Hepatocyte Growth Factor Gene Mutation in Human Colorectal Cancer: Causes and Consequences

by

Danushka S. Seneviratne

Bachelor of Science, Queen's University, 2008

Submitted to the Graduate Faculty of

The School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

This dissertation was presented

by

Danushka S. Seneviratne

It was defended on

April 3rd, 2014

And approved by

Marie DeFrances, MD, PhD, Associate Professor, Department of Pathology

Eric Lagasse, PharmD, PhD, Associate Professor, Department of Pathology

Daniel Johnson, PhD, Professor, Department of Pharmacology and Chemical Biology

Robert Sobol, PhD, Associate Professor, Department of Pharmacology and Chemical Biology

Dissertation Advisor: Reza Zarnegar, PhD, Professor, Department of Pathology

Hepatocyte Growth Factor Gene Mutation in Human Colorectal Cancer: Causes and Consequences

Danushka S. Seneviratne, PhD

Hepatocyte Growth Factor Gene Mutation in Human Colorectal Cancer:

Causes and Consequences

Danushka S. Seneviratne, PhD

University of Pittsburgh, 2014

Colorectal cancer (CRC) is currently the third most common form of cancer among developed nations. Given that nearly 50% of patients who undergo surgery and subsequent chemotherapy die of metastatic disease, novel therapeutic targets and more effective therapies are direly needed. Although genomic instability is known to promote colon carcinogenesis, many of the target genes that are mutated and drive tumorigenesis are yet to be elucidated. Based on a series of experiments and properties of hepatocyte growth factor (HGF), we hypothesized that *HGF* gene is a target of mutagenesis in human CRC. Therefore we aimed to test this hypothesis and determine the underpinning molecular mechanisms involved.

We show that genomic instability provoked by DNA mismatch repair (MMR) deficiency causes deletion mutagenesis of an important repressor element in the *HGF* gene proximal promoter in human CRC cells. This promoter element, which we have named DATE (DeoxyAdenosine Tract Element) consists of a polyA tract of 30 deoxyadenosine bases. We show that shortening of DATE leads to the activation of the *HGF* promoter (which otherwise is silent in normal colon epithelium) hence creating an HGF-Met autocrine loop in CRC cells. We show that acquisition of HGF-Met autocrine signaling endows cancer cells with growth advantages,

which promotes cell survival and confers resistance to both modes of programmed cell death (apoptosis and necroptosis).

We show that growth factor signaling like the HGF-Met axis can directly impact RIPK-1, a key mediator of necroptosis. This is achieved via tyrosine phosphorylation of RIPK-1 by activated Met, resulting in inhibition of RIPK-1 kinase activity. Furthermore, we discovered that Met activation causes RIPK-1 recruitment to the plasma membrane, its polyubiquitination (Lys-48 linked chain) and its subsequent proteasomal degradation. We also found that aberrant HGF expression in tumor tissues is significantly correlated with low RIPK-1 and with poor clinical prognosis. Collectively, our results shed important mechanistic insights into the molecular mechanisms of human colon carcinogenesis and warrant further investigation of HGF-Met as a viable therapeutic target in CRC, especially in those subsets harboring *HGF* gene mutation.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS **13**

1.0 INTRODUCTION **15**

1.1 COLORECTAL CANCER (CRC) **15**

1.1.1 CRC Epidemiology **15**

1.1.2 Molecular pathways involved in CRC **18**

1.1.3 Contemporary Treatment Strategies **22**

1.1.4 Predictive CRC biomarkers used to assess patient prognosis **24**

1.2 HGF-MET SIGNALING PATHWAY **26**

1.2.1 Overview of HGF-Met signaling **26**

1.2.2 Regulation of HGF-Met signaling **33**

1.2.3 HGF-Met signaling in cancer **39**

1.2.4 Therapeutic Strategies targeting HGF-Met signaling **40**

1.3 GENOMIC INSTABILITY IN CANCER **43**

1.3.1 Mechanisms of chromosomal instability **43**

1.3.2 Chromosomal instability in cancer **44**

1.3.3 Mechanisms of microsatellite instability **45**

1.3.4 Microsatellite instability in cancer **47**

1.3.5 The use of microsatellite instability as a diagnostic and a prognostic marker in cancer **48**

1.4 MAJOR PATHWAYS OF CRC CELL DEATH **50**

1.4.1 Apoptosis **50**

1.4.2 Autophagy 51

1.4.3 Necroptosis 52

1.4.4 RIPK-1 at the crossroads of cell survival and cell death 53

2.0 CAUSES OF *HGF* GENE MUTAGENESIS IN HUMAN CRC 58

2.1 ABSTRACT 58

2.2 INTRODUCTION 59

2.3 MATERIALS AND METHODS 61

2.4 RESULTS 65

2.4.1 An important regulatory region of the *HGF* gene promoter, DATE, undergoes deletion mutagenesis in human CRC 65

2.4.2 Defective mismatch repair is the underpinning cause of *HGF* promoter instability in human CRC 67

2.5 DISCUSSION 73

3.0 CONSEQUENCES OF *HGF* GENE MUTAGENESIS IN HUMAN CRC 77

3.1 ABSTRACT 77

3.2 INTRODUCTION 78

3.3 MATERIALS AND METHODS 80

3.4 RESULTS 88

3.4.1 DATE mutation reactivates the silenced *HGF* promoter 88

3.4.2 DATE truncation leads to autocrine HGF-Met signaling in human CRC cells 90

3.4.3 CRC cells with truncated DATE have operational HGF-Met signaling and are addicted to HGF for cell viability 97

3.4.4 Inhibition of autocrine HGF-Met signaling promotes induction of necroptosis in CRC 102

3.4.5 Autocrine HGF-Met signaling promotes escape from necroptosis via direct post translational modification and modulation of RIPK-1 107

3.4.6 Autocrine HGF-Met signaling associates with negative patient prognosis in CRC 116

3.5 DISCUSSION 117

4.0 IMPLICATIONS AND FUTURE DIRECTIONS..... 125

BIBLIOGRAPHY 130

LIST OF TABLES

Table 1: Patient demographics and genotype analysis of DATE in human CRC and corresponding adjacent normal tissues 63

Table 2: MSI status in human CRC and corresponding adjacent normal tissues in patients 69

Table 3: Human cancer cell lines known to have MMR deficiency and MSI also exhibit DATE instability 71

Table 4: **Primer sequences used in somatic gene targeting of wild-type DATE** 83

Table 5: Luciferase reporter assays are used to demonstrate that DATE truncation causes HGF promoter activation in CRC cells 90

Table 6: IC-50 of 5-Fluorouracil in CRC cells, alone and in combination with the Met inhibitor, SU11274 (20 μM) 100

LIST OF FIGURES

Figure 1. Progression from colorectal polyps to colorectal cancer 18

Figure 2. Structures of HGF and Met 28

Figure 3. Multiple signal pathways are activated by HGF-Met signaling 31

Figure 4. Roles of the HGF-Met signaling pathway 33

Figure 5. HGF-Met signaling is regulated in a cell-type specific manner 36

Figure 6. Regulation of cell-type specific HGF expression 36

Figure 7. Structure and function of DATE 38

Figure 8. Strategies currently being investigated to inhibit HGF-Met signaling 42

Figure 9. DNA mismatch repair system maintains genomic fidelity during replication 46

Figure 10. The structure of human RIPK-1 54

Figure 11. Multiple RIPK-1 mediated signaling complexes are initated downstream of TNFR-1 stimulation. 57

Figure 12. DATE is polymorphic in nature and undergoes deletion mutagenesis in human CRC 66

Figure 13. DATE mutagenesis results from genomic instability provoked by defective DNA Mismatch Repair (MMR) 70

Figure 14. Human CRC cell lines with microsatellite instability harbor mutant DATE ... 72

Figure 15. DATE truncation causes reactivation of the silenced *HGF* promoter 89

Figure 16. DATE truncation leads to HGF production and autocrine HGF-Met signaling in human CRC cells 92

Figure 17. DATE truncation is sufficient to activate endogenous *HGF* gene expression ... 94

Figure 18. DATE truncation correlates with HGF up-regulation in human CRC tissues . 96

Figure 19. DATE mutant CRC cells are addicted to HGF for cell growth 98

Figure 20. Inhibition of HGF-Met signaling causes significant reduction in CRC cell viability 101

Figure 21. Inhibition of Met causes necroptosis in CRC cells 103

Figure 22. Active HGF-Met signaling down regulates RIPK-1 in CRC cells and primary rat hepatocytes 104

Figure 23. HGF injection causes down regulation of RIPK-1 in vivo 105

Figure 24. HGF expression correlates with down regulation of RIPK-1 in human CRC patient tissues 106

Figure 25. HGF-Met signaling causes rapid recruitment of RIPK-1 to the plasma membrane 108

Figure 26. HGF-Met signaling results in down regulation of RIPK-1 in vivo 109

Figure 27. HGF induces polyubiquitination of RIPK-1 110

Figure 28. HGF stimulation causes the recruitment of TRAF-2 to plasma membrane and 112

Figure 29. Met directly tyrosine phosphorylates RIPK-1 decreasing its autocatalytic activity as indicated by the reduction in RIPK-1 serine phosphorylation 114

Figure 30. RIPK-1 can be phosphorylated by multiple tyrosine kinases 115

Figure 31. DATE mutation and low RIPK-1 levels associate with poor patient prognosis 116

Figure 32. Model of the causes and consequences of *HGF* gene mutation in human CRC 121

Figure 33. Proposed model outlining the role of autocrine HGF production in colon tumorigenesis 124

ACKNOWLEDGEMENTS

There are many individuals who have helped me reach this important milestone of my life. I am extremely grateful to each and every one of you, for your support and encouragement.

First and foremost I want to thank my advisor, Dr. Reza Zarnegar, for guiding me throughout my dissertation work. Thank you for always providing me with the tools and the necessary direction to explore my ideas and become an independent thinker. Your support, advice, and understanding nature, have all been crucial to realizing my goal of attaining a doctorate degree.

I am also extremely thankful to my thesis committee (consisting of Dr. Marie DeFrances, Dr. Eric Lagasse, Dr. Robert Sobol, and Dr. Daniel Johnson) for guiding me during the progress of my doctorate work. Your enthusiastic interest in my research and your experimental direction tremendously helped me focus and develop my work into its current form.

Members in the Zarnegar Lab have been indispensable during my dissertation work. I am very grateful to Dr. Ma in particular, for training me when I initially joined the lab, and for experimentally contributing to the work presented in this thesis. I am also thankful to Dr. Yong Kook Kwon, and Dr. Xinping Tan, who have both helped me many times with various lab matters.

I am also obliged to Dr. Wendy Mars, who has been vital to my achievements during my entire time as a doctorate student. I sincerely appreciate the support, understanding, and the encouragement you provided me during some of the more difficult times in the program.

My family has been fundamental to all my accomplishments to date. I am forever indebted to my parents, who have been my biggest influence in life. Thank you for continuously instilling in me the power and the importance of education, and constantly encouraging me to pursue my

interest in science. You always taught me to aim for greatness and attempt to become the best that I can be. I am certain that I would not have gotten to this point in my life without your unwavering support, guidance, and your unconditional love. I am convinced that no one on earth is happier about my PhD than you. I want you both to know how grateful I am for all the personal sacrifices you made, and all the hardships you endured, simply to ensure that your children are able to live their dreams. Everything I am, and everything I hope to achieve in the future will be the fruit of your labor.

I also want to thank my sister, who has been my best friend and confidante throughout my life. You have been there through all the good and the bad times, and I am so grateful for your constant companionship.

I am grateful to all the friends I made in Pittsburgh who have made this experience much more enjoyable than initially expected.

Last but definitely not least, I want to thank my husband who has been with me in Pittsburgh during the majority of my doctorate work. I have been extremely lucky to find both a loving spouse and the most wonderful friend in you. Thank you for always being encouraging and supportive of all my endeavors, holding steadfast through all the ups and the downs, and for helping me always see the silver lining in every dark cloud.

1.0 INTRODUCTION

1.1 COLORECTAL CANCER (CRC)

1.1.1 CRC Epidemiology

Colorectal carcinoma (CRC) is the third most common cause of cancer related death and the fourth leading cause of morbidity worldwide. CRC accounts for a staggering 9% of all human cancers, has an age standardized death rate of 18.8 per every 100,000 individuals, and affects both men and women equally [1]. During 2002, the most recent year for which international cancer statistics are available, an estimated 1 million new cases of CRC were diagnosed, and 529,000 global deaths were reported [2]. Recent records indicate that cancers of the colorectum, along with those of the lung, breast, and prostate, will account for approximately 50% of all new cancer diagnosis [3]. Distribution of CRC varies geographically, with 63% of all reported cases occurring in Australia, New Zealand, Canada, United States and Western Europe [4]. Developing nations such as China, India, and parts of Africa and South America exhibit the lowest rates of incidence [5]. Several risk factors, including age, personal medical history, and genetics, are associated with developing CRC. It is estimated that more than 90% of CRC diagnoses occur in individuals over the age of 50 and that the frequency is 50 times greater among those aged 60-79 in comparison to individuals under the age of 40 [6,7]. Incidence of CRC has however increased among younger persons over the last few decades and is now among the ten most commonly diagnosed cancers within the 20-49 age group [8].

As nearly 95% of sporadic CRCs develop from adenomas, an individual medical history of adenomas is considered to be a significant risk factor in developing CRC. Due to the long latent period of 5-10 years that is involved in the malignant transformation of benign adenomas, studies show the removal of adenomatous polyps and localized carcinomas can considerably reduce the risk of developing CRC at a future date [9]. The progression of benign adenomas to CRC is outlined in **Figure 1A** and the genes commonly altered at each stage of progression are shown in **Figure 1B**. A personal history of bowel disease (in the form of ulcerative colitis or Crohn's disease) that leads to inflammation of the colorectal mucosa or the bowel wall, is noted to increase the risk of developing CRC by 4-20 fold [4]. A family history of CRC is observed in approximately 20% of the new diagnoses [6]. The risk tends to be elevated in individuals with first degree relatives who developed adenomatous polyps prior to the age of 60, or those with first or second degree relatives who were diagnosed with CRC at any age [10]. Approximately 5-10% of CRC results from hereditary conditions such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis CRC (HNPCC) [1]. HNPCC, which accounts for 6% of CRC, is characterized by tumor development of at an early age (<46 years of age) and is also associated with cancers of the endometrium, stomach, pancreas-biliary tract, small intestine, urothelium and ovary [11,12]. FAP is caused by mutations in the *APC* tumor suppressor gene and is characterized by the early development of hundreds of polyps that may become malignant over time [11].

Despite the strong associations observed between CRC and the above described genetic and age related risk factors, environmental factors are believed to be the largest contributor in CRC tumorigenesis. For instance, a diet high in animal fat and meat, while low in fruits and vegetables, lack of physical activity and a tendency towards obesity, cigarette smoking, alcohol consumption, as well as long-term residence in urban regions, are strong predictors of CRC

development [13]. The linkage between diet and CRC largely stems from the role of animal fat and meat in exposing of the gut to large amounts of carcinogenic N-nitroso compounds, heme iron, and the potentially carcinogenic heterocyclic amines and polycyclic aromatic hydrocarbons produced during the high meat cooking temperatures. In contrast, high overall levels of physical activity and healthy body weight are associated with reduced risk of CRC. Preliminary studies regarding the underlying mechanisms have revealed that sustained physical activity raises metabolic rate, increases efficiency of oxygen uptake and gut motility, while reducing blood pressure and insulin resistance, contributing to lower overall tumorigenesis [1,14]. Further supporting this theory, individuals with high-levels of abdominal fat, lower gut motility and lower metabolic efficiency showed higher incidences of CRC [6]. Studies investigating the role of cigarette smoke in the onset of CRC showed that carcinogens in tobacco increase cancer growth within the colon and the rectum and that roughly 12% of CRC deaths are attributed to smoking. Heavy alcohol consumption is also believed to play a role in CRC tumorigenesis, as alcohol promotes the production of carcinogenic prostaglandins and free radicals and also acts as a solvent that increases the penetration of toxic compounds into the mucosal cells [1,15].

