Iron Uptake via DMT1 Integrates Cell Cycle with JAK-STAT3 Signaling to Promote Colorectal Tumorigenesis

Xiang Xue[1], **Sadeesh K. Ramakrishnan**[1], **Kevin Weisz**[1], **Daniel Triner**[1], **Liwei Xie**[1], **Durga Attili**[3], **Asha Pant**[4], **Balázs Gy rffy**[9,10], **Mingkun Zhan**[11], **Christin Carter-Su**[1,4], **Karin M. Hardiman**[2], **Thomas D. Wang**[4,7,8], **Michael K. Dame**[3], **James Varani**[3], **Dean Brenner**[3,4], **Eric R. Fearon**[3,4,5], and **Yatrik M. Shah**[1,4,*]

[1]Department of Molecular & Integrative Physiology, University of Michigan, Ann Arbor, MI, 48109

[2]Department of Surgery, University of Michigan, Ann Arbor, MI, 48109

[3]Department of Pathology, University of Michigan, Ann Arbor, MI, 48109

[4]Department of Internal Medicine, University of Michigan, Ann Arbor, MI, 48109

[5]Department of Human Genetics, University of Michigan, Ann Arbor, MI, 48109

[7]Department of Biomedical Engineering, University of Michigan, Ann Arbor, MI, 48109

[8]Department of Mechanical Engineering, University of Michigan, Ann Arbor, MI, 48109

[9]MTA TTK Lendület Cancer Biomarker Research Group, Budapest 1117, Hungary

[10]2nd Department of Pediatrics, Semmelweis University, Budapest 1085, Hungary

[11]Department of Plastic Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian, China, 350001

Summary

Dietary iron intake and systemic iron balance are implicated in colorectal cancer (CRC) development, but the means by which iron contributes to CRC are unclear. Gene expression and functional studies demonstrated that the cellular iron importer, divalent metal transporter 1 (DMT1), is highly expressed in CRC through hypoxia inducible factor 2α-dependent transcription. Colon-specific *Dmt1* disruption resulted in a tumor-selective inhibitory effect of

*Correspondence author: Tel: +1 734 6150567; Fax: +1 734 9368813; shahy@umich.edu.

proliferation in mouse colon tumor models. Proteomic and genomic analysis identified an iron-regulated signaling axis mediated by cyclin dependent kinase 1 (CDK1), JAK1 and STAT3 in CRC progression. A pharmacological inhibitor of DMT1 antagonized the ability of iron to promote tumor growth in a CRC mouse model and a patient-derived CRC enteroid orthotopic model. Our studies implicate a growth-promoting signaling network instigated by elevated intracellular iron levels in tumorigenesis, offering molecular insights into how a key dietary component may contribute to CRC.

eTOC

Although high iron levels, including dietary red meat, have been associated with increased cancer risk, the underlying mechanisms are unknown. Xue et al. show that the iron importer DMT1 is responsible for the intracellular iron accumulation in colorectal cancer and that iron activates CDK1-JAK1-STAT3 signaling to support tumor cell proliferation.
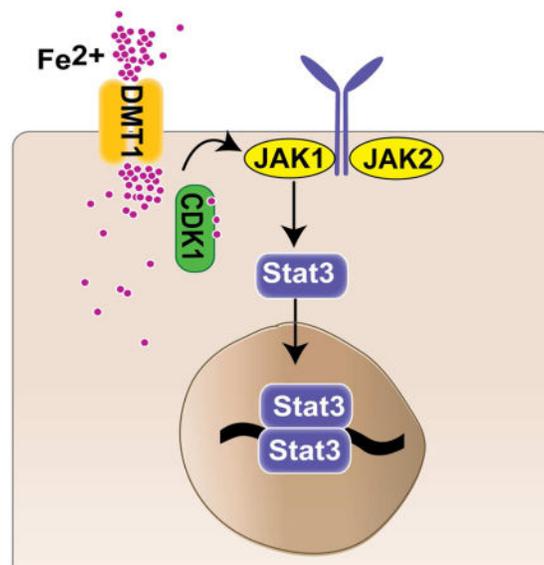
Introduction

Colorectal cancer (CRC) is the third most common type of cancer and the third leading cause of cancer-related death in the United States (Siegel et al., 2014). The mutations identified in CRC alter signaling pathways and gene expression as well as metabolite requirements, allowing cancer cells to acquire a growth and survival advantage. The metabolic differences between normal and cancer cells are being interrogated to uncover potential therapeutic targets and approaches. Nevertheless, at present, little is known about micronutrient metabolism in cancer. Several large epidemiological studies demonstrate a direct correlation between dietary iron intake and/or systemic iron levels and cancer risk in several cancers including CRC (Merk et al., 1990; Nelson, 2001; Osborne et al., 2010; Zacharski et al., 2008). Moreover, iron overload caused by mutations in the *HFE* gene (HFE C282Y and H63D homozygous) and/or high dietary heme iron consumption (red meat) is associated with increased CRC risk (Cross et al., 2010; Osborne et al., 2010). Consistent

with the human epidemiological data, in selected mouse models of intestinal tumor development, the tumor-prone mice fed low-iron diet had reduced tumor multiplicity, size, and progression; whereas, the mice fed high-iron diet had enhanced tumor phenotypes (Radulescu et al., 2012; Xue et al., 2012). The expression of multiple iron metabolism and transport genes is significantly altered in CRC relative to normal colon epithelium, leading to an accumulation of intratumoral iron (Brookes et al., 2006; Xue and Shah, 2013b; Xue et al., 2012). Some studies have shown iron can increase oxidative stress and WNT signaling (Brookes et al., 2006; Seril et al., 2003). Despite the data implicating iron is involved in colon tumorigenesis, the mechanisms through which iron metabolism might contribute to the development and progression of CRC remains unknown. In addition, it is unclear if targeting intratumoral iron might be an effective treatment or prevention strategy for CRC.

Understanding the role of intratumoral iron in colon tumorigenesis *in vivo* has been limited in part because most of the previously employed mouse intestinal tumor models, such as $Apc^{Min/+}$ mice, manifest mainly small intestinal adenomas (Hinoi et al., 2007). The small intestine and colon have markedly different roles in iron homeostasis. The small intestine is essential in maintaining intestinal iron absorption and systemic iron homeostasis. Thus, small intestine-preferential or intestinal-wide disruption of iron transporters and exporters lead to systemic and extensive changes in iron homeostasis (Donovan et al., 2005; Gunshin et al., 2005). Here, to address the role of iron in colon tumorigenesis, colon-selective gene targeting, and patient-derived colorectal tumor enteroids were used. We find that colorectal tumor epithelium, but not adjacent normal tissues, is dependent on elevated intracellular iron levels for growth, mediated via hypoxia inducible factor (HIF)-2α dependent regulation of the apical iron transporter divalent metal transpoter-1 (DMT1, also known as solute carrier family [SLC] 11, member 2 [SLC11A2]). In-depth characterization of the iron interactome in colon tumors highlighted a key-signaling axis. Iron increases cyclin-dependent kinase (CDK) 1 which activates the Janus kinase (JAK) 1/signal transducer and activator of transcription (STAT) 3 signaling pathway.

Results

Tumor epithelial DMT1 is increased in CRC patients

Prior work with a small collection of CRC patient samples indicated that expression of selected iron transport genes were increased in tumor tissues (Brookes et al., 2006). To investigate further potential factors and mechanisms involved in intratumoral iron accumulation in CRC, analysis of iron transport genes was undertaken using RNA-sequencing data from The Cancer Genome Atlas (TCGA) CRC analyses. This dataset contains over 200 CRC and adjacent normal epithelium samples (Cancer Genome Atlas, 2012). Several genes critical for iron regulation were differentially expressed, with reduced expression of aconitase 1 (*ACO1*, also known as *IRP1*), cytochrome b reductase 1 (*CYBRD1*), ferritin heavy chain (*FTH1*), hephaestin *(HEPH)* and ferroportin (*FPN,* also known as *SLC40A1*), and increased expression of hepcidin antimicrobial peptide (*HAMP*), hemochromatosis Type 2 Protein (*HFE2*), iron-responsive element binding protein 2 (*IREB2*), *SLC11A1, DMT1* and transferrin receptor *(TFRC)* (Figure 1A). The changes observed in DMT1 were further studied in 8 primary CRCs and paired adjacent normal

epithelium (Figure 1B). Indeed, increased *DMT1* expression in CRC compared to adjacent normal tissue is a consistent change that has been observed in several studies (Brookes et al., 2006; Xue and Shah, 2013b; Xue et al., 2012). However, it is unclear if protein expression correlates with increased *DMT1* mRNA and if DMT1 is localized to the tumor epithelium. We found the two-fold change in mRNA reflects a robust change in DMT1 protein expression, which goes from nearly undetectable to highly expressed and localized to the apical membrane in colorectal tumor epithelium (Figure 1C and 1D). These data suggest that DMT1 expression is increased and apical iron transport would be enhanced in CRC.

