPT
Figure 6. Pharmacologic inhibition of Notch signaling reduces LIN28B-induced liver metastasis. (A) qRT-PCR analysis of NOTCH3 mRNA in DLD-1 LIN28B<sup>high</sup> cells treated with DMSO, 10 µM DAPT, or 20 µM DAPT. (B) qRT-PCR analysis of HES1 mRNA in DLD-1 LIN28B<sup>high</sup> cells treated with DMSO, 10 µM DAPT, or 20 µM DAPT. (C) Representative images of the wound closure scratch assay performed using DLD-1 LIN28B<sup>high</sup> cells treated with DMSO, 10 µM DAPT, or 20 µM DAPT. Scale bar = 200 µm. Area of the wound was measured by using ImageJ. Data represented as means ± SEM (n=3). (D) Representative images of the aggregation assay performed using DLD-1 LIN28B<sup>high</sup> cells treated with DMSO, 10 µM DAPT, or 20 µM DAPT. The number of cell aggregates were counted using Keyence BZ-X810. Data represented as means ± SEM (n=3). (E) Schematic drawing of the workflow used to study the effects of DAPT injection in a portal vein injection model of CRC metastasis. (F) Representative images of RFP expressed by DLD-1 LIN28B<sup>high</sup> tumors in the liver. Images were taken using Keyence BZ-X810. Graphs in A, B, C, and D were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. Graph in F was analyzed by Fisher’s exact test.
Figure 7. Pharmacologic inhibition of NOTCH3 reduces LIN28B-induced liver metastasis. (A) qRT-PCR analysis of NOTCH3 and HES1 mRNA in DLD-1 LIN28Bhigh cells treated with vehicle or 10 µg/mL MOR20350 for 48 hours. (B) Representative images and quantification of the wound closure scratch assay performed using DLD-1 LIN28Bhigh cells treated with vehicle or MOR20350. Scale bar = 200 µm. Area of the wound was measured by using ImageJ. (C) Representative images and quantification of the 3D aggregation assay performed using DLD-1 LIN28Bhigh cells treated with the vehicle or MOR20350. The number of cell aggregates were counted using Keyence BZ-X810. (D) Representative images and quantification of 2D invasion assay. Cells that have invaded through the 8 µm pore and the ECM were counted using Keyence BZ-X810. (E) Representative images and quantification of 2D migration assay. Cells that have migrated through the 8 µm pore were counted using Keyence BZ-X810. (F) Representative images and quantification of the 3D invasion assay performed using DLD-1 LIN28Bhigh cells treated with the vehicle or MOR20350. Scale bar = 500 µm. (G) Representative images of RFP expressed by DLD-1 LIN28Bhigh tumors in the liver. Images were taken using Keyence BZ-X810. Data in graphs A-F are represented as means ± SEM and were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. Graph in G was analyzed by
Fisher’s exact test.
Primary CRC

\[ \text{Corresponding Liver mets} \]

\[ \text{Primary tumors from patients with no liver mets} \]
\[ \text{Primary tumors from patients with liver mets} \]
\[ \text{Corresponding liver mets} \]

Disease-free survival of all CRC patients

Proportion of patients with no liver mets (%)

\[ \text{LIN28B}^\text{high} \]
\[ \text{LIN28B}^\text{low} \]

\[ p<0.0001 \]

\[ n=39 \]
\[ n=87 \]

Disease-free survival of CRC patients with liver mets post hepatectomy

Proportion of patients with no recurring liver mets (%)

\[ \text{LIN28B}^\text{high} \]
\[ \text{CLDN1}^\text{high} \]
\[ \text{NOTCH3}^\text{high} \]
\[ \text{others} \]

\[ p=0.0484 \]

\[ n=25 \]
\[ n=41 \]

Corresponding liver metastasis

Primary tumors from patients with liver mets

Primary tumors from patients with no liver mets

\[ \text{LIN28B}^\text{high} \]
\[ \text{LIN28B}^\text{low} \]

\[ p<0.0001 \]

\[ n=25 \]
\[ n=41 \]
Figure 8. LIN28B-CLDN1-NOTCH3 axis positively correlates with metastatic progression of human colorectal tumors. (A) Primary colonic tumors and liver metastases were collected from CRC patients. Upon IHC staining, tumors were quantified based on their high or low expression. Data expressed as a proportion of all tumors (%). (B) Representative images of tumors stained with an anti-LIN28B antibody. Scale bar = 100 µm. (C) Representative images of primary colorectal tumors stained with LIN28B, CLDN1, and NOTCH3. Scale bar = 100 µm. (D) Representative images of corresponding liver metastases stained with LIN28B, CLDN1, and NOTCH3. Scale bar = 100 µm. (E) Graph depicting disease-free survival of all CRC patients. For each patient, frequency by which they develop liver metastases was tracked over five years. Data expressed as the proportion of CRC patients who do not have metastatic liver tumor (y-axis) at the time of analyses (x-axis). (F) Graph depicting disease-free survival of CRC patients that have undergone hepatectomy due to liver metastases. For each patient, frequency by which they develop a new liver tumor was tracked over five years. Data expressed as the proportion of CRC patients who do not develop another metastatic liver tumor (y-axis) at the time of analyses (x-axis). “Others” group includes patients with tumors that do not have high expressions of LIN28B, CLDN1, and NOTCH3. Graph in A and table in C and E were analyzed by Chi-square test. Graphs G and
H were analyzed by log rank test.