CRC survival rates can vary between 90%-10%, depending on the disease stage at the time of diagnosis (**Figure 1C**). The tumor may be localized to carcinoma in-situ (stage 0), or have invaded the submucosa (stage I), invaded the muscularis propria into pericolorectal tissues (stage II), invaded regional lymph nodes (stage III), or metastasized to distant organs and structures (stage IV) [16]. Due to increased access to healthcare, the overall 5-year survival for CRC has improved significantly over the last decade. The current five-year survival rate following CRC diagnosis is 60%, with only 6% of metastatic patients surviving this period [1,6]. Nearly 50% of all CRC patients who undergo surgery and subsequent chemotherapy die of metastatic disease [17,18].

Due to the bleak prognosis of colon cancer, novel therapeutic targets and more effective therapies are direly needed.

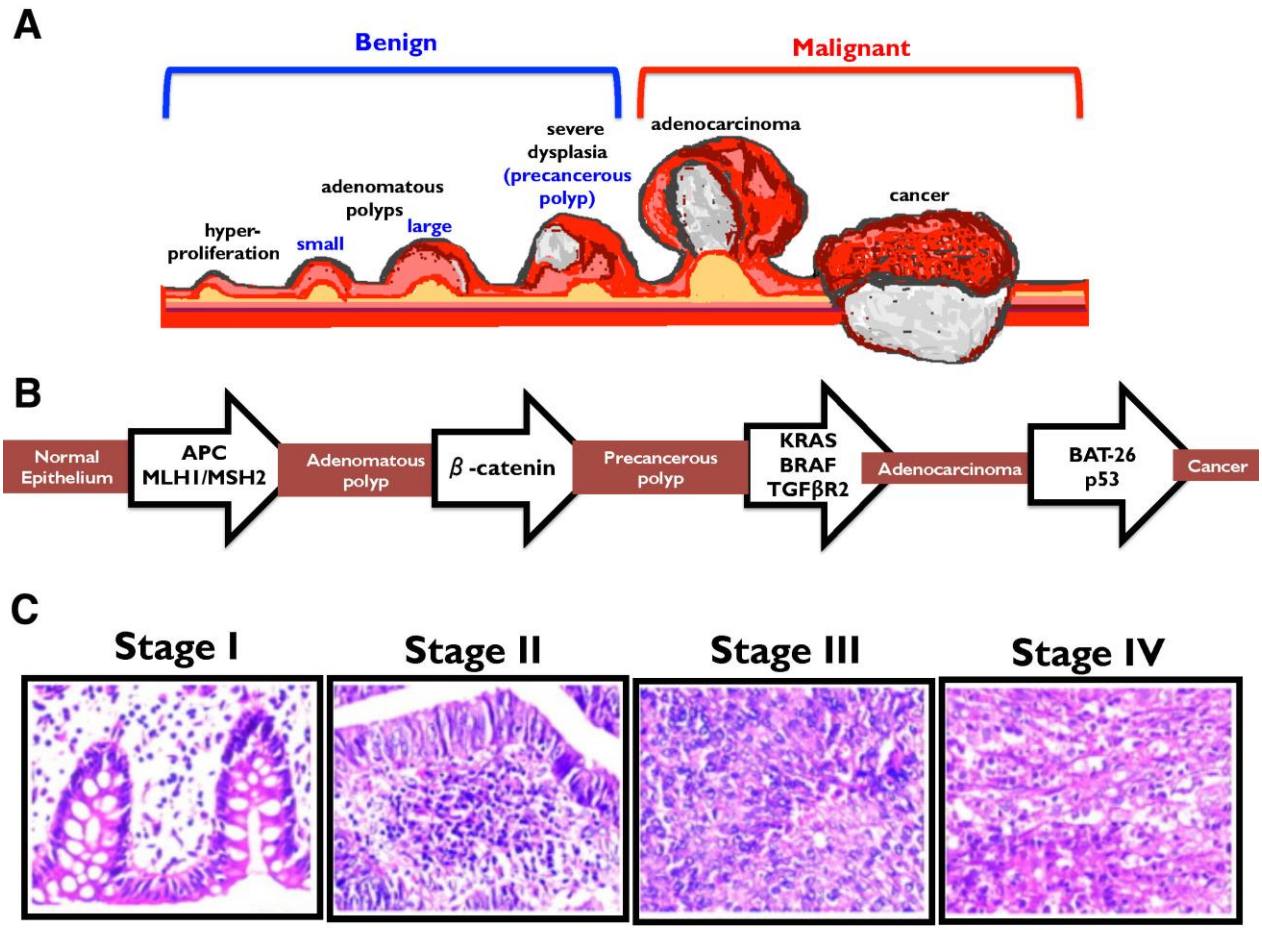

Figure 1. **Progression from colorectal polyps to colorectal cancer**
(**A**) Schematic indicating the development of colon polyps into cancer. (**B**) Vogelgram indicating the associated molecular alterations during the progression of CRC. While only 10% of all adenomas become malignant, more than 95% of CRC develop from adenomas. (**C**) Histological images depicting the progression of CRC from Stage I through Stage IV. Adapted from Johns Hopkins Medicine Colorectal Cancer Center.

1.1.2 Molecular pathways involved in CRC

Accumulation of genomic alterations facilitated by the loss of genomic stability has long been known contribute to tumorigenesis and the clinical progression of sporadic and familial

forms of CRC [19]. While chromosomal instability (CIN) is the form of genomic instability most commonly observed in CRC (85% of CRC), microsatellite instability (MSI) is also observed in a significant subset (15%–20%) of cancers [20]. Both forms of genomic instability result in the alteration of key regulatory genes, promoting dysregulated cell proliferation, survival, angiogenesis, and escape from cell death. The most commonly altered signaling pathways in human CRC are Wnt/β-catenin (*APC* gene), epidermal growth factor receptor, RAS/RAF/mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase/Akt (PI3K/Akt), and transforming growth factor β (TGFβ), and p53 [19-21].

Wnt/β-catenin pathway is primarily involved in developmental regulation and the postembryonic maintenance of intestinal stem cell populations. The protein product of the adenomatous polyposis coli (*APC*) gene participates in a destruction complex, which prevents activation of β-catenin in the absence of Wnt Signaling. Mutations in *APC* are observed in nearly all CRC patients harboring the hereditary familial adenomatous polyposis syndrome (FAP) as well as in 70-80% of the sporadic CRC cases. *APC* mutations are believed to be an early event during carcinogenesis. 95% of the known mutations in *APC* are nonsense or frameshift mutations that result in the formation of a truncated, nonfunctional version of the protein, which cannot participate in the β-catenin destruction complex. This leads to accumulation of β-catenin in the cytoplasm, allowing it to act as an unregulated transcriptional co-activator of genes important for cell survival and proliferation, ultimately contributing to colon carcinogenesis [21,22].

EGFR is a receptor tyrosine kinase that is activated by dimerization and autophosphorylation upon the extracellular binding of its ligand. EGFR signaling is important in cell growth, proliferation and differentiation. EGFR protein expression is significantly elevated in 80% of CRCs. While the cause is still unclear, it is speculated that the elevated *EGFR* gene

copy number due to gene amplification and polysomy at chromosome 7 is responsible for the *EGFR* overexpression in at least a subset of CRC patients exhibiting the phenotype. Although rare, mutations leading to the ligand independent activation of the EGFR receptor were also observed in some patients. The abnormal activation of the EGFR signaling pathway through these mechanisms is believed to play a causative role in colorectal carcinogenesis and metastasis. Activation of the EGFR receptor in cells initiates the onset of several oncogenic signaling pathways including RAS/MAPK, PI3-AKT, phospholipase C, STATs and SRC/FAK. These signaling networks generally involve protein phosphorylation, recruitment of adapters, and the activation of transcription factors, eventually leading to increased cell survival and proliferation. When the EGFR signaling network is dysregulated, it may alter cell metabolism, disrupt normal cell cycle progression and prevent apoptosis, triggering the malignant transformation of the affected cells [21,23]. Interestingly, several groups including ours have recently shown that other transmembrane growth factor receptors may also contribute to colorectal carcinogenesis. For instance, significant Met overexpression was found to provide a selective growth advantage to neoplastic colorectal cells at all stages of tumor growth and increase their metastatic potential [24-26].

The RAS family encodes small G-proteins, which are activated by the conversion of guanine diphosphate (GDP) to guanine triphosphate (GTP) and facilitates the downstream transduction of signals from extracellular receptors such as EGFR. *KRAS,* a prominent RAS family member, is mutated in 40% of sporadic CRCs. Point mutations in the codons 12 and 13 of *KRAS* are the most commonly observed lesions. These mutations cause the exchange of amino acids at the catalytic site, causing the protein to exist in the active GTP bound state and allowing

the recruitment of the serine/threonine kinase RAF, which phosphorylates and activates MAPK, promoting cell survival and proliferation [21,23,27].

The PI3-AKT signaling pathway is directly activated by the phosphorylation of receptor tyrosine kinases such as EGFR and Met, and is important for stimulating cell proliferation and promoting escape from apoptosis. Gain of function mutations in exons 1, 2, 9, and 20 within the *PIK3CA* gene are observed in 15-30% of CRCs. These mutations are more commonly noted to occur in female CRC patients [21,28]. Inactivating mutations in the tumor suppressor, *PTEN*, which encodes a phosphatase that antagonizes PI3-Akt signaling, are also observed in approximately 9% of CRCs [28].

TGF-β cytokines are involved in cell proliferation, differentiation, death, and extracellular matrix formation. The binding of TGF-β ligands to the transmembrane serine-threonine kinase receptor, TGF-β1, allows its interaction with TGF-βII (type II receptor) and activates the signaling pathway. TGF-β signaling causes the translocation of Smad 2/3/4 transcription factor complex to the nucleus, resulting in the transcription of cell cycle arrest genes such as the cyclin-dependent kinase inhibitor, *P21*. Frameshift mutations in the TGF-βII gene are observed in 30% of colorectal carcinomas, with a majority occurring in microsatellite unstable cases. These *TGF-βII* mutations promotes escape from growth suppression signaling, contributing to colon tumorigenesis and metastatic invasion [29-31].

The tumor suppressor, *P53*, which plays a role in facilitating cell cycle arrest, apoptotic cell death, cell differentiation, senescence, and angiogenesis, is also mutated in a wide variety of malignancies. Mutations in the *P53* gene are observed in over 50-70% of CRCs and are associated with more aggressive disease forms [32].

It is evident that multiple signaling pathways important for the regulation of cell proliferation, survival, death, angiogenesis and growth arrest are involved in the onset and progression of human CRC [21]. A schematic illustration outlining the process of colon tumorigenesis is shown in **Figure 1**. Targeted therapies directed at the above-discussed pathways are currently being heavily investigated and are entering the forefront of CRC treatment protocols.

1.1.3 Contemporary Treatment Strategies

The introduction of novel surgical techniques and the standard use of 5-Fluorouracil (5-FU) plus radiotherapy revolutionized CRC treatment during the 1980s and significantly reduced local recurrence rates. Currently, treatment strategies include preoperative chemotherapy, surgery, and postoperative adjuvant chemotherapy. Multiple new pre and postoperative chemo and radiotherapy combinations are currently being investigated for toxicity and efficacy in clinical trials in attempts to improve patient survival and quality of life, and reduce recurrence [33].

Surgical success largely depends on the accuracy of the preoperative tumor staging process. Tumor staging is used to assess the depth of tumor infiltration into the tissue and to determine whether the tumor has spread to nearby lymph nodes or to distant metastatic sites. Currently, endorectal ultrasound imaging and pelvic MRI scans are primarily used to determine tumor staging. After determining the depth of tumor infiltration, surgery may be performed [34]. While blunt digital resection has been the norm in colorectal tumor resection for many years, the use of novel, more precise surgical techniques, such as total mesorectal excision (TME) have dramatically reduced the local recurrence rate over the last two decades. Minimally invasive techniques such as lacroscopy and robotic surgery have also been utilized successfully to remove colorectal tumors and have resulted in low recurrence rates. These techniques have become

popular in the tumor surgical resection arena due to the fact that they are performed as outpatient procedures and minimize patient trauma by resulting in sharp dissections that preserve the sphincter, minimize blood loss, and allow for rapid recovery [33].

The use of pre and post-operative chemo-radiotherapy is utilized in order to reduce tumor volume when surgery alone is not a viable treatment option for the patient. 5-FU is the first-line of chemotherapy used in both pre and post-operative settings. In controlled studies, it was noted that in comparison to patients who received only radiotherapy, those who received 5-FU in combination with radiotherapy fared well and had significantly lower rates of local recurrence, local metastasis, distant metastasis, and cancer related deaths [33]. The chemotherapeutic agents, Oxiplatin and Irinotecan are often used in combination with 5-Fluouracil therapy and have shown improvements over single therapies in some patients. Phase III clinical trials, however, recently indicated that Oxiplatin does not significantly improve outcome of patients with locally advanced rectal cancer and thus should no longer be considered as a part of adjuvant chemotherapy [33]. Additionally, an orally administered Fluoropyrimidine known as Capecitabine, has shown significant promise in clinical trials [35]. Despite the clinical success of 5-FU in a majority of CRC patients, it is important to note that individuals exhibiting defective DNA mismatch repair and high microsatellite instability show little to no benefit from adjuvant 5-FU chemotherapy. (For detailed information on microsatellite instability please see the genomic instability subsection below). Following 5-FU administration, cells mistakenly incorporate the 5-FU metabolites into DNA and RNA. These lesions are generally recognized by the mismatch repair system as un-repairable base mismatches and apoptosis is initiated. The observed CRC resistance to 5-FU in microsatellite unstable cells is largely believed to result from the inability to recognize the 5-FU

metabolites that are incorporated into the DNA during replication as base pair mismatches, and initiate cell death [36,37].

Targeted therapies have also been investigated for the treatment of CRC with a particular focus on monoclonal antibodies against EGFR (such as Cetuximab or Panitumumab), and the VEGF receptor (bevacizumab). Cetuximab is approved for the treatment of EGFR expressing, *KRAS* wild-type CRC. Although Cetuximab remains the only targeted therapy approved for clinical use in human CRC, the low toxicity and promising anti-tumor activity observed in early clinical trials have encouraged further development of other targeted therapies for CRC treatment [33].

1.1.4 Predictive CRC biomarkers used to assess patient prognosis

A number of protein and genomic markers have been identified over the past two decades that have helped predict therapeutic response, chance of recurrence, metastasis and overall survival.

Microsatellite instability (MSI) is characterized by the expansion or the deletion of oligonucleotide repeat regions. It is observed in a large subset of hereditary and sporadic cancers and is caused by defects in the DNA mismatch repair pathway (MMR). Incidence of microsatellite instability has shown promise in predicting patient response to therapeutic agents, and in assessing patient prognosis at various stages of the disease. For instance, it is noted that MSI instability high phenotype is primarily observed among stage II CRC patients (22%) and occurs at a much lower frequency in stage III (12%) and stage IV (3.5%) patients [38,39]. Although controversial, several studies have reported that MSI-H subpopulation of CRC patients display more favorable, stage-adjusted prognosis in comparison to the MSS or the MSI-L patient subsets. For instance, initially

it was reported that a higher rate of disease-free survival was observed among 5-FU treated MSI-H cancers (only within the stage III group), and that MSI-H patients of all stages who received only surgery fare better than the corresponding MSS or MSI-L counterparts [40,41]. However, in contrast to these observations of favorable prognosis, a more recent large-scale study indicated that MSI-H patients who received 5-FU adjuvant chemotherapy showed significantly higher frequencies of intra-abdominal recurrence and local recurrence[37]. After multiple small and large-scale studies, MSI status is now considered to be a robust prognostic marker in certain aspects of the clinical decision making process. It is most important in identifying and determining treatment strategies for the MSI-H CRC patient group who do not appear to benefit from 5- FU adjuvant chemotherapy [42].

Another mutational signature that has shown predictive potential in CRC is widespread promoter methylation, a phenotype that is otherwise known as CIMP-high. The silencing of tumor suppressor promoters by the cytosine methylation of CpG islands is believed to be an early event in the process of carcinogenesis that is observed in 15-20% of sporadic cancers. Although conflicting, this phenotype was correlated with *BRAF* mutations, wild-type P53, right side tumors, older age, female sex, and poor differentiation [43,44]. Further studies are however needed to confirm the clinical value of assessing for CIMP in patients.