Colon-selective disruption of DMT1 decreases sporadic and colitis-associated colon tumors

To further address whether local colonic iron has an impact on tumor growth, $Dmt1^{F/F}$ mice were crossed with colon-selective caudal type homeobox 2 (CDX2) P-NLS Cre mice (Hinoi et al., 2007) to disrupt DMT1 in the colon (CDX2 $Dmt1^{F/F}$) (Figure S1A). Colon-selective disruption of DMT1 did not result in a systemic or tissue-specific decrease of iron (Figure S1B and S1C). Body weights, colon length, and Ki67 staining were not different under basal conditions or following DSS-induced injury and regeneration compared with wild-type littermates (Figure S1D–S1G). These findings demonstrate that DMT1 function is not essential in the colon. To evaluate the role of DMT1 in a colon tumorigenesis model, the CDX2 $Dmt1^{F/F}$ mice were crossed to $Apc^{F/+}$ mice (CDX2 $Dmt1^{F/F}/Apc^{F/+}$). This is a colon-preferential tumor model (Hinoi et al., 2007). DMT1 disruption in this tumor model led to significantly reduced tumor number, tumor burden, and tumor cell proliferation (Figure 2A–2D). CDX2-mediated Cre recombination can be seen as early as E9.5 in the caudal region of embryos (Hinoi et al., 2007). No clear colonic developmental issues were observed in the CDX2 $Dmt1^{F/F}$ mice. However, to further confirm the role of DMT1 in colon tumorigenesis temporally and conditionally, $Dmt1^{F/F}$ mice were crossed with a colon-specific CDX2-Cre^{ERT2} mouse (CDX2^{ERT2} $Dmt1^{F/F}$). Similarly, colon-specific disruption of DMT1 after tamoxifen (TAM) injection did not result in systemic or tissue-specific decreases in iron (Figure S1H–S1J). Also, susceptibility to DSS-induced acute colitis, as reflected by body weight, colon length, Ki67 staining, and histological analysis was not altered compared to littermate control animals (Figure S1K–S1N). CDX2^{ERT2} $Apc^{F/+}$ mice, following TAM treatment have a long latency period for colon tumor development and only a limited number of tumors arise spontaneously (Feng et al., 2013). This is likely due in part to a lower TAM-induced CDX2-Cre^{ERT2}-mediated targeting of one *Apc* allele in colon epithelium compared to the efficiency of *Apc* inactivation mediated by CDX2-NLS Cre. However, treatment of the CDX2^{ERT2} $Apc^{F/+}$ mice with DSS resulted in enhanced tumor formation, and disruption of colonic DMT1 significantly reduced the DSS-induced tumor development and iron accumulation (Figure S2A). The expression of DMT1 in tumors from colon-specific DMT1 inactivation was significantly reduced, albeit not to levels observed with the CDX2-Cre (Figure S2B and 2E). Tumor number, tumor size, and tumor cell proliferation in mice with colon-specific DMT1 inactivation was significantly reduced when maintained on an iron replete diet (35 mg/kg iron, 35 Fe) (Figure 2F and 2G). Moreover, we demonstrate that high-iron diet (1000 mg/kg iron, 1000 Fe) conditions increased tumor number and burden compared to iron-replete diet and this potentiation is also attenuated following DMT1 disruption (Figure 2F and 2G). Mice with a single copy deletion of DMT1

(CDX^{ERT2}*Dmt1*$^{F/+}$/*Apc*$^{F/+}$) had similar basal expression levels of *Dmt1* compared to wild type littermates, but the increase of *Dmt1* mRNA expression that is observed in tumors was lost (Figure 2E). A decrease in tumor number, tumor size, and tumor cell proliferation was observed in these mice, demonstrating that the increase in *Dmt1* observed in tumor tissue is critical to drive tumorigenesis (Figure 2F and 2G). Consistent with the Ki67 staining results, BrdU incorporation assays showed that tumor cell proliferation was decreased when DMT1 was disrupted (Figure S2C). In contrast, tumor apoptosis, vascularization and macrophage infiltration were not altered by DMT1 inactivation (Figure S2C and S2D). These results suggest intratumoral iron accumulation via DMT1 is an essential mechanism for neoplastic but not normal colon epithelium.

HIF-2α is essential in the activation of DMT1 in colon tumors

Previously, we have shown DMT1 is a direct target gene of HIF-2α (Shah et al., 2009). The increase in DMT1 in tumors has been suggested to be dependent on the β-catenin pathway (Radulescu et al., 2012). To further understand if HIF-2α is also involved, an intestine or colon specific disruption of HIF-2α in the *Apc*$^{min/+}$ mouse intestinal tumor model and the colon-specific sporadic CRC model were assessed. Interestingly, disruption of HIF-2α significantly reduced tumoral DMT1 (Figure 3A and 3B). In addition, colon-specific disruption of *Hif-2α* in *Apc*$^{F/+}$ tumor mouse model led to significantly reduced tumor number and intratumoral iron accumulation (Figure 3C and 3D). Thus, the lack of DMT1 induction following HIF-2α disruption may contribute to the reduced tumor burden. However, to be noted, other mechanisms may also explain or contribute to the reduction of tumor burden after loss of HIF-2α (Xue and Shah, 2013a). Mutations in oncogenes and tumor-suppressor genes such as *APC*, *KRAS*, *TP53*, *PIK3CA* as well as *MYC* expression have been well characterized during the progression of CRC (Cancer Genome Atlas, 2012). To understand if these defects contribute to DMT1 induction by HIF-2α, DMT1 promoter reporter assays were undertaken (Figure 3E–3H). Expression of β-catenin and c-MYC, but not control vector (pcDNA3.1), KRAS, P53 and PI3K, significantly increased HIF-2α mediated transcriptional activation of DMT1 (Figure 3E). These data are consistent with previous work demonstrating a role for β-catenin and c-MYC in mediating DMT1 increase in CRC (Radulescu et al., 2012). However, these data clearly demonstrate the central role of HIF-2α in the transcriptional regulation of DMT1. To fully elucidate genes critical for DMT1 activation, a high-throughput screening approach was used. HCT116 cells overexpressing HIF-2α and DMT1 promoter luciferase were tested with a siRNA-based screening using a druggable target library (Figure 3I). A panel of 230 genes were identified that led to more than 90% reduction of HIF-2α-induced DMT1 promoter activity. These genes were further filtered with the TCGA gene expression datasets and genes predictive of relapse free survival were validated with independent siRNAs, which identified a group of 15 genes involved in DMT1 activation. Together, these results provide insights for the mechanism of DMT1 activation and provide potential targets to limit DMT1 activation in tumors.

Iron is essential in JAK-STAT3 signaling in colon cancer

Previous reports have demonstrated that iron can increase WNT signaling and iron chelation can potently inhibit WNT signaling (Brookes et al., 2008; Song et al., 2011). Thus, we

investigated whether activation of WNT signaling could be the major mechanism for iron-driven colon tumorigenesis. Surprisingly and in contrast to the previous reports, iron treatment did not increase WNT signaling activity in nine different colon cancer cell lines studied (Figure S3A). To identify other mechanisms by which intratumoral iron regulates cellular proliferation and colorectal tumorigenesis, whole genome expression profiles were assessed in colorectal tumor tissue of CDX2 $Dmt1^{F/F}/Apc^{F/+}$ and their littermate controls. Several immune and inflammatory NF-κB responses were enriched in the tissues, based on gene set enrichment analysis (GSEA) (Figure 4A). Genes critical in the inflammatory response were confirmed by qPCR (Figure 4B). Iron did not directly activate NF-κB signaling in CRC cells, suggesting that other inflammatory transcription factors may be regulated by iron (Figure S3B and S3C). Mining the TCGA gene expression dataset in conjunction with the TCGA reverse phase protein arrays (Cerami et al., 2012) uncovered a connection indicating that expression levels of p-STAT3 were positively correlated with the expression level of iron transporters in colorectal tumors (Figure 5A). These data are consistent with the GSEA analysis in which several of the inflammatory genes identified were also STAT3-regulated target genes (Jarnicki et al., 2010). To further understand if STAT3 activation is dependent on DMT1 expression, p-STAT3 levels were assessed in tumors from colon-specific *Dmt1* knockout mice compared to littermate controls. Western blotting analysis and immunofluorescence staining of p-STAT3 clearly demonstrated that STAT3 activation was significantly attenuated in tumors where DMT1 was disrupted compared to tumors with intact DMT1 expression (Figure 5B and 5C). Moreover, treating colon-derived cell lines with high iron led to a dose- and time-dependent activation of STAT3 (Figure S4A–S4C). STAT3 can be activated by cytokines such as interleukin (IL) 6 via JAK signaling (O'Shea et al., 2013; Yu et al., 2009; Zhong et al., 1994) (Figure S4D). JAK1 and JAK2, but not JAK3, could potently activate STAT3 in two CRC-derived cell lines, and this activation was partially reversed by the iron chelator deferoxamine (DFO) (Figure 5D). DFO also decreased IL6-induced p-STAT3, whereas iron supplementation or DMT1 overexpression activated p-STAT3, in a JAK-dependent manner (Figure 5E–5G). Similarly, the STAT3 inhibitor (STAT3i) S3I-201 also abolished iron- and IL6-activated p-STAT3 (Figure 5H and S4E). To test whether STAT3 is critical for iron-driven tumor growth *in vivo*, we optimized a dose of STAT3i, which efficiently inhibited p-STAT3 in the dysplastic colon tissues from *Apc*-disrupted mice, but not p-STAT3 in normal colon tissues from wild-type littermate controls (Figure S4F). STAT3i was then tested in an iron-induced colorectal tumor model, whereby CDX2^{ERT2} $Apc^{F/+}$ tumor mouse model was placed on 35 Fe or 1000 Fe with or without STAT3i. The 35 Fe was used to assess the baseline STAT3 activity and the 1000 Fe was used to evaluate the role of STAT3i in iron-dependent colon tumorigenesis. High-iron treatment robustly increased colon tumor formation, tumor number, tumor size, tumor burden and tumor cell proliferation in this colitis-associated tumor mouse model (Figure 5I, 5J and S4G). STAT3i treatment dramatically reduced colon tumorigenesis in the high-iron treated mice. To test the potential translational application of STAT3 inhibition, the effect of STAT3 knockdown was assessed in patient-derived tumor enteroid models. Patient-derived colorectal tumor enteroids are an ideal model to study the effect of iron on intestinal epithelial cells. These cell models resemble colorectal tumors with respect to the spectrum of mutations that are observed (van de Wetering et al., 2015). The three-dimensional (3D) models show cell polarization, with apical localization of the