KRAS is currently the only CRC biomarker integrated into clinical practice. The rapid and nearly universal acceptance of *KRAS* as a valuable biomarker lies in the fact that *KRAS* mutations are highly prevalent in CRC and are strongly predictive of negative responses to anti- EGFR therapies (such as the anti-EGFR monoclonal antibody, Cetuximab). Screening for *KRAS* mutations are now routinely used to determine whether patients should receive anti-EGFR antibody therapy [42]. It was recently shown that *KRAS* mutant cells are dependent on C-RAF

signaling, and are thus hypersensitive to the kinase inhibitor, Sorafenib. Phase I/II clinical trials involving the combination of Sorafenib with Irinotecan have shown promise in the treatment of *KRAS* mutant metastatic CRC [45].

BRAF V600E mutations are mutually exclusive with *KRAS* mutations and occur in 8-15% of individuals with wild-type *KRAS*. It is strongly associated with low differentiation and poor patient prognosis. Clinical studies have shown that *BRAF V600E* mutant tumors are resistant to anti-EGFR therapies (such as Cetuximab and Panitumumab), and could be re-sensitized to Cetuximab when treated in combination with the kinase inhibitor, Sorafenib [42] .

Despite the large number of biomarkers recently identified in CRC, only *KRAS* mutations have been universally accepted and successfully incorporated into routine clinical practice. The lack of success observed with other biomarkers was largely a result of their inadequate predictive power and poorly defined study end points [42]. Our work, described below, identifies a novel *HGF* gene promoter mutation as a potential biomarker that is strongly predictive of negative patient prognosis in CRC.

1.2 HGF-MET SIGNALING PATHWAY

1.2.1 Overview of HGF-Met signaling

Hepatocyte Growth Factor (HGF) is a stromal cell derived stimulator of epithelial cell growth and tissue regeneration. Dysregulated HGF expression contributes to tumor promotion and progression. It is secreted initially as a functionally inert precursor and later converted into its active form by extracellular proteases. The active form of HGF consists of a 60 kDa alpha

chain consisting of an amino-terminal hairpin loop and four kringle domains, as well as a 30 kDa beta chain, harboring a serine protease homology domain that lacks protease activity. The alpha and the beta chains are held together by one disulphide bond. The structure of HGF is detailed in **Figure 2A.** The functionally inert form of HGF is distributed largely in the extracellular matrix where it is sequestered by heparin-like proteoglycans. HGF elicits its extensive biological effects by binding to and activating its cell surface receptor, Met, a tyrosine kinase that is highly expressed on epithelial cells. The high-affinity site located on the HGF alpha chain can bind to the extracellular region of Met even prior to HGF processing and activation. The low–affinity site (located on the HGF beta chain) is exposed only following processing, and its binding to Met promotes activation of Met signaling [46,47].

The Met receptor belongs to a unique subclass of receptor tyrosine kinases (RTKs) and is a widely expressed type I transmembrane tyrosine kinase cell surface receptor. It is highly expressed on epithelial cells. Met was initially classified as an oncogene due to its ability transform fibroblast cell lines. Met, like HGF, is proteolytically cleaved from a single chain precursor to form alpha and beta chains that are held by disulphide bonds. In its mature form, Met is a single-pass heterodimer with an extracellular region that encompasses that entire alpha subunit (50 kDA) linked to the beta chain (145 kDA), which traverses the plasma membrane. The intracellular portion harbors a juxtamembrane region, a kinase domain, and protein docking sites. The juxtamembrane domain contains a serine residue that down regulates kinase activity upon phosphorylation by protein kinase C or calcium/calmodulin dependent kinases, It also contains a tyrosine residue (Y1003), which binds the E3-ubiquitin ligase Cbl upon phosphorylation, targeting the Met receptor for ubiquitination mediated proteolytic degradation or receptor endocytosis [48,49]. The detailed structure of Met is depicted in **Figure 2B**.

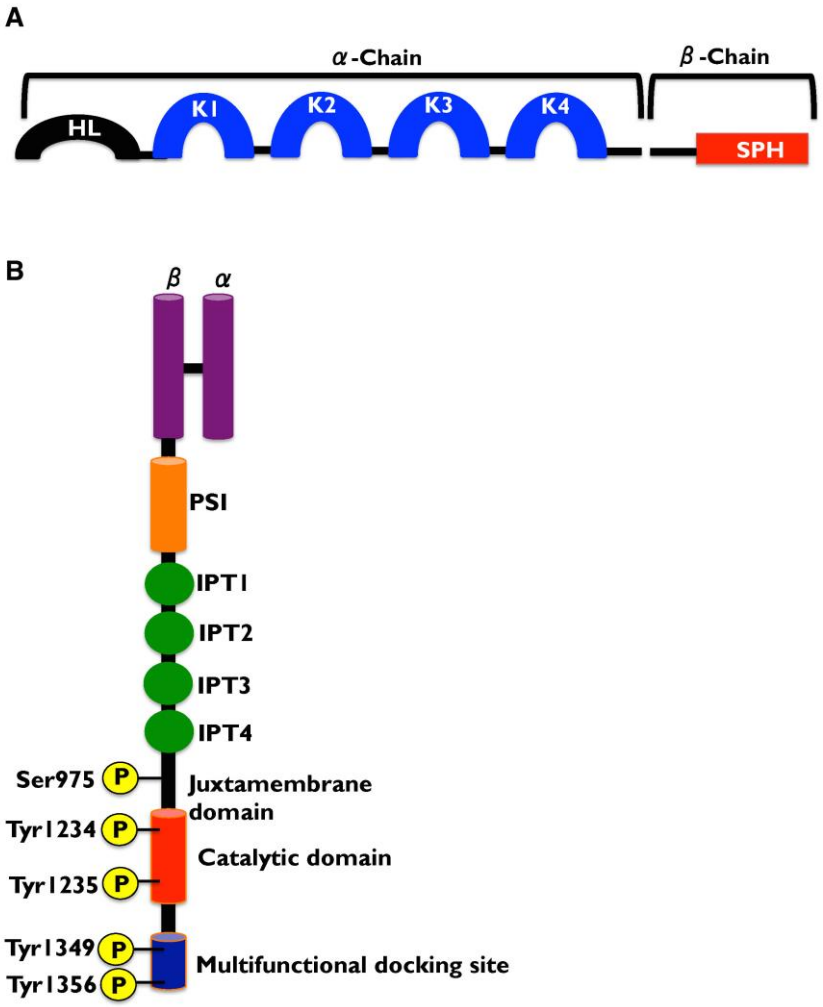

Figure 2. Structures of HGF and Met
(A) Hepatocyte growth factor is synthesized as a single chain precursor and later processed into an active α- and β-chain heterodimer. HGF consists of six domains: an amino-terminal hairpin loop (HL), four kringle domains (K1–K4), and a serine protease homology (SPH) domain that lacks proteolytic activity. (**B**) The Met receptor is a single-pass transmembrane heterodimer consisting of an extracellular α-subunit, which is linked by a disulphide bond to the transmembrane β-subunit, that harbors the catalytic region. The extracellular region of MET has several function domains, The sema domain encompasses the whole α-subunit and part of the β-subunit, the PSI domain contains four conserved disulphide bonds. There are four IPT domains that connects the PSI domain to the transmembrane helix. The intracellular region contains a juxtamembrane sequence, a catalytic region, and multifunctional docking sites. Adapted from [49].

Binding of the HGF heterodimer results in Met receptor dimerization and trans-phosphorylation of the kinase residues in the catalytic domain, followed by phosphorylation of the docking tyrosine residues in the carboxy-terminal. The C-terminal tail of Met is necessary and

sufficient for signal transduction. Met interacts with various scaffolding adapters such as Shc and Gab-1, and signaling proteins, to promote efficient downstream signaling activation. These adaptor proteins provide extra binding sites for PI3K, SHC, SHP2 and other signaling proteins and mediators [49].

Canonical signaling pathways activated by HGF-Met complex include mitogen-activated protein kinase 1 (ERK1) and ERK2, the phosphoinositide 3-kinase–Akt (PI3K–Akt) axis, signal transducer and activator of transcription proteins (STATs), Jun amino-terminal kinases (JNKs) and p38, and the nuclear factor-κB inhibitor-α (IκBα)–nuclear factor-κB (NF-κB) complex. In the activation of the ERK1/2, GRB2-SOS complex associates with the Met at the multifunctional C-terminal docking site, leading to Ras activation, followed by Raf, and then MEK1/2. The phosphorylation of ERK1/2 by MEK1/2 results in their translocation to the nucleolus where several transcription factors involved in the G1-S phase cell cycle phase transition are phosphorylated and stabilized. Activation of the PI3-Akt axis, which may be activated directly by Met or indirectly by Ras, results in the tethering of plextrin homology domain (PH) containing molecules to the plasma membrane. The most prominent of these molecules is the Ser/Thr kinase Akt, which suppresses the apoptosis activation by E3 ubiquitin ligase MDM2, inactivates the proapoptotic BCL-2 antagonist of cell death (BAD), and promotes survival by inactivating glycogen synthase kinase 3β that normally reduces the expression of cell cycle regulators such as Myc and cyclin D1. Activation of JNKs and p38 by a similar pathway results in alteration of a diverse variety of cellular processes including cell proliferation, differentiation, and apoptosis. Met-mediated phosphorylation leads to homodimerization of STATs (namely STAT3). The dimers then translocate to the nucleus and act as transcription factors, regulating the expression of cell proliferation and differentiation gens. Finally, Met activation also leads to the activation

of the NF-κB pathway, indirectly by way of the PI3-AKT and Src signaling pathways. Phosphorylation mediated degradation of the inhibitory proteins, IκBs, results in the release of NF-κB, which translocates to the nucleus and promotes transcription of various proliferation-associated and anti-apoptotic genes (**Figure 3**). Met signaling may be intensified, particularly in tumors, by the receptor's interaction with the α6β4 integrin. Integrin can act as a supplementary docking platform amplifying the Met receptors' ability to transduce cellular signals. Met can be dephosphorylated at the catalytic activation sites and the docking sites by several protein tyrosine phosphatases (PTPs), including the density enhanced phosphatase 1(DEP1) and leukocyte common antigen related phosphatases [49,50].

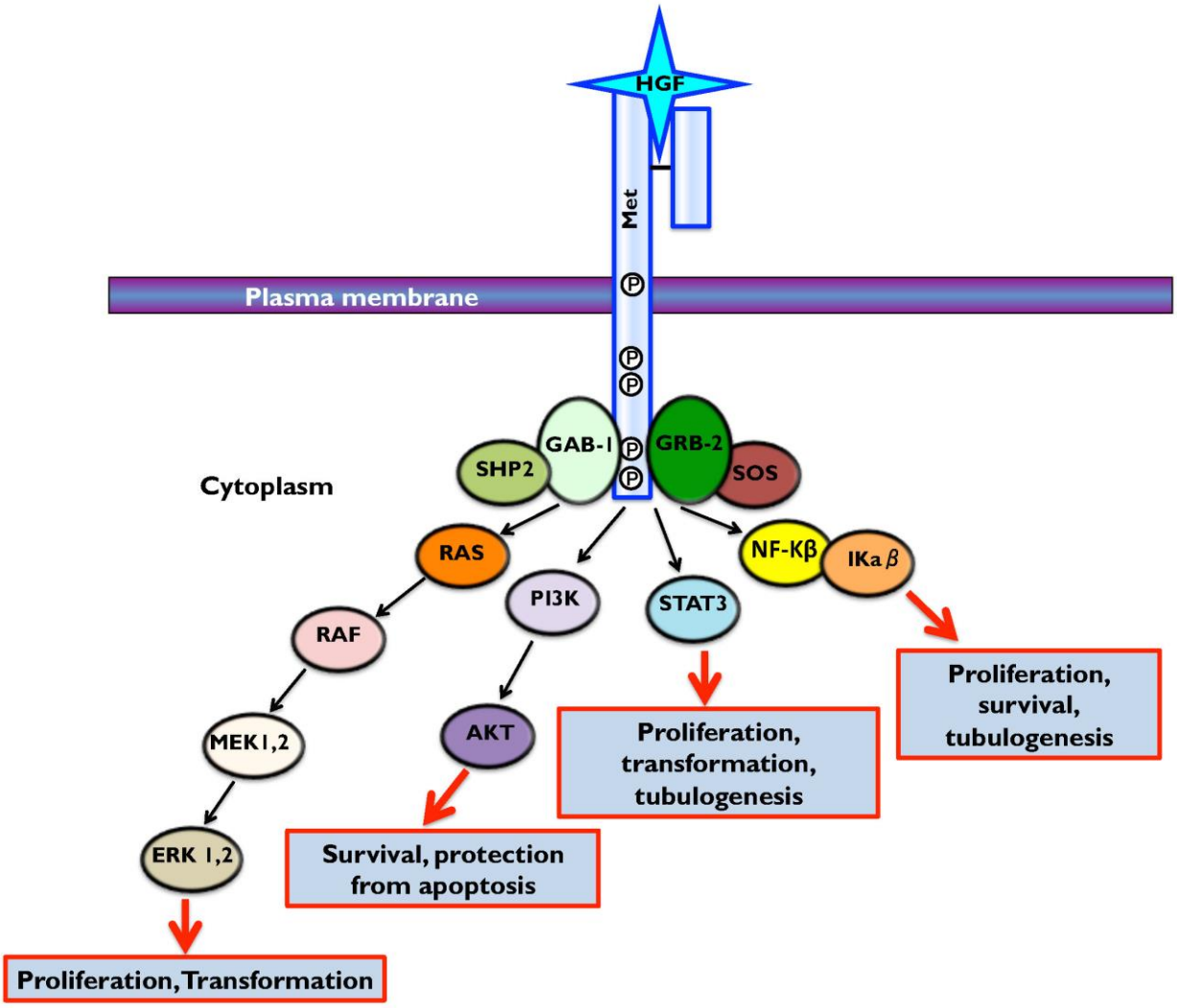

Figure 3. Multiple signal pathways are activated by HGF-Met signaling
Binding of HGF to the Met receptor activates multiple signaling pathways important for cell survival, proliferation and prevention of cell death. Adapted from [49].

HGF promotes cell growth, survival, proliferation, differentiation, and tumorigenesis (**Figure 4**). HGF-Met signaling is observed to be crucial for the growth and survival of hepatocytes and placental trophoblast cells during early development. The importance of HGF-Met signaling is evidenced by the fact that *HGF* or *MET* null embryos have significantly smaller livers, a hypomorphic placental phenotype, and compromised maternal and fetal gas exchange, that leads to *in utero* death of the organism by day 16 of embryonic development [49]. The absence

of HGF-Met signaling also results in complete ablation of proliferation and motility of a group of muscle progenitors, and significantly impairs the growth of the nervous system [51]. HGF-Met signaling also plays a crucial role in the regeneration of organs. *In vitro* studies of three dimensional collagen matrix culture conditions revealed that HGF promotes the partial epithelial-mesenchymal transition of normal epithelial spheroids, leading to cell re-differentiation and tubulogenesis that is necessary for organ regeneration. *In vivo* studies have shown that HGF-Met signaling is indispensable for the regeneration of the liver and kidney following both acute and chronic organ damage. HGF production is greatly increased following liver damage, providing hepatocytes with rapid mitogenic potential through Met-mediated proliferative and anti-apoptotic stimuli. In conditional mutant mice lacking *MET* in the liver, organ regeneration abilities are severely impaired following acute and chronic organ insults such as liver toxicity or partial hepatectomy [52]. HGF-Met signaling also has a protective role in the kidney. It prevents renal failure during tubular necrosis by promoting the survival and proliferation of renal cells, thus facilitating kidney regeneration [49,53]. HGF-Met signaling plays an important role during would healing. Mice harboring a conditional deletion of *MET* in the epidermis were unable to generate hyper-proliferative marginal keratinocytes necessary for repopulation of the wounded region. Furthermore, HGF is also believed to be important for cell locomotion to the de-epithelialized wounded region [49,54].

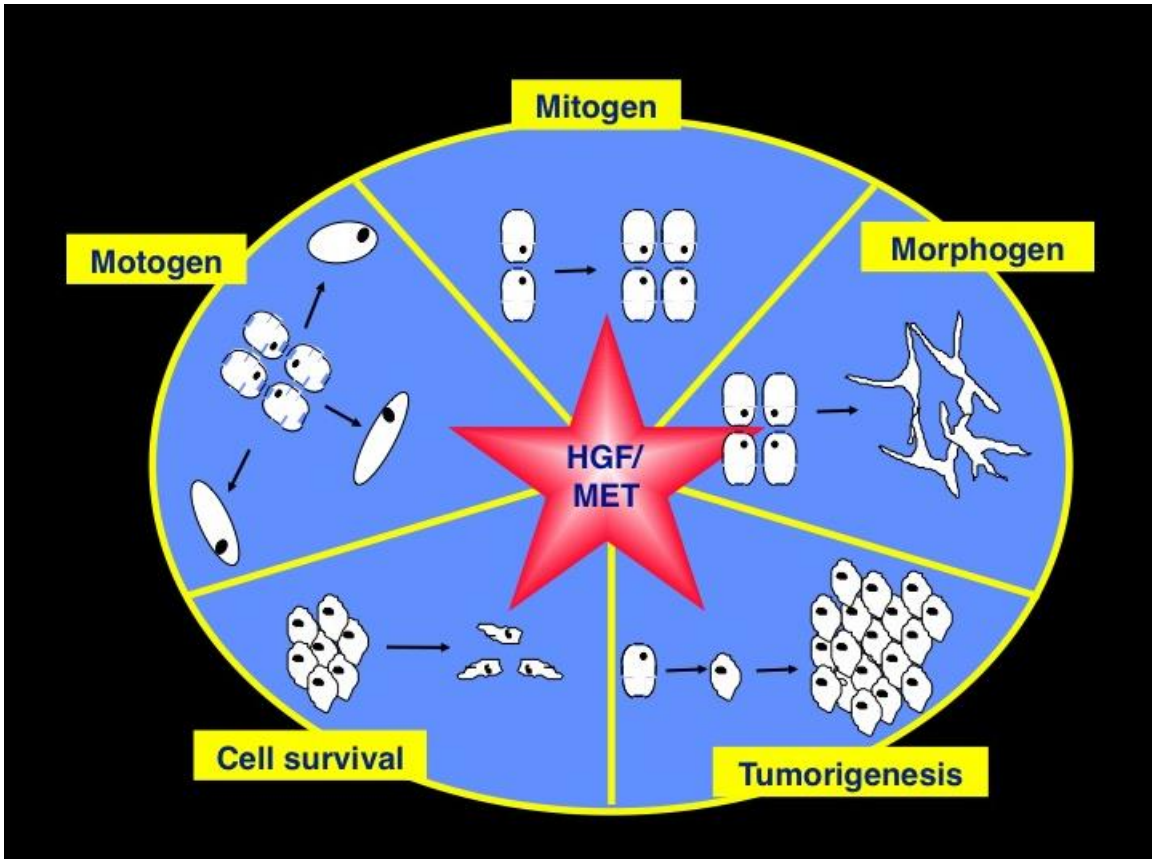

Figure 4. Roles of the HGF-Met signaling pathway
HGF-Met signaling is a crucial player in executing multiple cellular processes. HGF acts as a mitogen (promoting cell proliferation), motogen (promoting cell growth), and a morphogen (promoting cell differentiation), and stimulates cell survival. Dysregulated HGF-Met signaling is associated with malignant transformation and tumor progression.

1.2.2 Regulation of HGF-Met signaling

Given its crucial role in promoting cell survival and growth, the HGF/Met signaling axis is tightly regulated by a number of mechanisms at both the ligand and receptor levels. At the receptor level, internalization of the activated receptor and proteolytic cleavage are employed to attenuate Met signaling. Following ligand binding and activation, Met is monoubiquitinated by the E3 ubiquitin ligase, CBL, which targets the receptor for lysosomal degradation. Regulated

proteolysis of Met is also used as a mechanism to down-regulate Met levels to prevent excessive activation of the pathway [49,50].

HGF abundance is regulated primarily through gene expression in a cell type-specific manner. Inducible HGF production is limited to cells of mesenchymal origin while the *HGF* promoter is transcriptionally silenced in epithelial cells. HGF produced by the stromal cell compartment is largely sequestered in the extracellular matrix, allowing it to only act in a paracrine fashion on epithelial cells [46,55]. *In vitro* studies have revealed that *HGF* gene expression in stromal cells is inducible by a variety of signaling molecules, including estrogen, desamethasone, TGF-β, TNFα, as well as other cytokines and tissue loss (**Figure 5**). During wound repair for instance, interleukin-1 and 6, tumor necrosis factor-α and transforming growth factor-β (TGF-β) were observed to cause transcriptional up-regulation of HGF in fibroblasts and macrophages, but not epithelial cells [56].

Transcription factors like C/EBPβ, AP2, COUP-TF (chicken ovalbumin upstream promoter transcription factor), SP1 and SP3, and nuclear hormone receptors such as PPARγ and estrogen receptor were found to regulate *HGF* gene expression by binding to their associated sites in the *HGF* basal and proximal promoter [57-59]. *In vivo* studies using transgenic mice (harboring chimeric *HGF* consisting of mouse *HGF* promoter of varying lengths fused to a reporter gene) have identified a number of regulatory elements located within 1 kb upstream of the basal promoter that are necessary for *HGF* promoter activation. Several studies have confirmed that these upstream regulatory regions dictate *HGF* expression by directly binding nuclear factors and/or modifying chromatin dynamics, significantly altering the interaction between transcription factors and their cognate binding elements. These mechanisms allow for the tight regulation of inducible *HGF* expression in mesenchymal cells, and mediate the effective silencing of the *HGF*

gene in epithelial cells [57-64]. For instance, a DNA element located at 872−860 bp from the transcription start site contains an imperfect estrogen receptor element. Binding of COUP-TF, a nuclear orphan receptor belonging to the steroid/thyroid hormone receptor superfamily, to this DNA element silences *HGF* expression. On the other hand, the binding of the estrogen receptor to the site alleviates COUP-TF repression of transcription, causing activation of the *HGF* promoter. This was demonstrated using transgenic mice harboring either 2.7 kb or 0.7 kb promoter regions fused to a reporter. In contrast to mice harboring the entire 2.7 kb promoter region, mice with the shortened, 0.7 kb promoter lacking the estrogen receptor like regulatory element, did not show promoter activation in response to estradiol injection [58]. Additional mapping studies have revealed that in the absence of certain upstream regulatory elements, *HGF* core promoter within 100 bp upstream of the transcription start site is "promiscuous", indicating that it is active in both epithelial and mesenchymal cells (although to a lesser degree in epithelial cells). These works further validate the fundamental role played by upstream regulatory regions in controlling *HGF* promoter activity [57-59] (**Figure 6**).

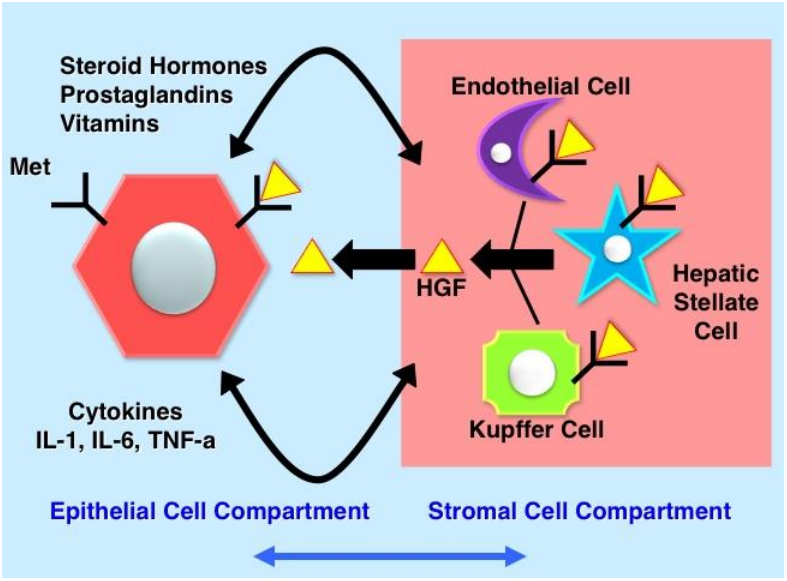

Figure 5. HGF-Met signaling is regulated in a cell-type specific manner
HGF gene expression is silenced in epithelial cells and HGF production is limited to stromal cells. Met is expressed in both epithelial and mesenchymal cells. *HGF* gene expression is induced in the stromal cell compartment by various signaling molecules including estrogen, desamethasone, TGF-β, TNFα, and a number of cytokines. HGF then activates Met signaling in epithelial cells in a paracrine manner.

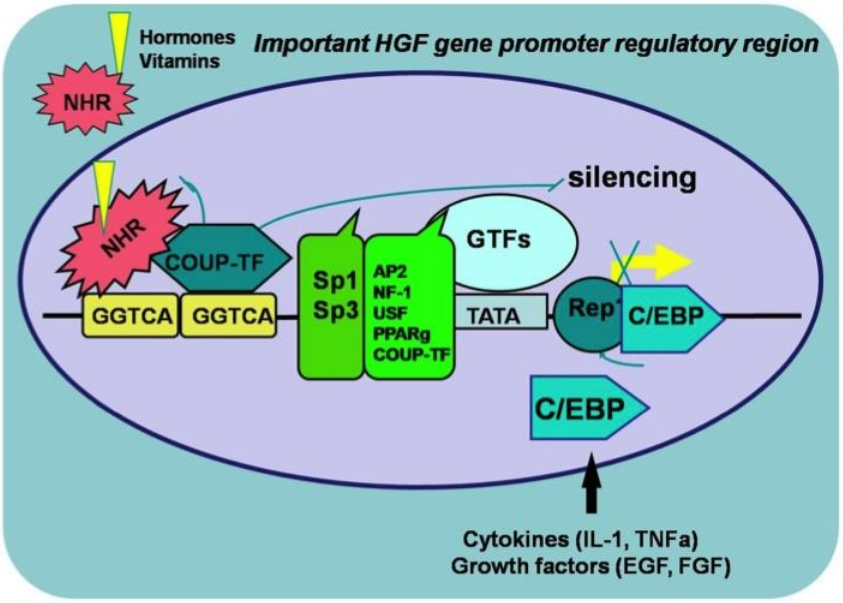

Figure 6. Regulation of cell-type specific HGF expression
Inducible *HGF* expression in mesenchymal cells is at least in part controlled by regulatory regions located within 1kb upstream of the basal promoter. These regulatory elements dictate the binding of various transcription factors, such as C/EBPβ, AP2, SP1,SP3 and nuclear hormone receptors such as PPARγ, estrogen receptor and COUP-TF to their cognate binding sites.

Recently, we identified novel regulatory cis-acting element located approximately 730 bp from the basal promoter of the human *HGF* gene. This element represses *HGF* gene transcription in epithelial cells by modulating chromatin structure and by regulating the recruitment of transcription enhancers to the promoter region. We named this regulatory element, as DATE (DeoxyAdenosine Tract Element) as it consists of a poly-A-tract of 30 deoxyadenosines (**Figure 7A**). We found that wild-type, full-length DATE (30 deoxyadenosines), fosters tight chromatin condensation and promote the binding of transcriptional repressors to the *HGF* promoter. When the fidelity of this region is compromised by reduction in the number of adenosines, the DATE region promotes release of repressors, chromatin remodeling and recruitment of transcriptional activators such as C/EBPβ and PARP1/2 to the *HGF* promoter, leading to *HGF* transcription. Notably, we reported that DATE is a subject of deletion mutagenesis in human breast ductal carcinoma tissues and its shortening leads to loss of its repressor function, leading to *HGF* promoter activation in these cells [65] (**Figure 7B**).

Collectively, these works indicate that in normal cells, HGF-Met signaling is tightly regulated in the stroma and entirely suppressed in the epithelia. Additionally, dysregulation of these regulatory mechanisms is associated with cancer. Although the detailed molecular mechanisms governing cell type-specific expression of *HGF* remain obscure to some degree, based on the above described promoter studies and other works, we now understand that silencing of *HGF* expression in epithelial cells is at least in part controlled by multiple cis acting regulatory elements in the region 1 kb upstream from the start site of the *HGF* promoter [57-64]. While these studies represent significant milestones in our understanding of *HGF* gene expression, the complete mechanisms responsible for HGF regulation remain to be elucidated.

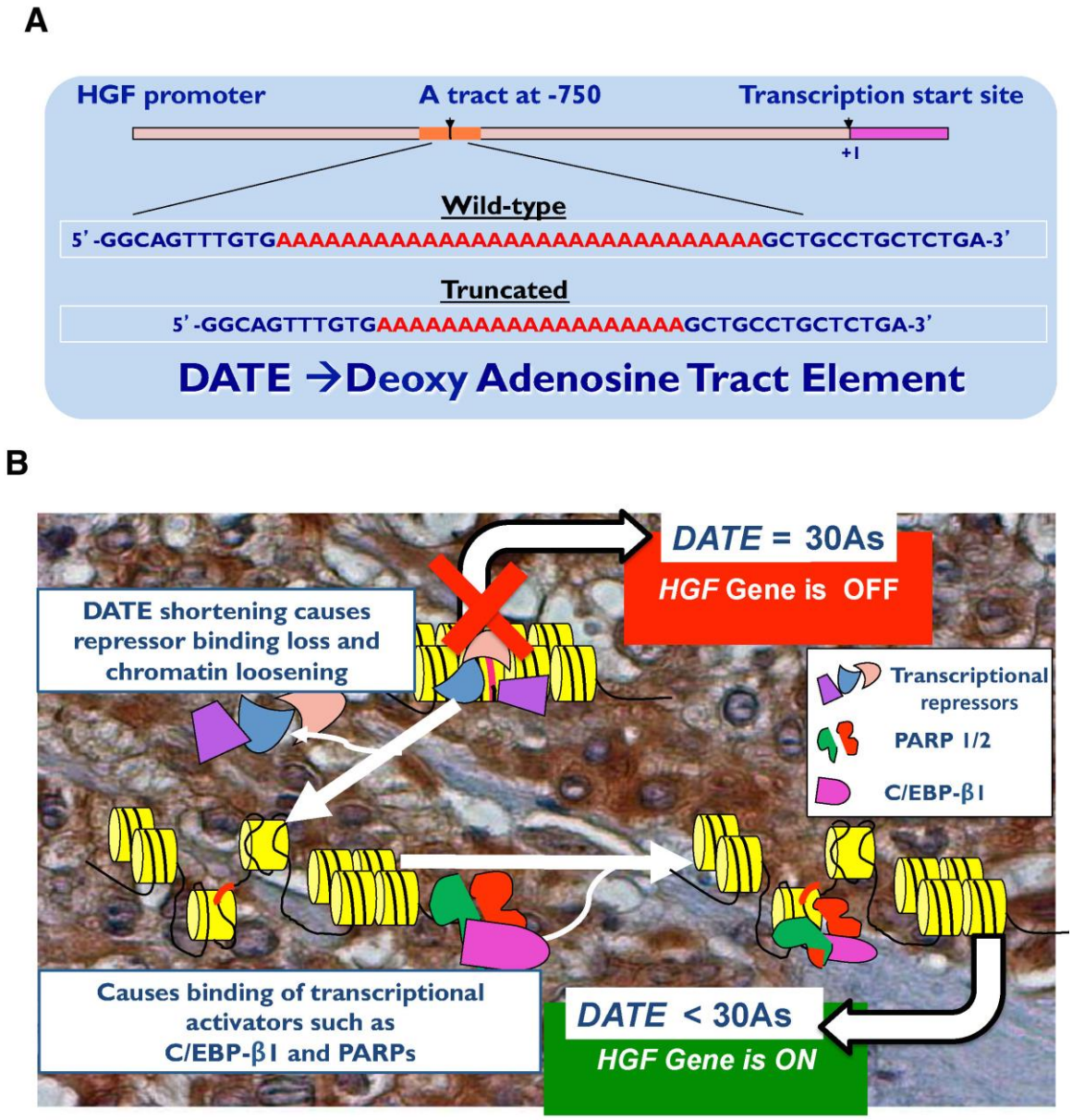

Figure 7. Structure and function of DATE

(**A**) DATE (deoxyadenosine tract element) is a regulatory cis-acting region located approximately 730 bp from the basal promoter of human *HGF* gene. In its wild-type form, DATE consists of 30 deoxyadenosines. (**B**) Wild-type DATE silences *HGF* expression by maintaining chromatin in a highly condensed form, promoting the binding of repressors to the promoter and by preventing the binding of transcriptional activators to their cognate promoter elements. DATE shortening causes repressor binding loss, chromatin remodeling, and the recruitment of transcriptional activators C/EBP-β1 and PARP1/2 to the promoter, leading to *HGF* promoter activation and gene expression. Adapted from [65].