iron transporter DMT1 and preserved tight junctions (Figure S5A), and the enteroid lines can survive long-term in defined serum- and transferrin iron-free conditions. To demonstrate that iron is essential for the growth of the tumor enteroids, two patient-derived enteroid lines, one isolated from a primary adenoma and the other from an adenocarcinoma were assessed. Equal number of enteroids were plated and established. The enteroids were treated with DFO. A dose-dependent decrease in the size of adenoma and adenocarcinoma enteroid was observed (Figure S5B). Ki67 staining confirmed a significant decrease in proliferation following DFO treatment. To assess the direct effect of apical iron transport, the enteroids were grown in iron-reduced RPMI 1640 media and supplemented with ferrous sulfate (FS, 10 or 100 μM) (Figure S5C). The size and cell proliferation of the FS-treated enteroids were robustly increased, compared to the untreated enteroids. This provides an optimal model to study downstream pathways essential in iron-induced growth. Stable tumor enteroids with STAT3 knockdown (shSTAT3) were confirmed by qPCR and Western blot analysis (Figure 5K). Consistent with the STAT3 inhibitor study, the growth of tumor enteroids was increased by iron treatment, whereas knockdown of STAT3 reduced the iron-enhanced growth and proliferation of the tumor enteroids (Figure 5L). These data demonstrate that activation of JAK-STAT3 signaling is essential for the effect of iron in enhancing colon tumorigenesis.

Iron-dependent CDK1 kinase activity is critical for JAK1-STAT3 activation

Our data has demonstrated that iron is essential and sufficient to activate STAT3 signaling. Iron is known to generate reactive oxygen species (ROS) through the Fenton reaction and oxidative stress is a known trigger for STAT3 activation (Carballo et al., 1999; Dixon and Stockwell, 2014). However, antioxidants such as butylated hydroxyanisole (BHA) and N-Acetyl-L-cysteine (NAC) could not inhibit the iron-mediated activation of STAT3 (Figure S5D). Furthermore, ROS levels, nitrosative marker nitrotyrosine and lipid peroxidation marker 4-Hydroxynonenal (4-HNE) in colon tumors were not changed in mice with colon-specific disruption of *Dmt1* compared to littermate control (Figure S5E and S5F). It is known that divalent metals such as magnesium (Mg) can directly associate with kinases and are critical for their function (Bao et al., 2011). To identify intracellular proteins that may be involved in iron signaling, ferrous iron-coated or metal-free beads were utilized. Several proteins will bind to beads coated with iron non-specifically through charge interactions, however we confirmed that the approach could recover transferrin, a known iron-interacting protein (Figure S6A). To identify additional proteins that could bind to iron, ferrous iron-coated or metal-free beads were incubated with HCT116 or SW480 cell lysates and associated proteins were identified by a mass spectrometry-based proteomics approach (Figure 6A). A total of 169 proteins including 17 kinases were identified and 7 kinases were further validated (Table S2 and S3). To understand if any of these kinases provide a mechanistic link to iron-induced STAT3 activity, luciferase reporter assays were performed. Interestingly, only CDK1 could potentiate the activation of STAT3 by IL6 (Figure S6B). We further confirmed that CDK1 could bind to iron in CRC-derived cell lines by Western blot analysis (Figure 6B). Furthermore, iron could bind to CDK1 from mouse colon tumor lysates (Figure 6C), and this binding was direct, as assessed using recombinant CDK1 (Figure S6C). To further demonstrate that iron and CDK1 resulted in a biologically relevant response, kinase assays were performed in the absence or presence of iron. The data clearly demonstrates that iron was critical for CDK1 activity (Figure 6D). To further understand if

iron via CDK1 was important for STAT3 activation we performed STAT3-dependent reporter assays. The increase in STAT3 reporter activity that was driven by IL-6 was potentiated by CDK1 (Figure 6E). The addition of iron further increased the activity, whereas chelation by DFO completely abrogated CDK1 induced potentiation of STAT3 activity. To assess if CDK1 is essential for iron and IL6 induced STAT3 activity, siRNA or CDK1/2 inhibitor (CDKi) Dinaciclib was used to inhibit CDK1 expression or function. In the STAT3 reporter assay, siRNA-mediated knockdown of CDK1 resulted in decreased STAT3 activity following IL6 treatment (Figure 6F). In addition, CDK1 siRNA attenuated IL6- and iron-mediated STAT3 activation as assesses by p-STAT3 in CRC-derived cell lines (Figure 6G and H). Consistent with the siRNA data, CDKi inhibited IL6- and iron-mediated STAT3 activation (Figure 6I and J). To understand the downstream JAKs that are critical for CDK1 potentiation, JAK1 and JAK2 were assessed (JAK3 had only minimal activity on the STAT3 reporter assay). CDK1 could potentiate JAK1- (but not JAK2-) STAT3 activity, whereas CDK1 siRNA reduced JAK1-dependent STAT3 activity on the STAT3 reporter assay (Figure 6K and L). This potentiation was shown to be iron-dependent as DFO treatment attenuated the increase (Figure 6M). Next, we performed a phospho-transfer assay, where we could monitor CDK1 mediated phosphorylation of JAK1 by a luminescence-based detection system. In the absence of iron, recombinant CDK1 did not increase phosphorylation of recombinant JAK1. However, the addition of iron increased this phosphorylation (Figure 6N). This provides direct evidence that iron is required for CDK1-mediated JAK/STAT3 activity. To test the potential translational application of CDKi, patient-derived enteroids were treated with Dinaciclib. At the indicated doses CDKi did not affect the growth and cell proliferation of enteroids under iron-reduced conditions. However, the CDKi reduced the iron-enhanced cell growth and proliferation in both colon adenoma and adenocarcinoma-derived enteroids (Figure 6O). These data indicate that CDK1 is an important downstream pathway for iron in CRC.

Pharmacological inhibition of DMT1 decreases colon tumor growth

To study whether DMT1 could be pharmacologically inhibited in colon tumors *in vivo* a DMT1-specific inhibitor (DMT1i) XEN 602 was utilized (Cadieux et al., 2012). DMT1i dose-dependently decreased iron absorption in a hyper-absorption mouse model *in vivo* (Figure S7A). To study whether DMT1 could be pharmacologically inhibited in colon tumors *in vivo*, CDX2^{ERT2} $Apc^{F/+}$ mice with established colon tumors were treated with DMT1i. Pharmacological inhibition of DMT1 significantly reduced tumor number and tumor burden (Figure 7A). Moreover, p-STAT3 activation, tumor iron accumulation, and tumor cell proliferation were also decreased following DMT1i treatment (Figure S7B and S7C). To test the translational application, patient-derived enteroids were assessed. DMT1i did not affect the growth and cell proliferation of patient-derived enteroids under iron-reduced conditions, but inhibited the iron-induced cell growth and proliferation in both colon adenoma (Figure 7B) and adenocarcinoma-derived enteroids (data unshown). To conclusively show that DMT1 is an effective therapeutic target in CRC, human tumor enteroids were intrarectally implanted in NOD/SCID mice. These tumors can engraft in the mouse colon, which is demonstrated by a mini colonoscope (Figure 7C). Moreover we confirmed the engrafted tumors by a human specific DMT1 staining at 2 weeks following intrarectal injections, which showed a robust signal in the engrafted tumor, but not in mouse

colon tumors (Figure 7D). Following engraftment at 2-weeks the mice were treated with DMT1i for an additional 2-weeks. The colons were stained with hDMT1 and PCNA to clearly indicate proliferation in our engrafted tumors. DMTi significantly and specifically decreased the tumor cell proliferation as observed by quantitating cells positive for hDMT1 and PCNA (Figure 7E). This not only establishes the role of DMT1 as a therapeutic target, but we have optimized a patient-derived orthotopic CRC model. Together, the findings highlight a DMT1-Fe^{2+}-CDK1-JAK1-STAT3 signaling axis essential in colorectal tumor growth (Figure 7F).

Discussion

Through loss- and gain- of function experiments we demonstrate that increased intratumoral iron is essential for colon tumorigenesis. Our studies and data offer in-depth insights into a key signaling axis initiated by tumor epithelial HIF-2α transcription, leading to an increase in apical iron transporter DMT1. Apical transport of iron leads to direct activation of CDK1, which in turn promotes the initiation and sustained signaling of the oncogenic JAK1-STAT3 pathway. Data from epidemiological studies, mouse models, and cell lines have supported the critical role of iron in CRC (Cross et al., 2010; Merk et al., 1990; Nelson, 2001; Osborne et al., 2010; Zacharski et al., 2008; Radulescu et al., 2012; Xue et al., 2012; Brookes et al., 2006; Seril et al., 2003). High dietary iron intake or patients with iron overload have increased risk for CRC (Cross et al., 2010; Osborne et al., 2010). Patients with lower iron or in mouse models treated with low iron diet exhibit a decrease in CRC (Ashmore et al., 2015; Bastide et al., 2015; Nelson, 2001). The majority of previously described mouse genetic models of intestinal tumorigenesis mainly develop small intestinal adenoma (Johnson and Fleet, 2013). The small intestine and colon vastly differ with respects to the relative levels of luminal iron (Blachier et al., 2007). Due to these issues, it has been difficult to fully understand the role and mechanisms of local and systemic changes in iron homeostasis in colon tumor progression in mice. Based on the use of colon-selective mouse tumor models, colon-derived cell lines and patient-derived enteroid models, the findings presented here offer a clear and convincing support for the role of iron in colon tumor development and progression. The present work demonstrates that STAT3 is an essential link between high iron levels and CRC progression. STAT3 signaling is frequently activated in malignant cells and capable of inducing a large number of genes that are crucial for promoting tumor cell proliferation, reducing cell apoptosis, increasing tumor invasion and suppressing anti-tumor immunity (Yu et al., 2009). Numerous carcinogens, growth factors and inflammatory cytokines activate STAT3. A well-characterized pathway for STAT3 activation in CRC is mediated via JAK family tyrosine kinases, following stimulation of cells by inflammatory cytokines, such as IL6 and IL11 (Putoczki et al., 2013). In mouse models, CRC-derived cell lines and CRC patients, we have shown that iron activates STAT3 signaling, which is essential for progression of colorectal tumors under high dietary iron. These data demonstrate high iron is sufficient in activating STAT3 signaling. Moreover, iron is also essential in the activation of STAT3 by IL6 in cell lines and tumors. Following iron chelation or disruption of DMT1 in the tumors, IL6-induced STAT3 signaling was significantly attenuated.