1.2.3 HGF-Met signaling in cancer

Transcriptional activation of Met in cancer is commonly induced by hypoxia, inflammatory cytokines, and the pro-angiogenic factors abundant in the tumor stroma. Activation of Met in these already transformed cells exacerbates the malignant phenotype by promoting increased cell proliferation, survival and migration. Cell lines that overexpress *MET* or *HGF* were noted to be tumorigenic and metastatic when introduced to nude mice, while down regulation of *MET* was noticed to decrease the tumorigenic potential of the cells [66]. Concurring with the model that invasive cells simply resume the behaviors of early development, Met driven aggravation of cancer involves dysregulated stimulation of nearly all of the signaling pathways normally operative during embryonic development and organ regeneration.

The oncogenic potential of the HGF-Met signaling axis is substantiated by the fact that a number of epithelial and mesenchymal human cancers exhibit HGF and Met mutations that lead to hyper or aberrant activation of the pathway. Although dysregulated Met activation is often a late event that intensifies tumor progression, genetic lesions in *HGF* or *MET* are observed to play a causative role in the onset and the maintenance certain malignancies. In these cases, persistent activation of the HGF-Met signaling pathway is required to maintain the hyper-proliferative phenotype, and thus the cancer cells are said to be oncogenically addicted to HGF-Met signaling. Somatic gene amplification leading to *MET* overexpression was observed in colorectal, gastro-esophageal, gastric, ovarian and non-small-cell lung cancers and was associated with adverse prognostic outcomes. Sequencing of *MET* from individuals with hereditary kidney cancer syndromes revealed the existence of germline missense point mutations in the kinase domain causing ligand independent activation of Met. Mutations in the intracellular region of the Met receptor have also been noted in sporadic hepatocellular carcinoma, papillary renal cells

carcinoma, and gastric tumors and head and neck squamous cell carcinomas. A chromosomal translocation leading to the production of Tpr-Met fusion protein (which lacks the inhibitory juxtamembrane portion of Met and is thus constitutively active) was observed in human gastric carcinomas. Autocrine Met signaling (which occurs when epithelial cells express both the HGF ligand and the Met receptor) due to aberrant activation of *HGF* transcription or the overexpression of HGF activator protein, are observed in osteosarcomas, rhabdomyosarcomas, gliomas, cancers of the thyroid and lung [48,66]. In congruence with these findings, we recently showed that a novel mutation of the *HGF* gene promoter region (designated as DATE) leads to the autocrine activation of HGF-Met signaling loop in a subset of breast cancers and contributes to breast carcinogenesis and tumor progression [65].

1.2.4 Therapeutic Strategies targeting HGF-Met signaling

The initial efforts to halt HGF-Met signaling in cancer primarily focused on preventing HGF ligand binding to Met using antagonist competitors such as NK4, a molecule which resembled the N-terminal hairpin region and the four kringle domains of HGF. NK4 showed robust antagonist and promising anti-tumor abilities in several mouse tumor models, however its large-scale therapeutic application was limited by its inability to induce significant tumor regression in cancers involving ligand-independent Met activation [67]. Similar to NK4, neutralizing HGF antibodies were explored for their potential to inhibit ligand-dependent Met activation in tumors. Although encouraging results were observed in mouse models, given that a minimum of three antibodies (with varying pharmacodynamics qualities) against different HGF

epitopes were necessary to completely inhibit Met, concerns about the practicality of the approach have delayed its clinical application [48].

Strategies directly targeting the Met receptor, which can be used in clinical situations involving both ligand dependent and independent activation of signaling, have shown promise in both mouse models and clinical trials. These approaches include inhibiting the tyrosine kinase activity of Met, blocking its interaction with downstream signaling effectors, decreasing cell surface expression and hindering receptor dimerization. Other potential therapeutic strategies include the introduction of polypeptides that compete with downstream effectors for access to the Met docking site, RNA interference to promote Met silencing, and the use of dominant-negative Met constructs that lead to nonfunctional Met dimerization. The low efficiency and difficulty in delivery associated with these strategies have led to reservations regarding their clinical relevance. To date, small molecule ATP-competitive and allosteric inhibitors of Met activation have shown the most promise due to their high efficacy, however, the lack of specificity and potential for systemic side effects have raised concerns. There are a number of Met kinase inhibitors and anti-HGF therapies in phase I and II clinical trials for the treatment of lung, renal, gastric, brain and CRC [48,68]. For instance, AMG 102 is a monoclonal HGF neutralizing antibody currently in phase I clinical trials for the treatment of patients with unresectable locally advanced or metastatic gastric adenocarcinomas, being used in conjunction with the chemotherapeutics agents Epirubicin and Cisplatin. AMG208 and AMG337 are two additional Met kinase inhibitors that are currently in clinical trials for the treatment of adults with a variety of advanced solid tumors [69].

The targetable nature of HGF-Met signaling in colorectal carcinoma was recently demonstrated using a selective small molecule Met kinase inhibitor, ARQ 197 (Tivantinib). *In*

vitro assays using ARQ 197 showed a dose and a time-dependent decrease in the proliferative capacity of CRC cell lines, and ARQ 197 injection in mice bearing CRC cell line xenografts caused inhibition of tumor growth [70]. In phase I clinical trials, Tivantinib was well tolerated and showed encouraging anti-tumor activity in patients with metastatic CRC in conjunction with the chemotherapeutics, Irinotecan and Cetuximab [71]. While targeting Met is relatively new and presents numerous challenges with regard to efficacy, specificity and delivery, further development and improvement of targeting approaches has vast therapeutic potential in both cancer and non-neoplastic pathologies. An overview of HGF-Met targeting strategies currently being investigated is depicted in **Figure 8**.

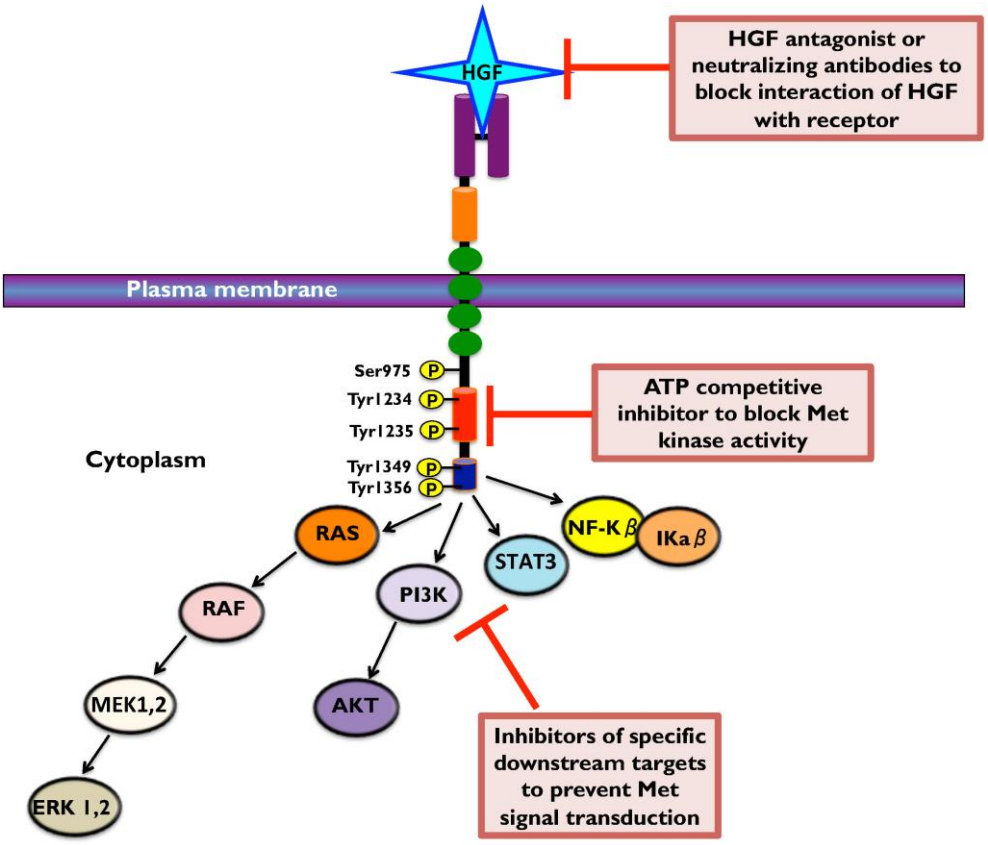

Figure 8. Strategies currently being investigated to inhibit HGF-Met signaling
Adapted from [72].

1.3 GENOMIC INSTABILITY IN CANCER

Originally outlined hallmarks of cancer include sustained proliferation, insensitivity to growth inhibitory and death signaling, replication immortality, sustained angiogenesis, ability to induce local and distant metastasis, abnormal metabolic pathways, immune system evasion, inflammation and genomic instability. Although only recently added to the list of cancer cell hallmarks, genomic instability is noted to be prominent in nearly all cancers, and can occur in the form of chromosomal instability (CIN) or microsatellite instability (MSI) [19,73].

1.3.1 Mechanisms of chromosomal instability

Fidelity of chromosome segregation is tightly controlled in normal cells, but is often dysregulated in cancer. CIN is hypothesized to result from alterations in classical caretaker genes such as DNA repair and mitotic checkpoint genes, or due to oncogene-induced DNA replication stress [19]. While the exact mechanisms of how caretaker gene mutations or oncogene- mediated replication stress eventually lead to the onset of CIN are not fully understood, it is generally believed that they induce multiple cellular defects, such as altered centrosome duplication, faulty spindle assembly, and telomere dysfunction. These defects cause inaccurate attachment of microtubules to chromosomes, resulting in the lagging of chromosomes in anaphase, mis-segregation, and eventually the presentation of the CIN phenotype. For instance, centrosome duplication increases the number of potential erroneous spindle attachment points, therefore rapidly overwhelms the cells' natural repair mechanisms, promoting chromosome mis-segregation during cell division. When there is aberrant activation of the spindle assembly

checkpoint, mis-segregation of chromosomes occurs, leading the formation of cells with the incorrect number of chromosomes and high tumorigenic potential [74].

1.3.2 Chromosomal instability in cancer

In cancer, CIN manifests itself in the form of aneuploidy, sequence changes, base substitutions, deletions, insertions, chromosomal rearrangements, and gene amplification. Given that 70% of solid tumors display these characteristics, it is strongly argued that chromosomal instability is a driver of tumorigenesis and an important player in cancer progression [74]. The CIN pathway is postulated to be a key player in the onset and progression of a majority of CRCs and is associated with poor patient prognosis. Large-scale sequencing analysis has revealed that cancers displaying CIN commonly show alterations in cell cycle caretaker genes including, the *TP53* tumor suppressor, the DNA damage checkpoint gene, *ATM*, and the cell cycle regulator, *CDKN2A*. Dysregulation of these caretakers alters cell cycle dynamics, resulting in the mis-segregation of chromosomes during replication and fostering instability throughout the genome. As mentioned above, CIN can also result from excessive DNA replication stress induced by activating mutations in classical oncogenes such as *EGFR,* the small GTPase, *RAS*, or the inactivation of the *PTEN* tumor suppressor gene, *RAS* mutations are frequently observed in pancreatic and lung carcinomas, while a large portion of glioblastomas present *PTEN* and *EGFR* mutations [74,75].

1.3.3 Mechanisms of microsatellite instability

Microsatellite instability (MSI), which is characterized by the expansion or the deletion of oligonucleotide repeat regions, is observed in a large subset of hereditary and sporadic cancers. Microsatellite instability is caused by defects in the DNA mismatch repair pathway (MMR) that may be caused by aberrant CpG methylation of *MLH1*, or point mutations in *MLH1*, *MSH2* or other MMR pathway genes. MMR system repairs the base-base mismatches and unmatched insertion-deletion DNA loops [76] (**Figure 9A**). When these lesions are left unrepaired, permanent mutations that may alter cell behavior and promote tumorigenesis are reported to occur. As such, the MMR system plays a key role in maintaining DNA replication fidelity and serves as a first line of defense against malignant transformation. Cells harboring MMR deficiency are unable to repair the nucleotide slippage errors that frequently occur during DNA replication and thus experience genomic alterations in the forms of frameshift or point mutations, as well the shortening and the lengthening of nucleotide repeat genomic regions. Repetitive nucleotide regions are particularly sensitive to mutagenesis in the absence of proficient MMR, as these regions commonly experience nucleotide slippage errors during replication (**Figure 9B**). While many repetitive genomic regions consist of noncoding DNA, when a mismatch or an erroneous deletion/insertion loop that occurs in a repetitive coding region of a key regulatory gene remains unrepaired, it may provide cells with an increased growth advantage and result in carcinogenesis [19,76]. This cause-effect relationship between MMR defects and tumorigenesis was demonstrated in experiments involving mice with knockout mutations in each of the MMR genes. Mice lacking functional MMR systems developed the expected mutator phenotype and exhibited a predisposition to develop various carcinomas, lymphomas and sarcomas [77].

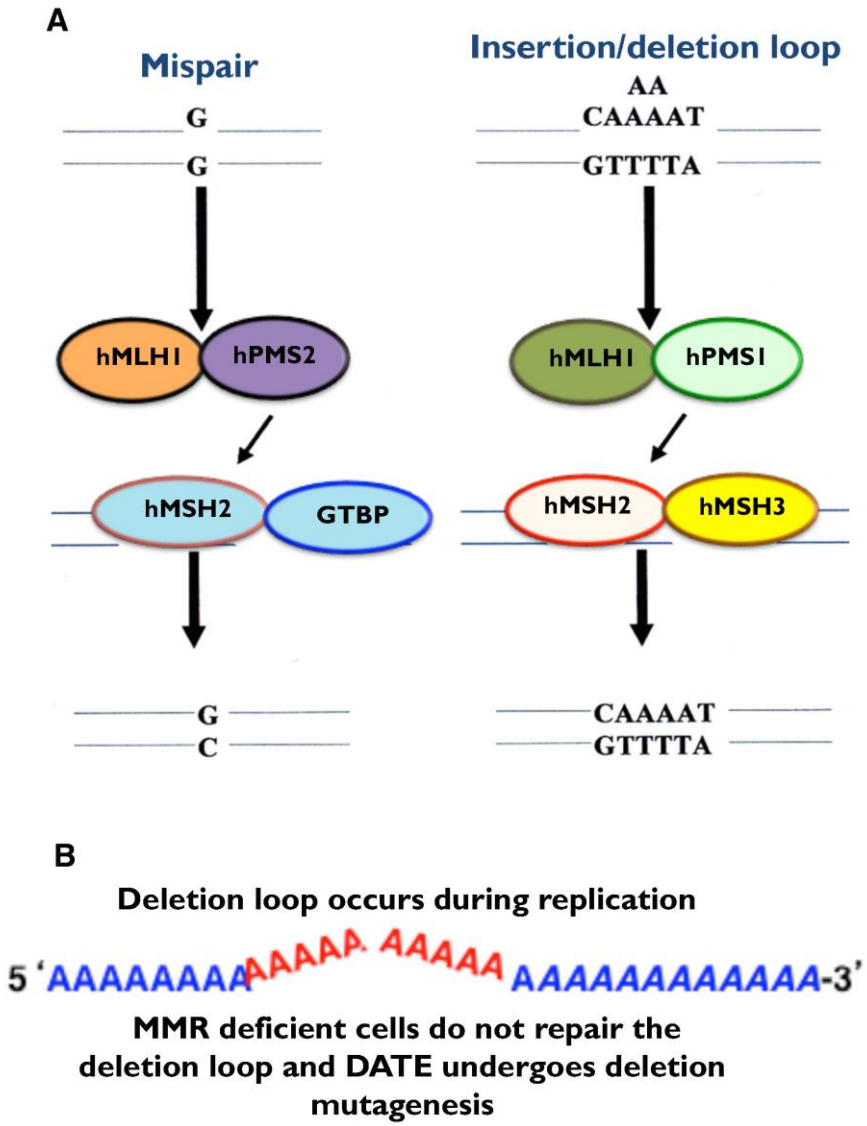

Figure 9. DNA mismatch repair system maintains genomic fidelity during replication
DNA mismatch repair (MMR) system (DNA repair system is utilized for recognizing and repairing (**A**) erroneous mis-incorporation of bases and (**B**) insertion/deletion loops and that can arise during DNA replication. The repair system consists of protein complexes involving MLH1, MSH2, among others. (**C**) Repetitive genomic regions such as DATE (see section 1.2.2) are particularly prone to the formation of insertion/deletion loops and thus replication errors. In MMR deficient cells, DATE deletion loop would be not repaired, leading to the formation of shortened, mutant DATE variant. Adapted from [78].

1.3.4 Microsatellite instability in cancer

Lynch syndrome, often referred to as hereditary nonpolyposis CRC (HNPCC), is a hereditary disorder that occurs due to germline mutations in the mismatch repair genes *MSH2*, *MLH1*, and *PMS2*, and is inherited in an autosomal dominant manner. MSI phenotype is observed in up to 90% of lynch syndrome associated diseases, including colorectal, stomach, small intestine, liver, gallbladder duct, upper urinary tract, brain and skin cancers, and fosters the early development of benign or precancerous polyps. Additionally, women with lynch syndrome have a significantly increased risk of developing ovarian, uterine and endometrial cancers [79,80]. Aside from its role in hereditary malignancies, MSI is also observed in a number of sporadic cancers, including colon, endometrial, gastric, and lung carcinomas, as well as several lymphomas. Approximately 15% of CRCs, 20-30% endometrial cancers, and 10% of gastric cancers, are characterized by MMR deficiency and exhibit high levels of MSI. While germline mutations in mismatch repair genes were causative of the hereditary lynch syndrome cancers, epigenetic silencing of *MLH1* via CpG methylation was found to be the most common lesion observed in sporadic cancers exhibiting the MSI phenotype [80,81].

Significant strides have been made in identifying the genomic targets of defective MMR, and in discerning the clinico-pathological behaviors of tumors harboring MSI. In MSI high cancers, the expansion and deletion of sequence-repeats leads to the accumulation of base substitutions and frameshift mutations in genes responsible for cell survival, proliferation, and apoptosis, such as transforming growth factor receptor type II, insulin-like growth factor type II receptor, pro-apoptotic *BAX* genes, transcription factor *E2F-4*, and tumor suppressor genes such as *APC* and *PTEN*. Unlike tumors exhibiting CIN, microsatellite unstable tumors do not show gross genomic lesions or aneuploidy, and instead have diploid genomes, display proximal tumor

location, a mucinous phenotype, and are poorly differentiated with heavy lymphocyte infiltration [76,82].

1.3.5 The use of microsatellite instability as a diagnostic and a prognostic marker in cancer

MSI has been used as a helpful cancer diagnostic tool in detecting lynch syndrome, as nearly all patients presenting lynch syndrome tumors show mismatch repair defects and instability at nucleotide repeat genomic regions. Furthermore, in sporadic human CRC, MSI is maintained to have prognostic significance at particular disease stages and is believed to predict the patient response to several commonly utilized chemotherapeutic agents [82]. As described in above sections, MMR system detects DNA damage and promotes apoptosis in cells with irreparable levels of damage. Therefore, MMR deficiency predisposes cancer cells to be resistant to treatment with DNA damaging alkylating agents such as Temozolomide, or Procarbazin and Cisplatin, by failing to detect the formation of DNA adducts and consequently preventing the onset of apoptosis. Furthermore, MMR deficient CRC patients are predicted to receive little benefit from 5-FU adjuvant chemotherapy that is commonly utilized in clinical settings. The resistance to 5-FU is believed to result from the inability of MMR deficient cells to recognize the 5-FU metabolites that are incorporated into DNA as mismatches, and self-eliminate via apoptosis [82,83].

Despite the fact that hundreds of microsatellite markers have been identified to date, it is yet unclear how many and which loci should be used to robustly evaluate MSI in clinical settings. In order standardize the screening of MSI internationally, a set of parameters known as Bethesda guidelines were established during the last decade. These guidelines specify that at least five loci must be assessed for instability. If one of the five loci were unstable, then the tumor is classified

as MSI-low (MSI-L), and if two or more loci were altered, then the tumor is determined to be MSI-high (MSI-H). Cancers exhibiting MSI can be further divided into two groups: those that show instability primarily at mono- and di-nucleotide markers, and those that exhibit instability at larger tri- or tetranucleotide repeats. It has been observed that endometrial colonic, and gastric tumors generally belong the first group, while bladder and lung cancers with MSI belong the latter group [84]. As investigators were using many different microsatellite markers to assess instability and thus obtaining varying frequencies of MSI in the same cancer type, in an attempt to standardize the protocol, the National Cancer Institute (NCI) recently recommended a five-marker reference panel. The panel consists of two mononucleotide loci, *BAT-25* and *BAT-26*, and three dinucleotide loci *D2S123*, *D5S346*, and *D17S250,* and were chosen due to the fact that these loci were ubiquitously altered in nearly all MMR deficient cancers [84,85]. The recommended reference panel is expected to change over time as more sensitive and clinically applicable markers are identified through novel research.

As we stride forward into the era of personalized medicine, it is important to recognize that MMR deficient tumors are a clinico-pathologically distinct subset that must be identified and treated accordingly. Given that MMR deficiency antagonizes many commonly utilized chemotherapeutic agents [83], it is pertinent that we continue to uncover novel, more reliable markers for MSI detection, and identify clinically relevant targets of defective MMR, in order to improve the therapeutic strategies available for this patient population.

1.4 MAJOR PATHWAYS OF CRC CELL DEATH

1.4.1 Apoptosis

Apoptosis is a form of programmed cell death that was first described by Kerr et al. in 1972 [86]. The ability of certain cells to escape apoptotic cell death is now identified as a hallmark of cancer and is believed to play a major role in malignant transformation [87]. Cells undergoing apoptotic cell death are characterized by cell shrinkage, nuclear condensation, aggregation of chromatin at the nuclear membrane, and fragmentation of the cell into membrane bound vesicles, known as apoptotic bodies. Apoptosis is implemented by the proteolytic enzymes known as cysteinyl aspartate-specific proteases (Caspases). Caspases are classified as either initiators (Caspases 8,10) or executioners (Caspases 3,6, and 7), and participate in the extrinsic and intrinsic death pathways. In the extrinsic, otherwise known as the death receptor pathway, tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand treatment (TRAIL), or Fas ligands bind to their cognate receptors, and in congruence with adapter molecules such as Fas-associated death domain (FADD) or TNF receptor–associated death domain, causes the cleavage and the activation of the initiator Caspases. These Caspases then cleave and activate the executioner Caspases, resulting in apoptotic cell death. In the intrinsic mitochondrial pathway, apoptotic signaling results in the release of cytochrome c into the cytosol. Cytochrome C then interacts with the apoptosis-activating factor-1 (Apaf-1), ATP, and pro-Caspase 9 to form a complex known as the apoptosome. This complex then cleaves and activates Caspase 9, which then cleaves and activates the executioner Caspases 3,6, and 7, causing the onset of apoptosis [88].

1.4.2 Autophagy

Autophagy involves the self-mediated lysosomal degradation of cellular contents. During this process, a portion of the cytoplasm becomes enveloped in a double membrane vesicle known as the autophagosome, which then fuse with the lysosome to form the autolysosome. Within this membrane bound structure, the cellular contents undergo hydrolytic degradation. While autophagy occurs in normal cells during tissue remodeling and stress conditions, it is particularly associated with certain pathologies, including viral infection, neurodegenerative diseases and cancer [89].

Autophagy may be utilized to promote both cell survival and death. In survival, autophagy may be used by cells to relieve the burden of excess protein and damaged organelles, as well as to conserve energy during nutrient depravation conditions by facilitating the recycling of amino acids for new protein production [90]. It was shown that induction of extreme stress (nutrient depravation and hypoxia) in human CRC cells results in the onset of autophagy and cell death in response to chemotherapeutic agents. The molecular depletion of the autophagy executing proteins, beclin-1 or ATG5, enhanced the sensitivity of the colorectal tumor cells to oxiplatin [91]. Furthermore, the term autophagy dependent resistance has been coined to describe a form of therapeutic resistance exhibited by CRC cells to 5-FU therapy, as it can be rapidly reversed following the molecular ablation of autophagy stimulating proteins or treatment with Chloroquine (which has anti-autophagy properties) [92]. Despite its role in promoting cell survival, widespread autophagy that depletes a majority of cellular organelles can result in programmed cell death. Interestingly, there is evidence indicating that 5-FU treatment and a number of more novel experimental therapeutic agents, can induce autophagy-mediated cell death in human CRC cells, particularly when apoptotic mechanisms are compromised [93-95]. Given the dual and antagonizing

roles of autophagy, it is currently being heavily investigated as a potential therapeutic target in cancer [96].

1.4.3 Necroptosis

While apoptosis remains the most widely studied and understood form of regulated cell death, ordered necrosis, dubbed as necroptosis, is now accepted as an important contributor to programmed cell death. Until relatively recently, necrosis was considered to be an unregulated, accidental and passive form of cell death. However, accumulating evidence indicating that cells can actively undergo a series of characteristic molecular and morphological changes in response to particular stimuli (such as TNF treatment) has led to the acknowledgment of necroptosis as a regulated form of death [97]. Morphologically, necrotic cell death is contrasted from apoptosis by the occurrence of features such as the translucent cytoplasm, swelling of organelles, condensation of chromatin into irregular patches, the disintegration of the plasma membrane, and leakage of intracellular contents into the extracellular space. Additionally, unlike apoptosis, nuclei of necrotic cells remain intact, no membrane bound vesicle formation occurs, and eventually the cell undergoes total lysis [97].

Although its existence is no longer intensely debated, the complete underlying mechanisms responsible for the onset of necroptosis in cells are yet to be elucidated. The onset of necroptosis is believed to involve a number of proteins, classified as either initiators or modulators/executioners. [98]. Necroptosis can be initiated through the ligation of death receptors such as TNF-R1, FAS, and TRAIL-R, particularly when apoptosis is blocked through the inhibition of Caspases. The serine-threonine kinases, receptor interacting protein kinase-1 and 3

(RIPK-1, RIP-3), were found to play critical roles in the progression of necroptosis via the death receptor pathway [97].

Necroptosis can also be instigated by the pathogen recognition receptor (PRR) family. Important members of the PPR family include the plasma membrane or endosome membrane-associated Toll- like receptors, cytosolic NOD-like receptors, and retinoic acid-inducible gene I-like receptors. These receptors are expressed on the surface of immune cells to detect pathogen-associated particles, such as viral or bacterial nucleotides, lipoproteins, and peptidoglycans. Upon detection of viral or bacterial molecules by the PRR members, necrotic cell death may be induced [97]. Other more marginally studied initiators of necroptosis include reactive oxygen species, ubiquitin E3 ligases, certain deubiquitinating enzymes, mitochondrial and lysosomal membrane permeabilization, hyper-activation of PARP-1, and rapid lipid degradation that increases cytosolic calcium burden and alters mitochondrial dynamics [98].

1.4.4 RIPK-1 at the crossroads of cell survival and cell death

RIP serine/threonine kinase family members play crucial roles in detecting stress and in regulating cellular decisions regarding survival and death. RIP family members have structurally homologous N-terminal kinase domains, but possess vastly differing recruitment, and intermediate domains. The focus of the current work, RIPK-1, consists of a N-terminal serine-threonine kinase domain and a homotypic interaction motif comprised of an intermediate domain and carboxy-terminal death domains. RIPK-1 contains several sites that may be post-translationally modified, particularly Lys377 which can be ubiquitinated to allow interaction of RIPK-1 with a large number of proteins. The death domain of RIPK-1 is fundamental in the onset of cell death, while the intermediate domain participates in cell survival signaling via NF-κB. The

death domain of the RIPK1 interacts with death receptors such as TNF-R1 and Fas, as well as TRAIL1/2, TRADD, FADD, and Casapse-8, and is necessary for the induction of Caspase-8 dependent apoptosis. The homotypic interaction motif of RIPK-1 facilitates the interaction with its sister necroptotic initiator, RIP-3 (**Figure 10**) [99].

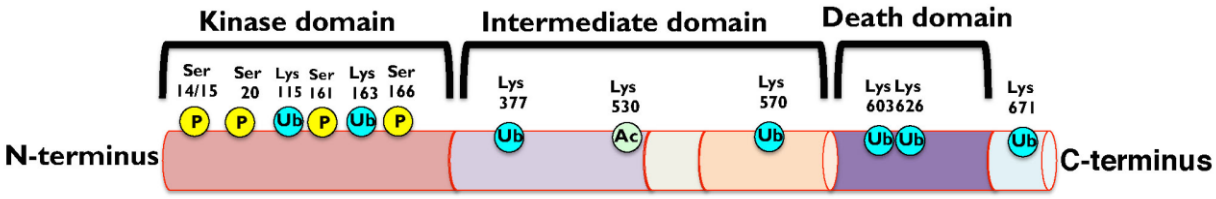

Figure 10. The structure of human RIPK-1
Adapted from [99].

RIPK-1 regulates cell survival and death through its participation in multiple cellular complexes described below. The binding of TNFα results in the ligation of the TNF-R1 death receptor, causing the formation of one of three complexes. Complex I mediates cell survival and involves the rapid association of the molecules TRADD (TNFR type 1 associated death domain protein), TRAF2/5 (TNFR associated factor 2/5), cIAP1/2 (cellular inhibitor of apoptosis 1/2) and the polyubiquitinated RIPK-1 at the plasma membrane. Ubiquitination of RIPK1 determines whether it participates in pro-survival or pro-death signaling. In complex I, RIPK-1 becomes polyubiquitinated (Lys63-linked chain type) in lipid rafts by TRAF-2, and acts as a docking site for the recruitment and activation of various components of the NF-κB survival pathway. This results in the subsequent induction of a number of anti-apoptotic proteins such as c-FLIP, cIAP1 and cIAP2, as well as the activation of MAPKs such as p38, MAPK, JNK, and ERK. It is important to note however that kinase activity of RIPK-1 is not required for the activation of most MAPKs or NF-κB signaling. NF-κB survival signaling is negatively regulated via the depletion

of cIAP1 and cIAP2 proteins and the binding of the inhibitory proteins, A20 and Cyld to RIPK-1. The Lys63-linked chain on RIPK-1 is edited by A20 and Cyld, switching its polyubiquitin moiety to the Lys48-linked form, thus, targeting RIPK-1 for degradation via the 26S proteasome complex, [99-103].

If the cellular environment is more conducive to death, RIPK-1 participates in molecular complexes that lead to apoptosis (Complex IIA) or necroptosis (Complex IIB). During the induction of death receptor mediated apoptosis, RIPK-1 was shown to be deubiquitinated by Cyld, and translocated to the death inducing signaling complex (DISC) known as complex IIA. This signaling moiety consisting of the molecules RIPK-1, FADD, TRADD and Caspase-8, rapidly activates Caspase driven apoptotic cell death. Alternatively, this apoptotic-signaling complex may also be formed following the binding of the FAS ligand to the FAS death receptor. While the exact means of how RIPK-1 transitions from complex I to complex II remain ambiguous, deubiquitination is believed to play a crucial role in the dissociation of RIPK-1 from the pro-survival complex I [99,101,104]. For instance, the depletion of the deubiquitinase, Cyld, using siRNA methods was found to prevent the formation of complex II and inhibit both apoptosis and necroptosis [99,101]. Interestingly, apoptotic signaling is actively maintained by the prevention of survival and necroptotic signaling via the cleavage of RIPK-1 by Caspase-8 [105]. Complementary studies indicate that overexpression of the Caspase antagonist, FLIP, prevents the onset of apoptosis by failing to inhibit the survival and necroptotic signaling arms that are facilitated by RIPK-1 [106].