JAKi completely abolished iron- and DMT1-induced STAT3, demonstrating that iron-mediated activation of STAT3 is dependent on JAKs. To identify pathways which link iron to STAT3 signaling in tumor epithelium, an unbiased proteomic approach was utilized to identify proteins that could directly bind to iron. We believe ferrous iron to be the main form of iron that acts as a signaling co-factor, since ferrous iron is the preferred form to be transported intercellular via DMT1. However, free iron is very low in cells and metalation of kinase substrates could be through a chaperone-mediated mechanism. The iron chaperone poly(rC) binding proteins (PCBPs) can bind to ferrous iron with low micromolar affinity and deliver iron to substrates (Shi et al, 2008). Future work is needed to better understand the mechanisms of iron mobilization to substrates identified in the present work. Here we offer findings indicating that iron may be a key and direct signaling co-factor. CDK1 was one of the 17 protein kinases that could bind with iron. CDK1 is a kinase important in promoting cell mitosis (Satyanarayana and Kaldis, 2009). CDK1 activity is highly elevated in colorectal tumor tissues compared to normal tissues (Salh et al., 1999) and predicts distant metastasis risk in stage II CRC (Zeestraten et al., 2012). JAK1 contains 8 canonical CDK1 phosphorylation motifs (Ser/Thr–Pro–X–Lys/Arg) (Tak et al., 2006; Xue et al., 2008), and future studies are needed to validate the importance of these sites in JAK1-STAT3 signaling. The direct regulation of CDK1 activity by iron provides mechanistic data integrating iron levels to cell cycle regulation, and is consistent with previous work, demonstrating an essential role of iron in regulating cell cycle progression (Le and Richardson, 2002; Nurtjahja-Tjendraputra et al., 2007). Similar integration of divalent metals to cell proliferation has been described for copper. Copper can directly bind MEK1 and this interaction is essential for activation of ERK1/2 (Brady et al., 2014). Currently, it is not clear if direct binding of iron to CDK1 is essential to increase activity. We are assessing mechanisms and motifs required for iron to bind to proteins. Phosphorylation is the most commonly studied post-translational modification of proteins, with a major role in mediating extracellular and intracellular signals and enabling cells to adapt to a rapidly changing environment. The significance of iron in the activation of the other kinases and proteins in addition to CDK1, needs to be determined and future studies will focus on understanding specific pathways regulated by iron.

The present work identifies a pathway by which iron can contribute to CRC progression. However, previous work has demonstrated that iron is critical in WNT signaling, DNA synthesis, ROS-induced cell damage, and P53 modulation (Brookes et al., 2008; Dixon et al., 2012; Dixon and Stockwell, 2014; Le and Richardson, 2002; Shen et al., 2014). Studies to discern the contribution of each pathway are required to understand their importance and the possible crosstalk among pathways. However, the present work clearly indicates an important role of DMT1 in regulating intratumoral iron levels. Interestingly, DMT1 has no apparent role in regulating normal colon epithelial homeostasis, demonstrating that intestinal epithelial cells can acquire iron for local use through transferrin-bound iron or through other iron transporters such as SLC39A14 (also know as ZIP14) (Jenkitkasemwong et al., 2015). The tumor epithelium is highly dependent on DMT1 for iron acquisition and growth. Disruption of DMT1 in the small intestine leads to progressive and severe anemia (Gunshin et al., 2005). However, using a DMT1i we demonstrated a therapeutic dosing scheme that results in decrease of colon tumors and tumor cell proliferation with minimal systemic

effects. Moreover, the possibility of combining DMT1i with a colon-specific delivery agent may even further increase the efficacy of DMT1 inhibition for possible CRC prevention and treatment.

In conclusion, our findings support the notion that local depletion of iron, inhibition of DMT1 function and iron influx, and/or inhibition of JAK-STAT3 activity in colon tissues might be powerful therapeutic approaches for the treatment of CRC and perhaps even prevention in certain subsets of patients at elevated risk of CRC.

EXPERIMENTAL PROCEDURES

Patient-derived colorectal tumor enteroids

Patient-derived colorectal tumor enteroids were generated as previously described (Dame et al., 2014).

Animals and treatments

Full details on the mouse lines and treatments are found in Supplemental Information. All animal studies were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Quantitative real-time reverse transcriptase PCR

mRNA was measured by Real Time RT-PCR (Life Technologies, Carlsbad, CA; primers listed in Table S1). Quantification cycle (Cq) values were normalized to β-actin and expressed as fold difference from controls.

Determination of serum and tissue iron, tissue ROS

Serum and tissue non-heme iron was quantitated as previously described (Anderson et al., 2013). ROS was determined using dichlofluorescein diacetate (DCF).

High-throughput screening

HCT116 cells overexpressing HIF-2α and DMT1 promoter luciferase were tested with the siRNA druggable target screen. 230 genes were identified to inhibit more than 90% of HIF-2α-induced DMT1 promoter luciferase activity. Genes that demonstrated increased expression in CRC tissues and predicted decreased tumor free survival were validated with independent siRNAs.

Microarray

Microarray analysis was done as previously described (Xue et al., 2014). The full data set is available on the Gene Expression Omnibus database accession number GSE65104.

Proteomic analysis of the iron interactome

Fe^{2+} conjugated beads or metal-free beads were obtained from Affiland (Liege, Belgium). Transferrin or lysates from HCT116, SW480 cell or mouse colon tumor were incubated with Fe^{2+} conjugated beads or metal-free beads for 2 hours and washed three times with EDTA-

Author Manuscript

free Triton lysis buffer. Proteins were eluted and used for Westerns or trypsin digestion and mass spectrometry analysis.

Data analysis

Data are presented as individual data points and error bars represent the standard error of the mean (SEM). P values were calculated by independent t test, paired t-test, one-way ANOVA, Dunnett t test, and two-way ANOVA. Organoid size, immunofluorescence staining and western blot analysis were quantified with Image J.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

Anderson ER, Taylor M, Xue X, Ramakrishnan SK, Martin A, Xie L, Bredell BX, Gardenghi S, Rivella S, Shah YM. Intestinal HIF2alpha promotes tissue-iron accumulation in disorders of iron overload with anemia. Proc Natl Acad Sci U S A. 2013; 110:E4922–4930. [PubMed: 24282296]

Ashmore JH, Rogers CJ, Kelleher SL, Lesko SM, Hartman TJ. Dietary Iron and Colorectal Cancer Risk: A Review of Human Population Studies. Crit Rev Food Sci Nutr. 2015

Bao ZQ, Jacobsen DM, Young MA. Briefly bound to activate: transient binding of a second catalytic magnesium activates the structure and dynamics of CDK2 kinase for catalysis. Structure. 2011; 19:675–690. [PubMed: 21565702]

Bastide NM, Chenni F, Audebert M, Santarelli RL, Tache S, Naud N, Baradat M, Jouanin I, Surya R, Hobbs DA, et al. A central role for heme iron in colon carcinogenesis associated with red meat intake. Cancer Res. 2015; 75:870–879. [PubMed: 25592152]

Blachier F, Vaugelade P, Robert V, Kibangou B, Canonne-Hergaux F, Delpal S, Bureau F, Blottiere H, Bougle D. Comparative capacities of the pig colon and duodenum for luminal iron absorption. Can J Physiol Pharmacol. 2007; 85:185–192. [PubMed: 17487259]

Brady DC, Crowe MS, Turski ML, Hobbs GA, Yao X, Chaikuad A, Knapp S, Xiao K, Campbell SL, Thiele DJ, Counter CM. Copper is required for oncogenic BRAF signalling and tumorigenesis. Nature. 2014; 509:492–496. [PubMed: 24717435]

Brookes MJ, Boult J, Roberts K, Cooper BT, Hotchin NA, Matthews G, Iqbal T, Tselepis C. A role for iron in Wnt signalling. Oncogene. 2008; 27:966–975. [PubMed: 17700530]

Brookes MJ, Hughes S, Turner FE, Reynolds G, Sharma N, Ismail T, Berx G, McKie AT, Hotchin N, Anderson GJ, et al. Modulation of iron transport proteins in human colorectal carcinogenesis. Gut. 2006; 55:1449–1460. [PubMed: 16641131]

Cadieux JA, Zhang Z, Mattice M, Brownlie-Cutts A, Fu J, Ratkay LG, Kwan R, Thompson J, Sanghara J, Zhong J, Goldberg YP. Synthesis and biological evaluation of substituted pyrazoles as blockers of divalent metal transporter 1 (DMT1). Bioorg Med Chem Lett. 2012; 22:90–95. [PubMed: 22154351]