The execution of necroptosis is arbitrated via the signaling Complex IIB, consisting of RIPK-1 and RIP-3. Unlike its non-essential role in apoptosis, RIPK-1 is indispensable for the induction of programmed necroptosis [99,101,104]. Furthermore, elimination of RIPK-1 kinase

activity using the specific allosteric inhibitor, Necrostatin, maintained NF-κB survival signaling in cells, while prohibiting complex II formation and the onset of necroptosis, highlighting the fundamental role played by RIPK-1 in necroptotic cell death [98]. RIP-3 has also recently emerged as a critical regulator of necroptosis, as knockdown of RIP-3 hindered the onset of TNFR-1 mediated necroptosis in some cell types [107]. Importantly, necroptosis is primarily observed when Caspase activity is inhibited via the loss of Caspase-8/Fas-associated death domains, or through the inhibition of Caspase-8 enzymatic activity by the pan-Caspase inhibitor zVAD or viral proteins. In the absence of Caspase-8 activity, a functional DISC cannot form to induce apoptosis. RIPK-1 is therefore directed to form the necroptosome (RIPK-1 and RIP-3 complex). RIPK-1 dimerization and autophosphorylation at Ser161 (which is believed to activate the kinase) are thought to play crucial roles in enabling necroptosis [105]. Following the assembly of complex IIB, RIP-3 phosphorylates RIPK-1. Although a substrate for RIPK-1 is yet to be definitively identified, the inhibition of RIP-3 phosphorylation at Ser199 when cells are treated with the RIPK-1 kinase inhibitor, Necrostatin, suggests that RIP-3 serves as a substrate for RIPK-1. Further research is however necessary to confirm the precise role of RIPK-1 kinase activity and its downstream targets. Given that necroptosis involves massive production of reactive oxygen species, it has been speculated that RIPK-1 directly targets the mitochondria. This notion is supported by data indicating that in TNF-stimulated cells, RIPK-1 translocates to the mitochondria. Despite the strides made over the last decade, the major gaps in our knowledge that remain regarding the onset of necroptotic cell death, underscore the need for extensive future research in this field [99,101].

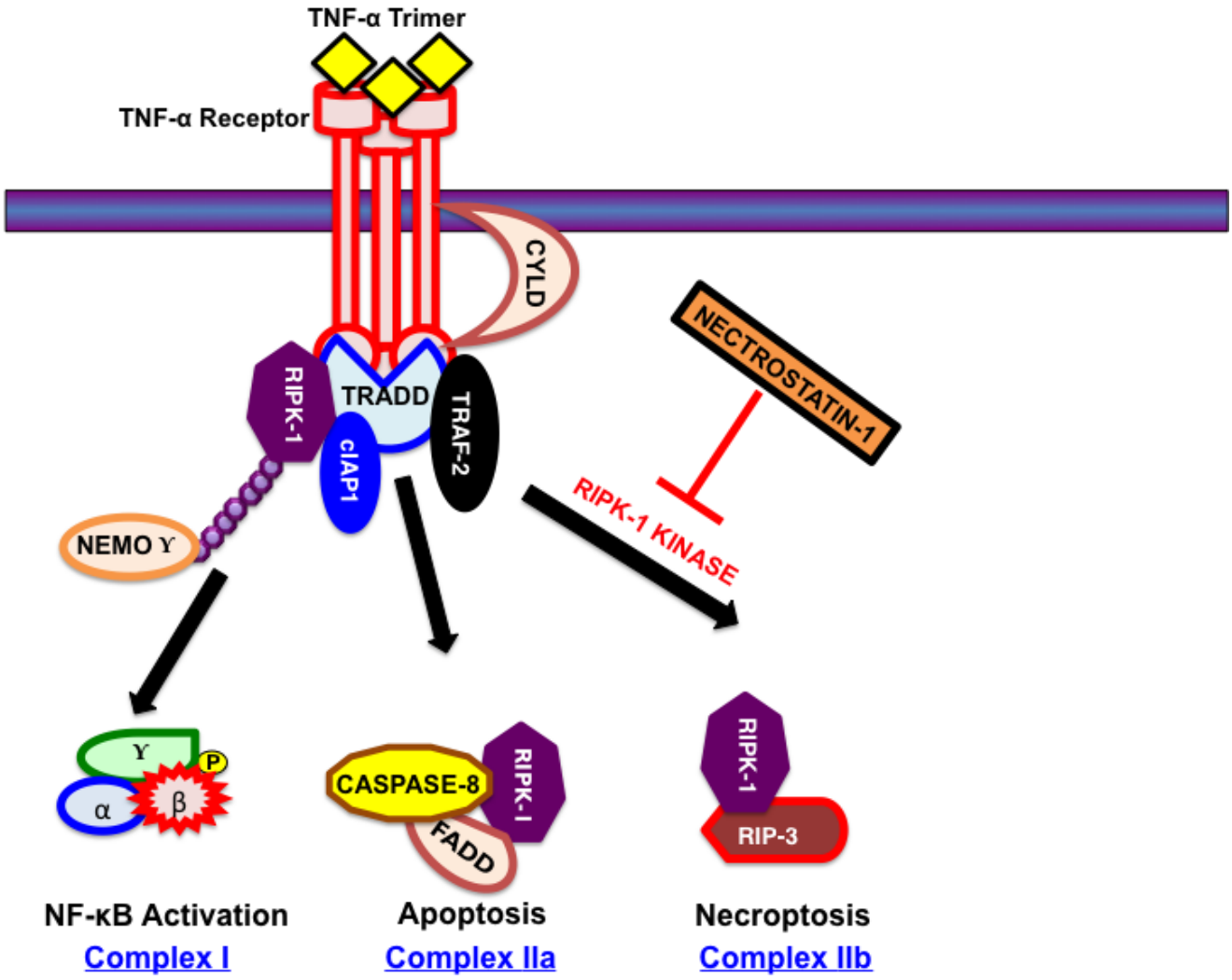

Figure 11. Multiple RIPK-1 mediated signaling complexes are initated downstream of TNFR-1 stimulation.
Adapted from [104].

2.0 CAUSES OF *HGF* GENE MUTAGENESIS IN HUMAN CRC

2.1 ABSTRACT

It is known that genomic instability, (both chromosomal and microsatellite instability) promote carcinogenesis by causing mutations in key growth regulatory genes, which consequently drive the tumorigenic process. The identity of these "so called driver genes" affected by genomic instability, however, has not yet been completely identified. Here, we report that microsatellite instability (MSI) provoked by DNA mismatch repair (MMR) deficiency causes deletion within an important repressor element in the *HGF* gene proximal promoter region. As this region consists of 30 deoxyadenosines, we have named it DATE (DeoxyAdenosine Tract Element]. We determined that DATE is polymorphic in nature, occurs in approximately 30% of sporadic human CRC, and significantly associates with the incidence of CRC, suggesting that DATE mutation plays a functional role in CRC pathogenesis. Furthermore, we noted that DATE truncation was significantly more prevalent in the normal tissues of African Americans in comparison to Caucasians. We postulated that this high incidence of germline DATE truncation may underlie the observed predisposition of African Americans to develop more aggressive forms of CRC and experience lower overall survival rates. We also noted that DATE truncation in tumor was a highly sensitive marker of MMR deficiency and MSI in human CRC. Collectively, our results revealed that in human CRC, *HGF* gene promoter is a target of mutagenesis provoked by defective MMR. Moreover, our findings encourage the adaptation of DATE as a highly sensitive marker for detecting MSI in the clinical arena, and warrant further investigation of the prognostic significance of DATE in human CRC.

2.2 INTRODUCTION

Receptor tyrosine kinase (RTK) signaling is important in the regulation of a variety of processes including development, cell function and tissue maintenance. Despite these crucial roles, aberrant activation of RTK pathways has been implicated in the onset and progression of a variety of cancers. The HGF-Met signaling pathway is exemplary of this phenomenon. HGF is normally produced by stromal cell compartment of various tissues and elicits its effects by activating its cell surface receptor, Met [46,49]. Binding of HGF to the Met receptor activates a number of complex signaling networks leading to cell growth, proliferation, and escape from cell death. Due to its critical role in a vast number of cellular processes, HGF-Met signaling pathway is tightly regulated through mechanisms such as receptor internalization, receptor degradation, and the maintenance of paracrine signaling. The loss of these regulatory measures is associated with a variety of human cancers [48].

We recently showed that in a subset of human breast cancers, *HGF* gene promoter undergoes mutagenesis at a novel cis-acting regulatory element located approximately 700 bp from the basal promoter transcription start site. This element, which we have named DATE, consists of a poly-A tract of 30A, represses *HGF* gene transcription in epithelial cells by modulating chromatin structure and by regulating the binding of transcriptional repressors and activators to the *HGF* promoter [65]. Upon mutagenesis of DATE, repressor activity of the region is lost, leading to promoter activation and transcription of the *HGF* gene in epithelial cells. The molecular basis underlying DATE mutagenesis, and whether its mutation occurs in other human carcinomas remained unknown. The overall goal of this study was to address these issues.

DNA mismatch repair (MMR) system is indispensable for maintaining genomic fidelity, particularly at microsatellite regions that are subject to frequent strand slippage during replication

[76]. MSI resulting from MMR deficiency is documented to contribute to many malignant conditions, including hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome and its associated cancers), sporadic human colorectal carcinoma (CRC), as well as cancers of the endometrium, stomach, pancreas-biliary tract, small intestine, urothelium and the ovary [76,82,108,109]. As DATE consists of a repetitive tract of deoxyadenosines and resembles a microsatellite, we predicted that DATE mutagenesis could arise from MSI provoked by deficient MMR. We also hypothesized that that DATE truncation is a result of defective MMR and therefore could be observed in sporadic human CRC, which exhibits MMR defects and MSI in a significant patient subset.

Our results indicated that the *HGF* promoter is indeed a target of genomic instability in CRC harboring MMR deficiency. We found that the frequency of DATE truncation significantly associated with the incidence of human CRC, suggesting that DATE mutagenesis plays a functional role in the onset and/or maintenance of the carcinogenic phenotype. DATE was noted to be polymorphic, and the incidence of DATE truncation in the normal population significantly differed between Caucasian and African American populations. Truncated DATE was also a highly sensitive indicator of MSI in CRC. Together, our results shed new lights on the potential molecular mechanisms of human CRC pathogenesis and support further development of DATE as a clinically relevant marker for detecting MSI in CRC.

2.3 MATERIALS AND METHODS

CRC tissues and cell lines

Archival human colon tumor tissues and paired normal adjacent tissues from 78 patients were obtained from Health Sciences Tissue Bank, University of Pittsburgh according to an approved IRB. Informed consent was obtained from all subjects prior to using tissue samples. These patients were of either Caucasian or an African American ethnic background as documented in Table 1. An Additional 50 human CRC tissues and corresponding adjacent normal tissues were purchased in the form of a tissue microarray (IMH-359) from IMGENEX. This particular cohort, consisting of patients of Asian ethnicity from Korea, were chosen as it was likely to be relatively homogenous in terms of genetic background. Additional information regarding the tissue microarray is provided in the following website: http://www.imgenex.com. All cell lines were purchased from American Type Culture Collection (ATCC) and cultured according to the supplier's instructions.

							Length of DATE	
Case #	**Type of tumor**	**Tumor size (cm)**	**Lymph node metastasis**	**Gender**	**Age (yr)**	**Race**	**Tumor**	**Normal Adjacent**
1	AI	7	-	M	76	AA	29	29
2	**AI**	**2.5**	+	**M**	**65**	**AA**	**25, 30**	**25, 30**
3	**AI**	**3**	+	**F**	**45**	**AA**	**19,30**	**19, 30**
4	AI	7	-	F	55	AA	30	30
5	AI	5	-	M	85	AA	30	30
6	AI	11	-	F	75	AA	30	30
7	**AI**	**8.5**	**-**	**F**	**65**	**AA**	**21,29**	**29**
8	**A**	**5.4**	**-**	**M**	**55**	**AA**	**24,30**	**24, 30**
9	AI	1.5	-	F	85	AA	30	30
10	AI	2.5	+	F	45	AA	30	30
11	AI	4.6	-	M	65	CC	29	29
12	A	4.5	+	M	55	CC	30	30
13	AI	5.2	+	M	N/A	CC	30	30
14	AI	N/A	-	M	86	CC	30	30
15	**AI**	**8**	**+**	**F**	**75**	**CC**	**20, 30**	**30**
16	AI	5	+	F	75	CC	30	30
17	**A**	**3.5**	**-**	**M**	**85**	**CC**	**22,29**	**29**
18	A	4	-	F	75	CC	30	30
19	A	3.5	-	F	75	CC	29	29
20	AI	5	-	M	75	CC	31	31
21	**A**	**3.7**	**-**	**F**	**75**	**CC**	**21, 30**	**30**
22	A	5	-	F	85	CC	30	30
23	**AI**	**9**	**+**	**M**	**75**	**CC**	**21, 30**	**30**
24	AI	5.5	-	F	85	AA	31	31
25	A	7.2	-	M	45	CC	30	30
26	A	4	+	F	95	AA	30	30
27	**AI**	**4.2**	**-**	**M**	**75**	**CC**	**28**	**28**
28	**AI**	**10**	**-**	**F**	**85**	**CC**	**18, 30**	**30**
29	AI	2	+	F	55	CC	30	30
30	**AI**	**3**	**-**	**F**	**75**	**CC**	**21, 30**	**21, 30**
31	AI	3	+	M	85	CC	30	30
32	A	3	-	F	65	CC	30	30
33	**AI**	**13.5**	**+**	**M**	**45**	**CC**	**17, 22**	**30**
34	AI	4.5	+	F	65	CC	30	30
35	AI	3.5	+	F	45	CC	30	30
36	AI	4	-	F	85	CC	29	30
37	AI	8	+	M	55	AA	30	30
38	AI	4	-	F	75	CC	30	30
39	A	6	-	M	45	AA	30	30
40	A	6	-	M	85	CC	30	30
41	AI	5.5	+	F	85	CC	30	30
42	AI	5	-	F	85	CC	30	30
43	A	9.3	-	M	45	N/A	30	30
44	AI	3	-	F	55	CC	29	29
45	**AI**	**5**	**-**	**F**	**75**	**AA**	**21, 30**	**30**
46	A	5.2	-	M	75	CC	30	30
47	A	7	+	F	65	CC	30	30
48	AI	4	-	M	65	CC	30	30
49	AI	4	+	M	55	N/A	30	30
50	AI	4	+	M	61	CC	30	30

							Length of DATE	
Case #	**Type of tumor**	**Tumor size (cm)**	**Lymph node metastasis**	**Gender**	**Age (yr)**	**Race**	**Tumor**	**Normal Adjacent**
51	AI	4.7	+	M	42	CC	29	29
52	**AI**	**4.3**	**+**	**M**	**78**	**CC**	**18,21**	**30**
53	AI	6	+	M	58	CC	30	30
54	**AI**	**4**	**+**	**M**	**75**	**CC**	**28**	**28**
55	**AI**	**10**	**-**	**M**	**62**	**CC**	**27**	**27**
56	**AI**	**6**	**-**	**M**	**60**	**CC**	**28**	**28**
57	**AI**	**3**	**N/A**	**M**	**72**	**CC**	**28**	**28**
58	AI	5.5	-	M	72	CC	29	29
59	AI	8	+	M	67	CC	30	30
60	AI	2.5	-	M	60	CC	30	30
61	**AI**	**5**	**N/A**	**M**	**67**	**CC**	**28**	**28**
62	**AI**	**6**	**N/A**	**M**	**80**	**CC**	**28**	**28**
63	AI	3	-	M	70	CC	30	30
64	AI	8	-	M	87	CC	30	30
65	**AI**	**6**	**+**	**M**	**70**	**CC**	**27**	**27**
66	AI	3.5	+	M	66	CC	30	30
67	AI	3.5	+	M	84	CC	30	30
68	A	3.5	-	M	86	CC	29	29
69	AI	4	+	M	68	CC	30	30
70	AI	3.5	-	M	78	CC	30	30
71	AI	3.5	-	M	62	CC	29	29
72	A	2.5	N/A	M	79	CC	29	29
73	**AI**	**5**	**-**	**M**	**55**	**CC**	**28**	**28**
74	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**19,25**	**30**
75	N/A	N/A	N/A	N/A	N/A	N/A	30	30
76	N/A	N/A	N/A	N/A	N/A	N/A	30	30
77	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**22,29**	**22,29**
78	N/A	N/A	N/A	N/A	N/A	N/A	30	30

Table 1: Patient demographics and genotype analysis of DATE in human CRC and corresponding adjacent normal tissues
(A refers to adenocarcinoma, and AI refers to adenocarcinoma invasion.)