Cancer Genome Atlas N. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012; 487:330–337. [PubMed: 22810696]

Carballo M, Conde M, El Bekay R, Martin-Nieto J, Camacho MJ, Monteseirin J, Conde J, Bedoya FJ, Sobrino F. Oxidative stress triggers STAT3 tyrosine phosphorylation and nuclear translocation in human lymphocytes. J Biol Chem. 1999; 274:17580–17586. [PubMed: 10364193]

Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov. 2012; 2:401–404. [PubMed: 22588877]

Cross AJ, Ferrucci LM, Risch A, Graubard BI, Ward MH, Park Y, Hollenbeck AR, Schatzkin A, Sinha R. A large prospective study of meat consumption and colorectal cancer risk: an investigation of potential mechanisms underlying this association. Cancer Res. 2010; 70:2406–2414. [PubMed: 20215514]

Dame MK, Jiang Y, Appelman HD, Copley KD, McClintock SD, Aslam MN, Attili D, Elmunzer BJ, Brenner DE, Varani J, Turgeon DK. Human colonic crypts in culture: segregation of immunochemical markers in normal versus adenoma-derived. Lab Invest. 2014; 94:222–234. [PubMed: 24365748]

Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer AJ, Cantley AM, Yang WS, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell. 2012; 149:1060–1072. [PubMed: 22632970]

Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. Nat Chem Biol. 2014; 10:9–17. [PubMed: 24346035]

Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S, Andrews NC. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. Cell Metab. 2005; 1:191–200. [PubMed: 16054062]

Feng Y, Sentani K, Wiese A, Sands E, Green M, Bommer GT, Cho KR, Fearon ER. Sox9 induction, ectopic Paneth cells, and mitotic spindle axis defects in mouse colon adenomatous epithelium arising from conditional biallelic Apc inactivation. Am J Pathol. 2013; 183:493–503. [PubMed: 23769888]

Gunshin H, Fujiwara Y, Custodio AO, Direnzo C, Robine S, Andrews NC. Slc11a2 is required for intestinal iron absorption and erythropoiesis but dispensable in placenta and liver. J Clin Invest. 2005; 115:1258–1266. [PubMed: 15849611]

Hinoi T, Akyol A, Theisen BK, Ferguson DO, Greenson JK, Williams BO, Cho KR, Fearon ER. Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. Cancer Res. 2007; 67:9721–9730. [PubMed: 17942902]

Jarnicki A, Putoczki T, Ernst M. Stat3: linking inflammation to epithelial cancer - more than a "gut" feeling? Cell Div. 2010; 5:14. [PubMed: 20478049]

Johnson RL, Fleet JC. Animal models of colorectal cancer. Cancer Metastasis Rev. 2013; 32:39–61. [PubMed: 23076650]

Le NT, Richardson DR. The role of iron in cell cycle progression and the proliferation of neoplastic cells. Biochim Biophys Acta. 2002; 1603:31–46. [PubMed: 12242109]

Merk K, Mattsson B, Mattsson A, Holm G, Gullbring B, Bjorkholm M. The incidence of cancer among blood donors. Int J Epidemiol. 1990; 19:505–509. [PubMed: 2262240]

Nelson RL. Iron and colorectal cancer risk: human studies. Nutr Rev. 2001; 59:140–148. [PubMed: 11396694]

Jenkitkasemwong S, Wang CY, Coffey R, Zhang W, Chan A, Biel T, Kim JS, Hojyo S, Fukada T, Knutson MD. SLC39A14 Is Required for the Development of Hepatocellular Iron Overload in Murine Models of Hereditary Hemochromatosis. Cell Metab. 2015; 22:138–150. [PubMed: 26028554]

Nurtjahja-Tjendraputra E, Fu D, Phang JM, Richardson DR. Iron chelation regulates cyclin D1 expression via the proteasome: a link to iron deficiency-mediated growth suppression. Blood. 2007; 109:4045–4054. [PubMed: 17197429]

O'Shea JJ, Holland SM, Staudt LM. JAKs and STATs in immunity, immunodeficiency, and cancer. N Engl J Med. 2013; 368:161–170. [PubMed: 23301733]

Osborne NJ, Gurrin LC, Allen KJ, Constantine CC, Delatycki MB, McLaren CE, Gertig DM, Anderson GJ, Southey MC, Olynyk JK, et al. HFE C282Y homozygotes are at increased risk of breast and colorectal cancer. Hepatology. 2010; 51:1311–1318. [PubMed: 20099304]

Putoczki TL, Thiem S, Loving A, Busuttil RA, Wilson NJ, Ziegler PK, Nguyen PM, Preaudet A, Farid R, Edwards KM, et al. Interleukin-11 is the dominant IL-6 family cytokine during gastrointestinal tumorigenesis and can be targeted therapeutically. Cancer Cell. 2013; 24:257–271. [PubMed: 23948300]

Radulescu S, Brookes MJ, Salgueiro P, Ridgway RA, McGhee E, Anderson K, Ford SJ, Stones DH, Iqbal TH, Tselepis C, Sansom OJ. Luminal iron levels govern intestinal tumorigenesis after Apc loss in vivo. Cell Rep. 2012; 2:270–282. [PubMed: 22884366]

Salh B, Bergman D, Marotta A, Pelech SL. Differential cyclin-dependent kinase expression and activation in human colon cancer. Anticancer Res. 1999; 19:741–748. [PubMed: 10216486]

Satyanarayana A, Kaldis P. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene. 2009; 28:2925–2939. [PubMed: 19561645]

Seril DN, Liao J, Yang GY, Yang CS. Oxidative stress and ulcerative colitis-associated carcinogenesis: studies in humans and animal models. Carcinogenesis. 2003; 24:353–362. [PubMed: 12663492]

Shah YM, Matsubara T, Ito S, Yim SH, Gonzalez FJ. Intestinal hypoxia-inducible transcription factors are essential for iron absorption following iron deficiency. Cell Metab. 2009; 9:152–164. [PubMed: 19147412]

Shen J, Sheng X, Chang Z, Wu Q, Wang S, Xuan Z, Li D, Wu Y, Shang Y, Kong X, et al. Iron metabolism regulates p53 signaling through direct heme-p53 interaction and modulation of p53 localization, stability, and function. Cell Rep. 2014; 7:180–193. [PubMed: 24685134]

Shi H, Bencze KZ, Stemmler TL, Philpott CC. A cytosolic iron chaperone that delivers iron to ferritin. Science. 2008; 320:1207–1210. [PubMed: 18511687]

Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014; 64:9–29. [PubMed: 24399786]

Song S, Christova T, Perusini S, Alizadeh S, Bao RY, Miller BW, Hurren R, Jitkova Y, Gronda M, Isaac M, et al. Wnt inhibitor screen reveals iron dependence of beta-catenin signaling in cancers. Cancer Res. 2011; 71:7628–7639. [PubMed: 22009536]

Tak YS, Tanaka Y, Endo S, Kamimura Y, Araki H. A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2-Dpb11. EMBO J. 2006; 25:1987–1996. [PubMed: 16619031]

van de Wetering M, Francies HE, Francis JM, Bounova G, Iorio F, Pronk A, van Houdt W, van Gorp J, Taylor-Weiner A, Kester L, et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. Cell. 2015; 161:933–945. [PubMed: 25957691]

Xue X, Ramakrishnan S, Anderson E, Taylor M, Zimmermann EM, Spence JR, Huang S, Greenson JK, Shah YM. Endothelial PAS domain protein 1 activates the inflammatory response in the intestinal epithelium to promote colitis in mice. Gastroenterology. 2013; 145:831–841. [PubMed: 23860500]

Xue X, Ramakrishnan SK, Shah YM. Activation of HIF-1alpha does not increase intestinal tumorigenesis. Am J Physiol Gastrointest Liver Physiol. 2014; 307:G187–195. [PubMed: 24875099]

Xue X, Shah YM. Hypoxia-inducible factor-2alpha is essential in activating the COX2/mPGES-1/PGE2 signaling axis in colon cancer. Carcinogenesis. 2013a; 34:163–169. [PubMed: 23042097]

Xue X, Shah YM. Intestinal iron homeostasis and colon tumorigenesis. Nutrients. 2013b; 5:2333–2351. [PubMed: 23812305]

Xue X, Taylor M, Anderson E, Hao C, Qu A, Greenson JK, Zimmermann EM, Gonzalez FJ, Shah YM. Hypoxia-inducible factor-2alpha activation promotes colorectal cancer progression by dysregulating iron homeostasis. Cancer Res. 2012; 72:2285–2293. [PubMed: 22419665]

Xue Y, Ren J, Gao X, Jin C, Wen L, Yao X. GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. Mol Cell Proteomics. 2008; 7:1598–1608. [PubMed: 18463090]

Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer. 2009; 9:798–809. [PubMed: 19851315]

Zacharski LR, Chow BK, Howes PS, Shamayeva G, Baron JA, Dalman RL, Malenka DJ, Ozaki CK, Lavori PW. Decreased cancer risk after iron reduction in patients with peripheral arterial disease: results from a randomized trial. J Natl Cancer Inst. 2008; 100:996–1002. [PubMed: 18612130]

Zeestraten EC, Maak M, Shibayama M, Schuster T, Nitsche U, Matsushima T, Nakayama S, Gohda K, Friess H, van de Velde CJ, et al. Specific activity of cyclin-dependent kinase I is a new potential predictor of tumour recurrence in stage II colon cancer. Br J Cancer. 2012; 106:133–140. [PubMed: 22108518]

Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science. 1994; 264:95–98. [PubMed: 8140422]

Highlights

- Iron accumulation in CRC is dependent on HIF-2α-induced iron importer DMT1.

- Genetic disruption or pharmacological inhibition of DMT1 reduces CRC.

- Iron-CDK1 interaction activates JAK1-STAT3 signaling in CRC.

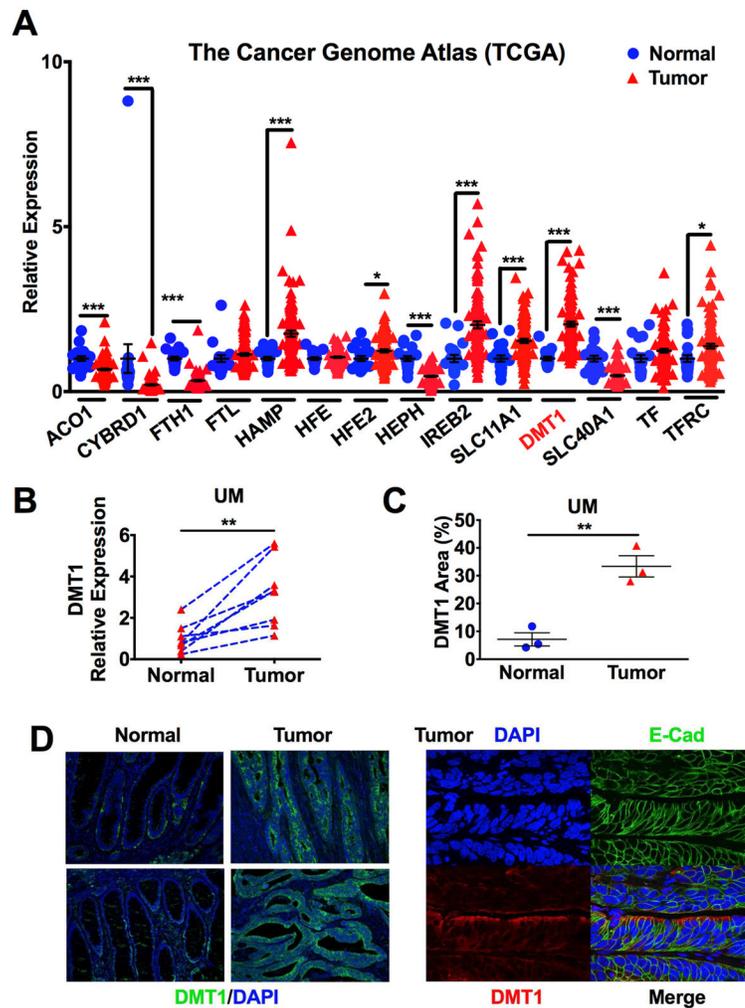- Inhibition of CDK1 or STAT3 antagonizes iron-driven cell proliferation in CRC.

Figure 1. DMT1 is overexpressed in CRC

(**A**) Studies of iron regulatory genes determined by RNA-Seq in the TCGA database and (**B**) gene expression analysis of *DMT1* by qPCR in 8 pairs of primary human CRCs and adjacent normal tissues collected at University of Michigan (UM). (**C**) Quantification of DMT1 staining and (**D**) representative fluorescent microscopy in CRCs and adjacent normal tissues (left), and confocal images demonstrating apical colocalization of DMT1 and E-Cadherin (E-cad) co-staining (right). *p<0.05, **p<0.01 and ***p< 0.001 and error bars represent SEM.
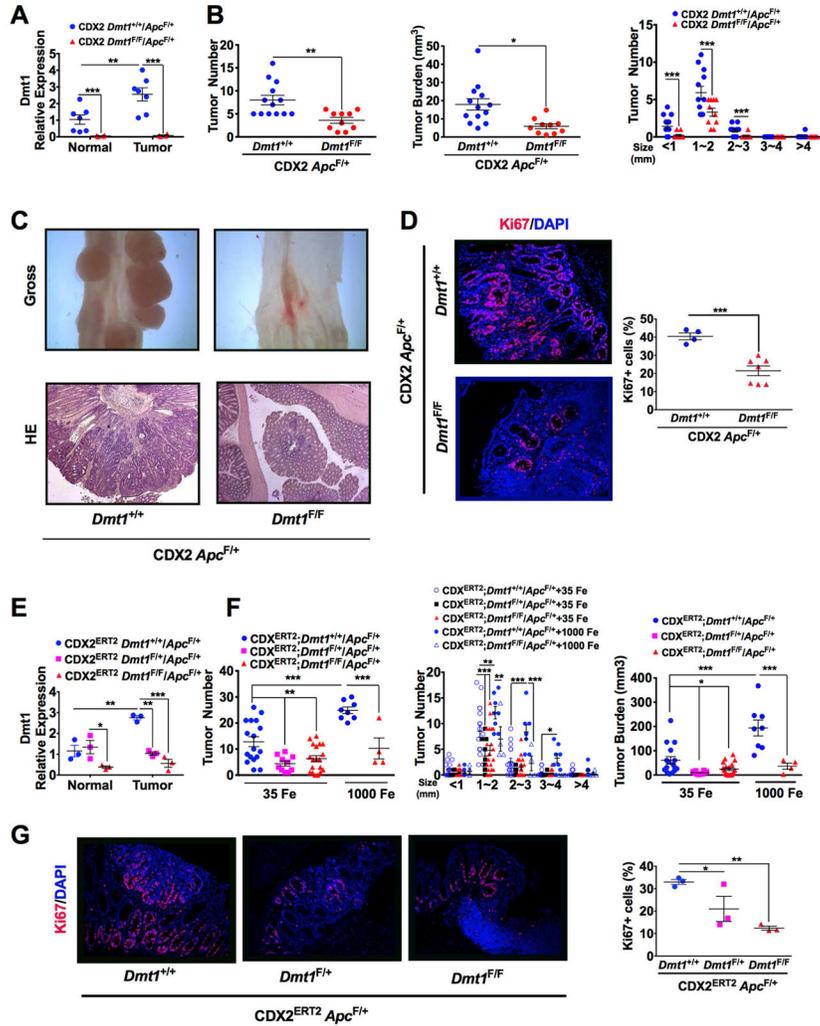
Figure 2. Colon-selective disruption of DMT1 decreases sporadic and colitis-associated colon tumors

(**A**) Gene expression of *Dmt1* in CRCs and their adjacent normal tissues, (**B**) tumor number and burden, (**C**) gross image and H&E staining, and (**D**) representative images and quantification of Ki67 staining of CRCs from CDX2 *Dmt1*F/F/*Apc*F/+ mice and their littermate controls at 3 months old. (**E**) Gene expression of *Dmt1* in CRCs and their adjacent normal tissues, (**F**) tumor number and burden, (**G**) quantification of Ki67 staining of colon tumors from CDX2ERT2 *Dmt1*F/F/*Apc*F/+, CDX2ERT2 *Dmt1*F/+/*Apc*F/+ mice and their littermate controls. *p<0.05, **p<0.01 and ***p< 0.001 and error bars represent SEM. See also Figure S1 and S2.
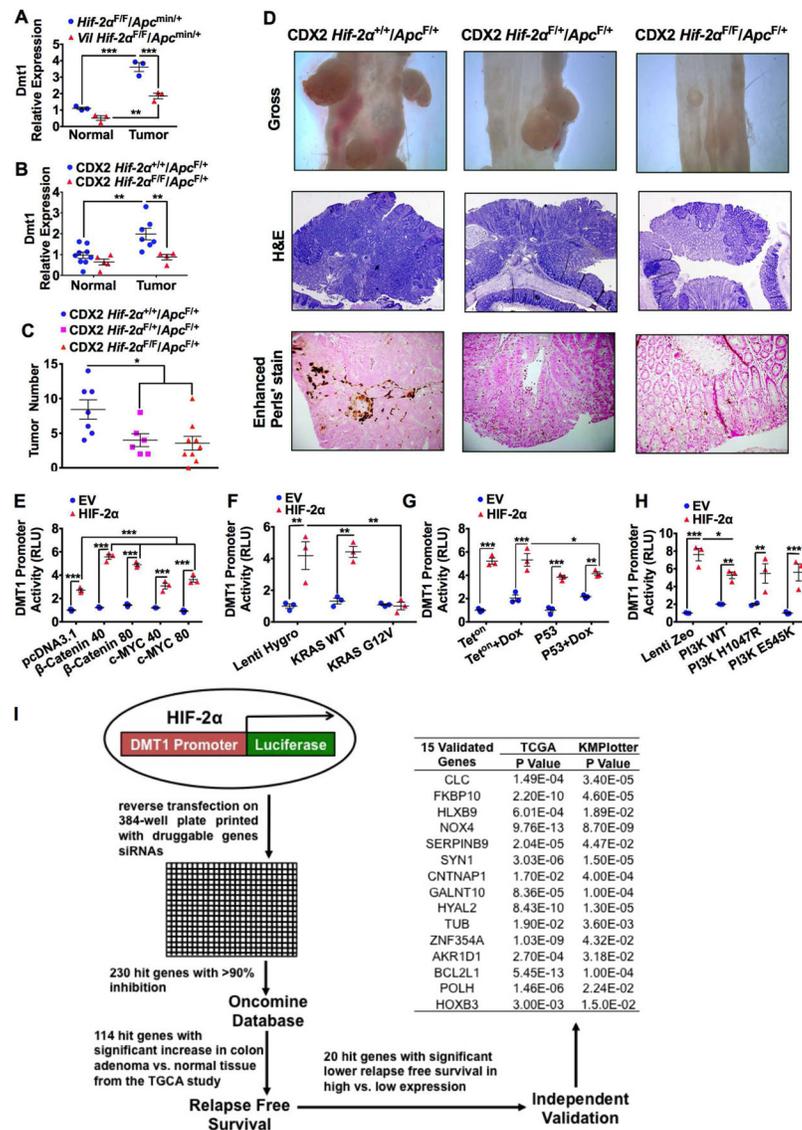
Figure 3. HIF-2α is essential in the transcriptional regulation of DMT1

Dmt1 expression in CRCs and adjacent normal tissues from Vil *Hif-2α*^F/F^/*Apc*^min/+^ mice (**A**), CDX2 *Hif-2α*^F/F^/*Apc*^F/+^ mice (**B**) and their littermate controls. (**C**) Tumor number, (**D**) gross image, H&E and enhanced Perls' staining of CRCs, from CDX2 *Hif-2α*^F/F^/*Apc*^F/+^, CDX2 *Hif-2α*^F/+^/*Apc*^F/+^ mice and their littermate controls at 3 months old. (**E–H**) DMT1 promoter luciferase assay in HCT116 cells. (**I**) High throughput screening for genes critical for DMT1 activation. *p<0.05, **p<0.01 and ***p< 0.001 and error bars represent SEM.

Figure 4. Inflammatory responses are increased in iron-driven colon tumorigenesis
(**A**) Enrichment plot and heatmap derived by gene set enrichment analysis (GSEA) for genes in regulation of immune response and inflammatory response. (**B**) Gene expression of inflammatory genes in colon tumors (T) and adjacent normal tissues (N) from CDX2; $Dmt1^{F/F}/Apc^{F/+}$ mice and their littermate controls. *p<0.05, **p<0.01and ***p< 0.001 error bars represent SEM. See also Figure S3.
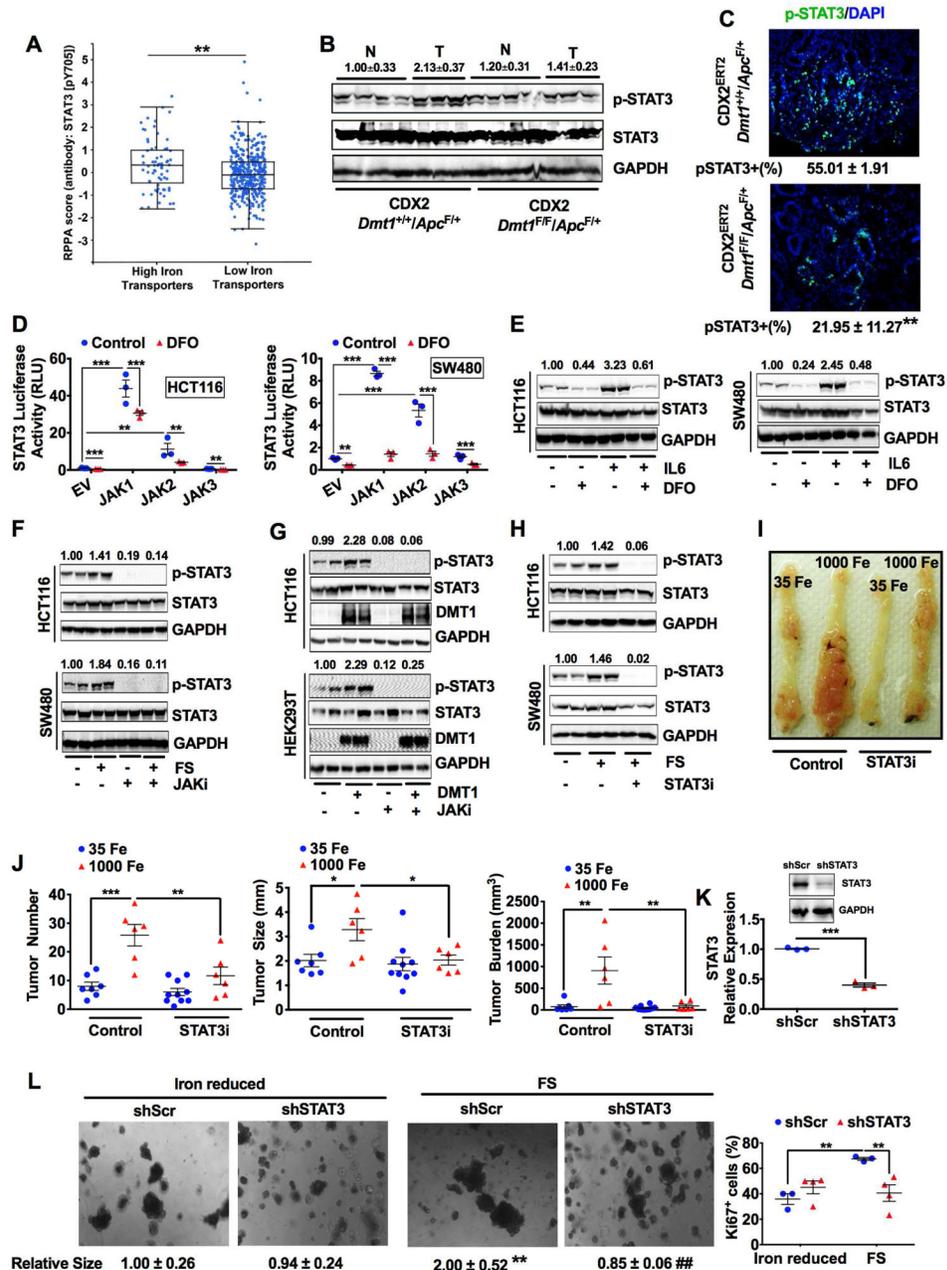
Figure 5. Iron is essential for STAT3 activation in colon tumorigenesis
(**A**) TCGA reverse phase protein array (RPPA) analysis based on iron transporter expression in tumor tissues (high iron transporters, n=85; low iron transporters, n=531). (**B**) Western blotting analysis of in colon tumors (T) and adjacent normal tissues (N) from CDX2 *Dmt1*$^{F/F}$/*Apc*$^{F/+}$ mice and littermate controls. (**C**) Immunofluorescence staining and quantification for p-STAT3 in CRCs from CDX2^{ERT2} *Dmt1*$^{F/F}$/*Apc*$^{F/+}$ mice (n=3). (**D**) STAT3 activity reporter assay in cells transfected with empty vector (EV), JAK1, JAK2 or JAK3 and treated with deferoxamine (DFO) 100 μM or control for 24 hours. Western blot analysis in HCT116 or SW480 cells treated (**E**) with DFO (100 μM) and IL6 (10 ng/mL) for

24 hours, or (**F**) with ferrous sulfate (FS, 100 μM) and JAK1/2 inhibitor (JAKi, 3 μM Ruxolitinib) for 24 hours. (**G**) Western blot analysis in HCT116 or HEK293T stable cells overexpressing DMT1 or parental controls treated with JAKi (3 μM) for 24 hours. (**H**) Western blot analysis in HCT116 or SW480 cells treated with FS (0 or 100 μM) and STAT3 inhibitor (STAT3i, 0 or 100 μM S3I-201) for 24 hours. (**I**) Representative gross image of CRCs, (**J**) tumor number, size, burden and number grouped by size from CDX2^{ERT2} $Apc^{F/+}$ mice maintained on regular iron diet (35 mg/kg iron, 35 Fe) or high iron diet (1000 mg/kg iron, 1000 Fe). (**K**) STAT3 expression level in stable adenoma enteroids with STAT3 knockdown (shSTAT3) or scramble shRNA (shScr). (**L**) Representative bright-field images and quantification of enteroid size and Ki67 staining of adenoma enteroids grown in iron-reduced RPMI media or RPMI media supplemented with FS 100 μM for 7 days. *p<0.05, **p<0.01 and ***p<0.001. ##p<0.01 compared with FS treated shScr stable adenoma enteroids. Errors bars represent SEM. Western blot data was quantitated and the values above the blots represent p-STAT3/STAT3. See also Figure S4 and S5.
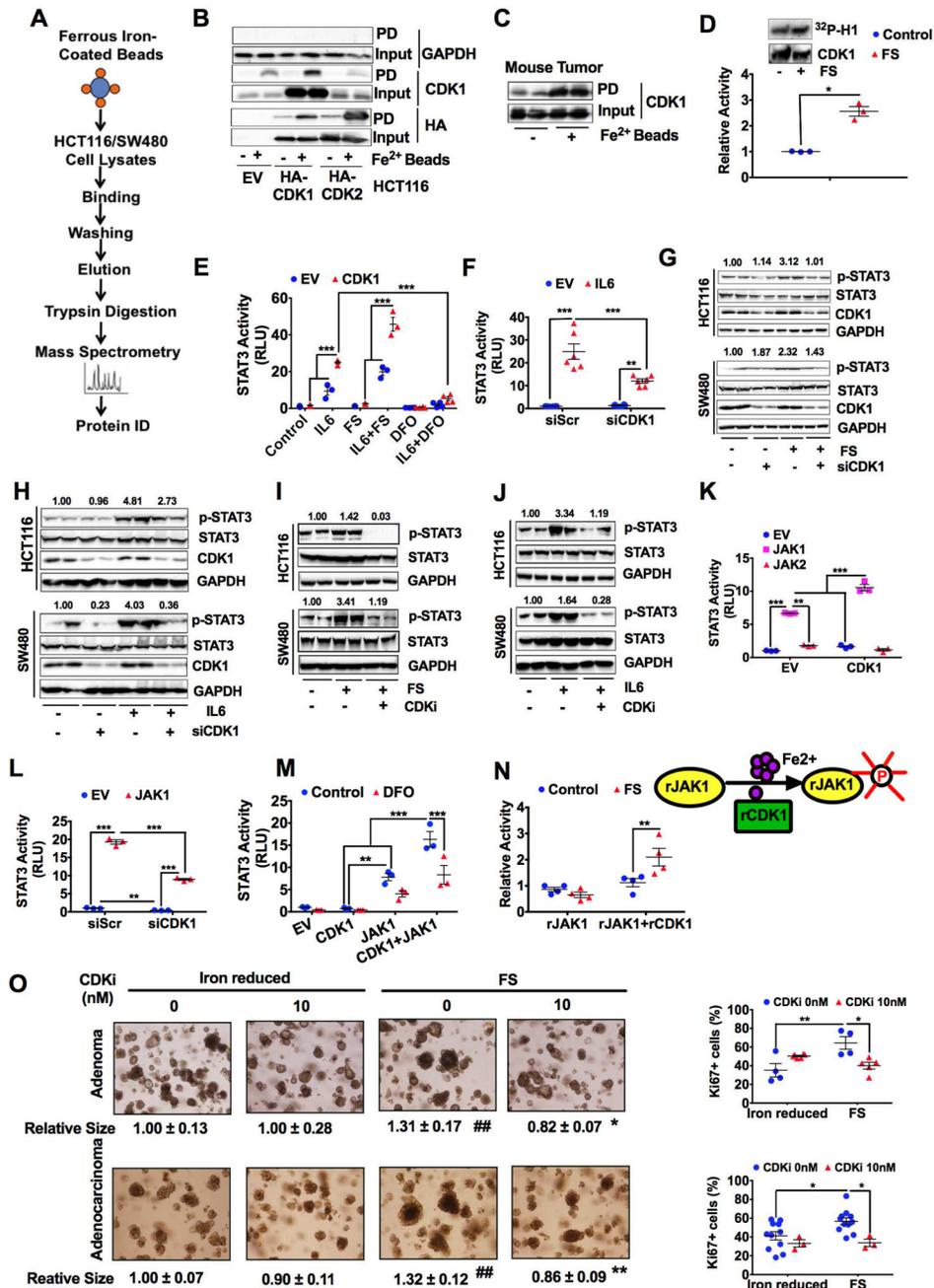
Figure 6. Iron-dependent CDK1 kinase activity is critical for JAK-STAT3 activation
(**A**) Schematic diagram for identification of Fe^{2+} binding proteins with LC-MS/MS based proteomics analysis. (**B**) Immunoblot analysis of proteins pulled down (PD) by Fe^{2+} or empty beads in HCT116 cells transfected with HA-tagged CDK1 or empty vector (EV). (**C**) Immunoblot analysis of CDK1 protein PD by Fe^{2+} or empty beads from mouse colon tumors lysates. (**D**) Kinase assay assessing histone H1 (H1) phosphorylation by purified recombinant (r) CDK1 in the presence or absence of FS 10μM. (**E**) STAT3 activity reporter assay in HCT116 cells transfected with EV or CDK1 and treated with IL6 10 ng/mL, FS 100

μM, DFO 100 μM or vehicle control for 24 hours. **(F)** STAT3 activity reporter assay in HCT116 cells transfected with 50 nM siRNA for CDK1 (siCDK1) or scrambled control (siScr) and treated with vehicle control or IL6 10 ng/mL. Western blot analysis in HCT116 or SW480 transfected with siCDK1 for 24 hours and then treated with **(G)** FS or **(H)** IL6 for 24 hours. Western blot analysis in HCT116 or SW480 cells treated with **(I)** FS and CDK1/2 inhibitor (CDKi, 0 or 10 nM Dinaciclib), or **(J)** with IL6 and CDKi for 24 hours. STAT3 activity reporter assay in HCT116 cells transfected with **(K)** EV, CDK1, JAK1 or JAK2, **(L)** siCDK1, siScr and/or JAK1, or **(M)** EV, CDK1, JAK1 and/or DFO for 24 hours. **(N)** Phospho-transfer assay assessing JAK1 phosphorylation by rCDK1 in the presence of FS (0 or 10 μM). **(O)** Representative bright-field images and quantification of enteroid size and Ki67 staining of adenoma enteroids grown in iron-reduced RPMI media or RPMI media supplemented with FS 100 μM after treatment with CDKi (0 or 10 nM) for 7 days. $*p<0.05$, $**p<0.01$ and $***p<0.001$. $\#\#p<0.01$ compared with iron-reduced conditions. Error bars represent SEM. Western blot data was quantitated and the values above the blots represent p-STAT3/STAT3. See also Figure S6, Table S2 and S3.
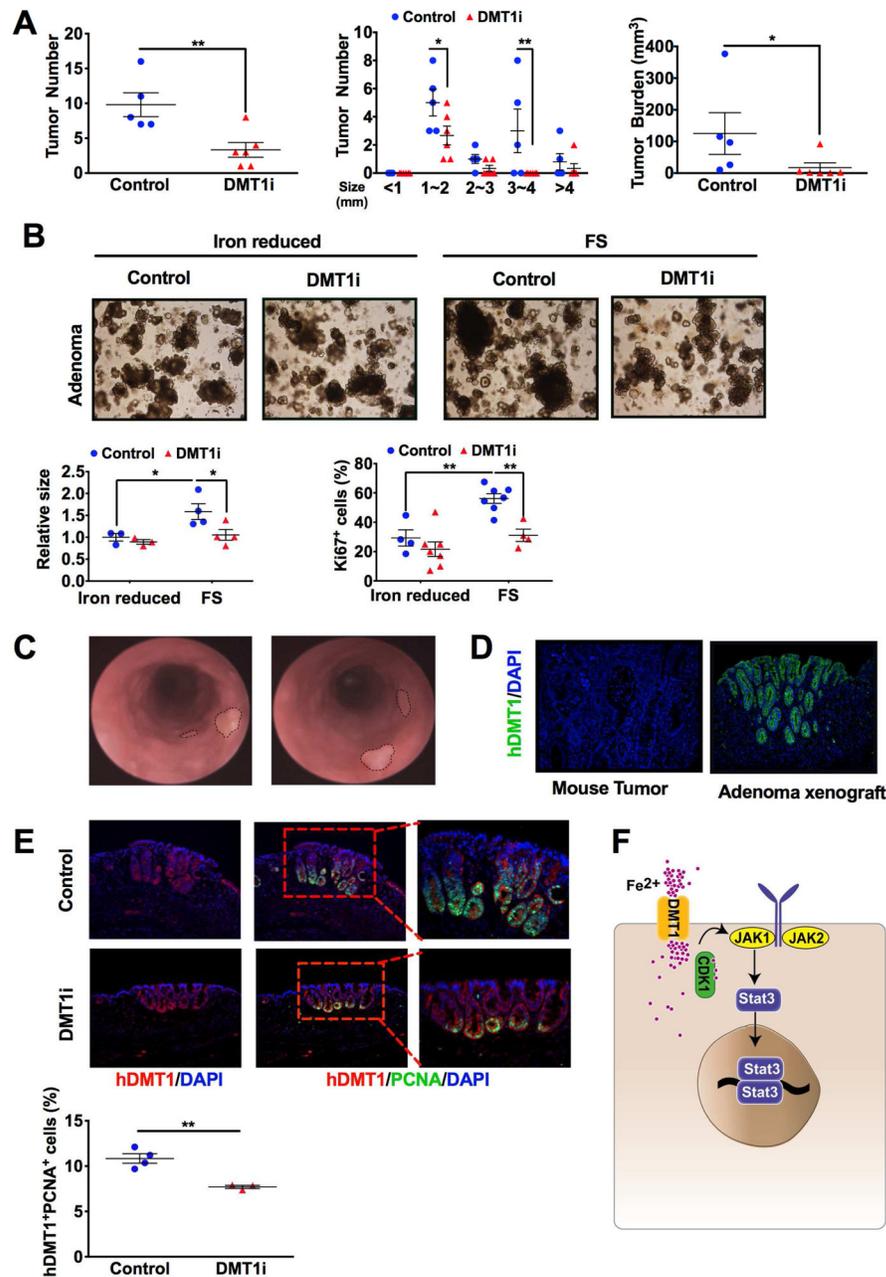
Figure 7. Pharmacological inhibition of DMT1 decreases growth of colon tumors

(**A**) Tumor number and burden from CDX2^{ERT2} $Apc^{F/+}$ mice orally administered with 50 mg/kg DMT1i, or vehicle. (**B**) Representative bright field images, and quantification of size and Ki67 staining of adenoma enteroids grown in iron-reduced RPMI media or RPMI media supplemented with FS 100 μM after treatment with DMT1i (0 or 3 μM) for 7 days. (**C**) Intrarectal implantation of adenoma enteroid in NOD/SCID mice visualized by endoscopy examination. Dotted line represents the established adenoma xenograft. (**D**) Immunofluorescent staining of a mouse colon tumor and adenoma xenograft from human specific DMT1 antibody (hDMT1). (**E**) Immunofluorescent staining and quantification of

co-localization of hDMT1 and PCNA for established tumors from human adenoma enteroids grown in NOD/SCID mice for 2 weeks and then mice orally administered with 50 mg/kg DMT1i, or vehicle for 2 weeks. (**F**) Proposed mechanism for iron-driven colon tumorigenesis. Fe^{2+} is transported by DMT1 into intestinal epithelial cells and binds with CDK1, which increases CDK1 kinase activity to phosphorylate JAK1 kinase and activates STAT3 signaling pathway for tumor growth. *$p < 0.05$ and **$p < 0.01$ and error bars represent SEM. See also Figure S7.