Analysis of DATE and MSI status

Genomic DNA was prepared from human cell lines and tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The DATE region was amplified by PCR according to the protocol described in [65]. The sequences of the fluorescent labeled (5'-FAM) primers used to amplify the DATE fragment are as follows: forward primer 5'–TATTTCGTGAGTTTGGCAGTTTGTG and reverse primer; 5'–

AACAAAAGCACGCAGATTGTCAGATG which will yield a 121 bp DNA product. For MSI determination, we used an assay kit from Promega Corporation which contain labeled primers for seven markers; five mononucleotide repeat markers: NR-21 SLC7A8; 103 bp), BAT-25 (c-kit; 124 bp), BAT-26 (hMSH2; 120 bp), NR-24 (ZNF-2; 132 bp), and MONO-27 and two Penta repeats; Penta C and Penta D. The system allows amplification and detection of all markers in a single reaction. All PCR products were separated by capillary electrophoresis using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Data analysis was performed using the GeneScan Analysis software. MSI status was determined by assessing each of the mononucleotide markers for instability. Samples were classified as being either MSI-high (MSI-H) if at least two of the five markers showed instability, MSI-low (MSI-L) if one marker showed instability, and MSI-stable (MSS), in the absence of instability.

Immunohistochemical analysis of CRC tissues for MMR defects

MLH1 and MSH2 immunostaining on human CRC tissue sections was performed by the Department of Pathology, Development Laboratory (University of Pittsburgh, School of Medicine) that performs this assay for clinical purposes. Tissues were classified as MMR proficient or deficient by observing for the presence of nuclear staining under light microscopy.

2.4 RESULTS

2.4.1 An important regulatory region of the *HGF* gene promoter, DATE, undergoes deletion mutagenesis in human CRC

We genotyped 78 cases of sporadic human CRC to detect the status of a novel *HGF* promoter regulatory region, DATE. We discovered that DATE undergoes deletion mutagenesis in the tumors, while remaining intact in the corresponding matched adjacent normal colon tissues, indicating that DATE is unstable in cancer. Representative genotyping results are depicted in **Figure 12A**. (DATE exhibiting loss of at least 2 deoxyadenosines was considered truncated, as per our findings regarding the functional consequences of DATE mutagenesis, described in Figure 15 and Table 5 in Section 3.4). We found DATE instability occurs at a frequency of 13% (10/78) in CRC. Notably, we uncovered that DATE is polymorphic in nature, as 18% (14/78) of the CRC patients harbored the short DATE variant in both their tumor samples as well as in their corresponding normal tissues **(Figure 12B, and Table 1**). Thus, the overall incidence of the truncated DATE variant in CRC patients was observed to be 24/78 (31%). We verified the polymorphic nature of DATE using genomic DNA isolated from peripheral blood lymphocytes of healthy individuals and found that the short DATE variant exists in the normal population (**Figure 12C**) at a frequency of 7.7% (46/592). We compared the frequency of occurrence of truncated DATE variant in CRC cases versus the normal population and found that DATE truncation significantly associates with the incidence of human CRC (**$p < 0.001$, 2- tailed Fisher's Exact test**), suggesting that DATE mutagenesis may be playing a role in colon carcinogenesis and tumor cell survival/proliferation.

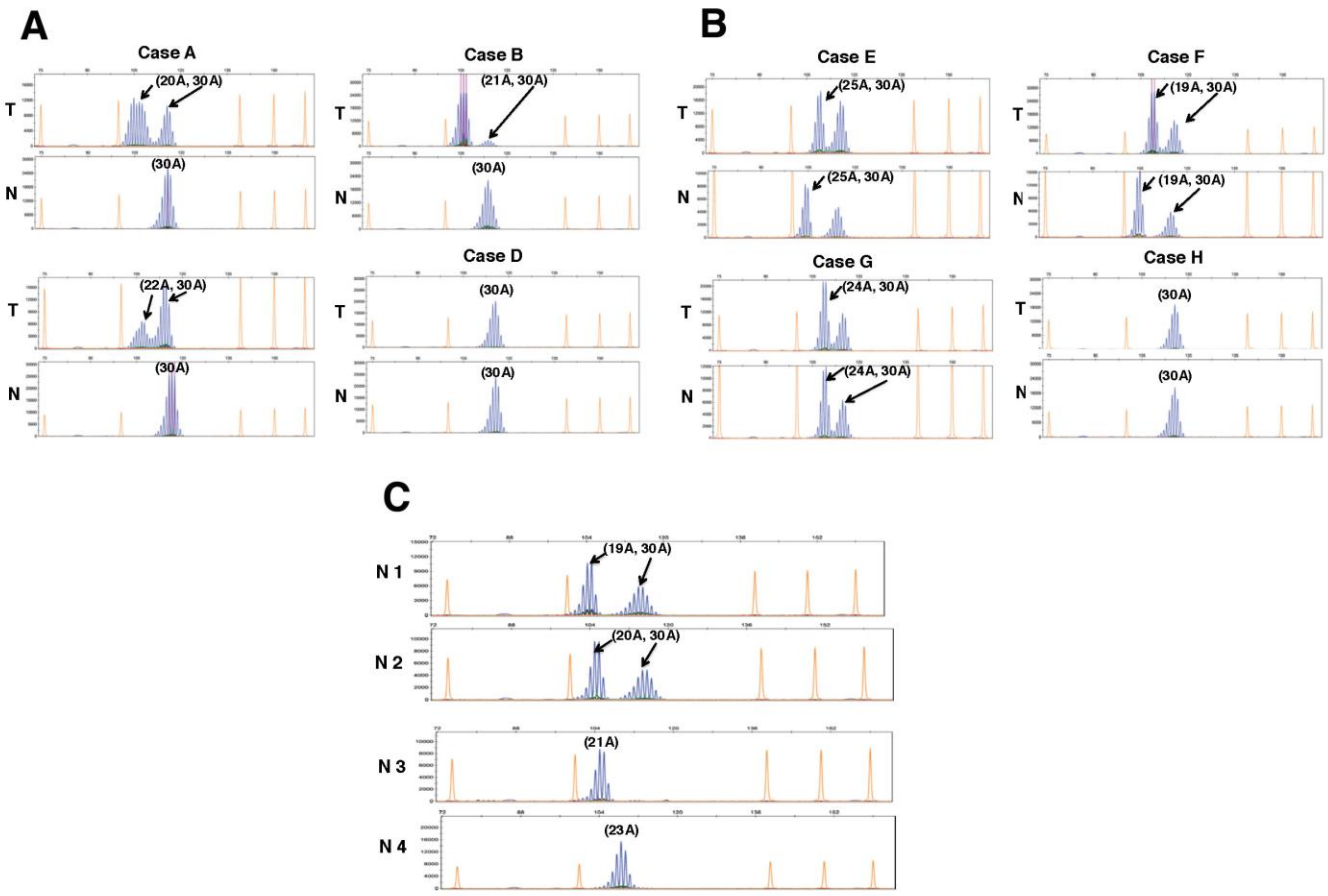

Figure 12. DATE is polymorphic in nature and undergoes deletion mutagenesis in human CRC

(A) Representative genotyping from different human CRC cases showing DATE instability in tumors (T) as compared to the corresponding adjacent normal (N) tissues. A DATE wild-type case is also shown. Arrows indicate DATE length. (**B**) Representative genotyping results demonstrating that DATE is polymorphic in colorectal tissues. (**C**) Representative genotyping data of genomic DNA from blood lymphocytes of healthy individuals showing DATE is polymorphic in normal tissues and that the truncated DATE occurs in a heterozygous (N1, N2) or homozygous manner (N3, N4).

We had previously shown that the incidence of the truncated DATE variant is significantly higher in the adjacent normal breast tissues of African Americans in comparison to that of Caucasians [65]. We found that the same trend occurred in the adjacent normal tissues of CRC patients: the short DATE variant occurred at a frequency of about 3% (15/473) in normal tissues

of Caucasians, compared to a frequency of 26% (31/119) in the normal tissues of African Americans. Statistical analysis indicated that African American subjects have a significantly higher incidence of DATE truncation in their adjacent normal colon tissues (**$p < 0.00001$, 2- tailed Fisher's Exact test**).

2.4.2 Defective mismatch repair is the underpinning cause of *HGF* promoter instability in human CRC

The mismatch repair (MMR) system is indispensable for maintaining fidelity of DNA replication and genomic integrity, particularly at microsatellite regions [76]. Microsatellite instability (MSI) resulting from MMR deficiency is documented to play important roles in a number of malignancies, including hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome and its associated cancers) and sporadic colon cancer. [76,82,108,109]. As DATE resembles a microsatellite, we hypothesized that its mutation could be due to genomic instability provoked by deficient MMR. Accordingly, we analyzed the CRC cases that exhibited truncated DATE variant for MSI using the well-established NCI-recommended MSI marker panel, which consists of five mononucleotide markers, NR-21, NR-24, BAT25, BAT26, MONO27. Samples were classified as microsatellite instability high (MSI-H) if at least two of the five markers showed instability, microsatellite instability high (MSI-L), if one marker showed instability, and microsatellite stable (MSS), in the absence of instability. The results revealed that nearly all of the cases with unstable DATE in the tumor (as compared to normal adjacent tissue) exhibited the MSI-H phenotype (**Figure 13A**, **and Table 2**), indicating a strong correlation between DATE mutagenesis and MSI. Interestingly however, two CRC cases that harbored mutant DATE were classified as MSS according to the MSI markers panel. These cases are discussed below.

The underlying cause of MSI is known to be MMR deficiency, and is characterized by an increased frequency of mismatches and alterations (deletion of mononucleotide repeats and deletion or expansion of oligonucleotide repeat regions) [76]. In human CRC, MMR deficiency is most often due to the loss-of-function of "caretaker" genes, *MLH1* or *MSH2*, (which govern the process of DNA mismatch repair during replication). *MLH1* or *MSH2* expression is routinely assessed in the clinical arena by IHC. Therefore, we performed IHC on our CRC samples to assess for the expression status of these MMR proteins. Our data clearly showed that all DATE unstable tumors have deficient MLH1 or MSH2 expression, while DATE stable tumors have robust expression of both proteins (**Figure 13B**).

Of note, the above-mentioned two DATE mutant tumor cases that were classified as microsatellite stable according to the MSI marker panel, interestingly exhibited defective MLH1 protein expression as detected by IHC (**Figure 13C**). These findings suggested that DATE could serve as a sensitive marker of MSI, even in cases where standard MSI markers fail to detect instability.

Additionally, we also examined the MSI and DATE status of several human breast cancer cases and noted that those with a MSI-H phenotype expectedly harbored DATE truncations, further indicating that DATE instability in cancer likely results from defective MMR (**Figure 13D**).

Case #	Tissue type	NR21	BAT26	BAT25	NR24	MONO27	DATE status	MSI status
2	Tumor Normal	101 101	115 115	124 124	131 131	151 151	25,30 25,30	MSS
3	Tumor Normal	101 101	104,115 104,115	123,127 124,127	131 131	153 153	19,30 19,30	MSS
7	Tumor Normal	93,100 101	101,105 104,115	119,123 123	126 131	143,151 152	21,29 29	MSI-H
8	Tumor Normal	101 101	115 115	124 124	131 131	153 153	24,30 24,30	MSS
15	Tumor Normal	91,96 96,101	103,115 115	116,125 125	128,131 131	147,151 152	20,30 30	MSI-H
17	**Tumor** **Normal**	**101** **101**	**115** **115**	**125** **125**	**132** **132**	**152** **152**	**22,29** **29**	**MSS**
21	Tumor Normal	92,101 101	105,115 115	119,124 124	131 131	153 143	21,30 30	MSI-H
23	**Tumor** **Normal**	**101** **101**	**115** **115**	**125** **125**	**131** **131**	**153** **153**	**21,30** **30**	**MSS**
28	Tumor Normal	94,101 101	103,114 114	124 124	131 131	144,152 152	18,30 30	MSI-H
30	Tumor Normal	95,99 101	104,115 115	121,125 125	132 132	143,153 153	21,30 21,30	MSI-H
33	Tumor Normal	94 101	106 115	114 124	123 131	146 152	17,22 30	MSI-H
45	Tumor Normal	101 101	104 114	120 124	132 132	146,152 152	21,30 30	MSI-H
52	Tumor Normal	93,101 101	105,115 115	124 124	131 131	139,146 152	18,21 30	MSI-H
74	Tumor Normal	92,101 101	104,115 115	115,119 124	129 132	141,151 152	19,25 30	MSI-H

Table 2: MSI status in human CRC and corresponding adjacent normal tissues in patients harboring truncated DATE

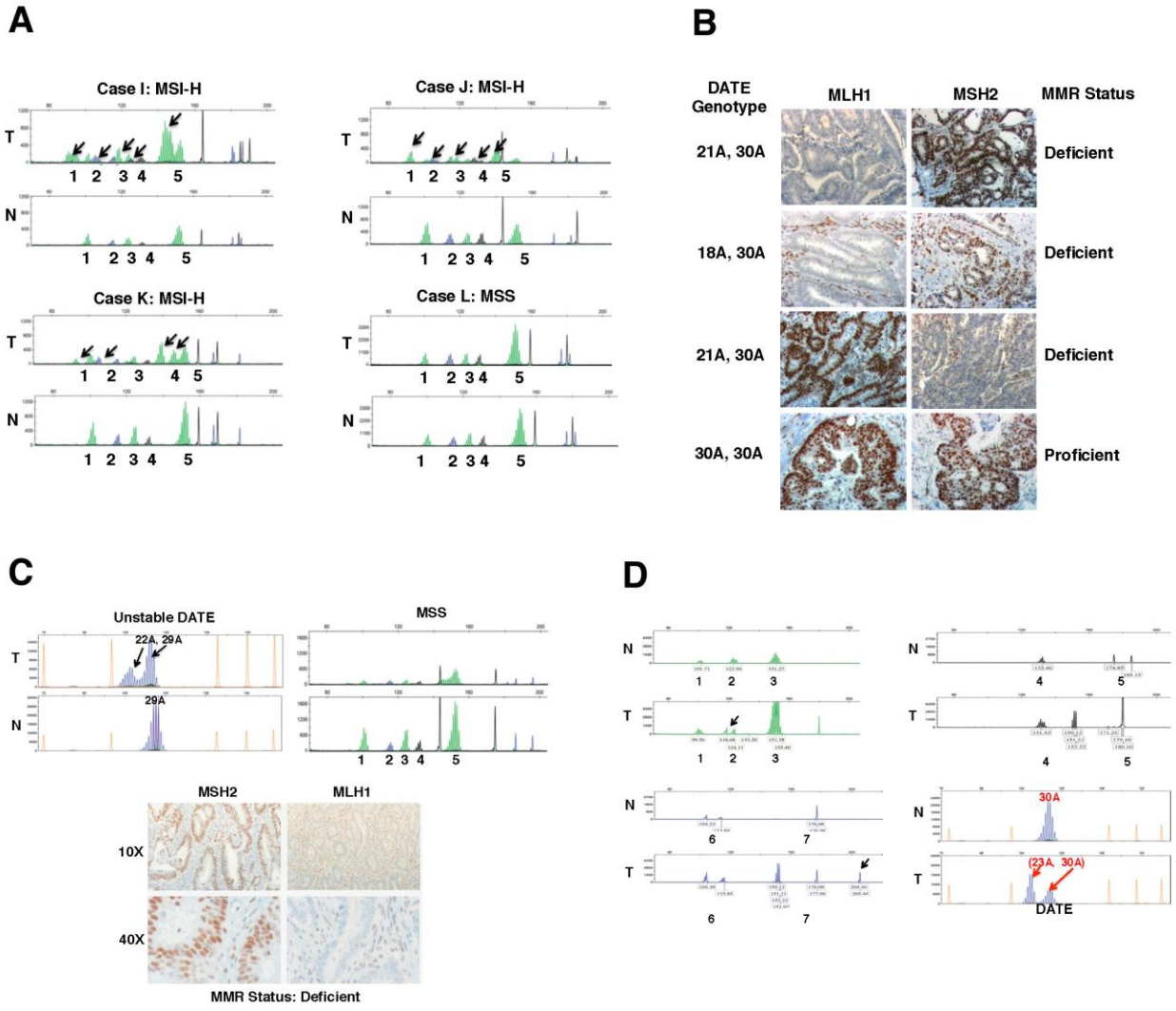

Figure 13. DATE mutagenesis results from genomic instability provoked by defective DNA Mismatch Repair (MMR)

(**A**) MSI status of DATE mutant CRC cases and their corresponding normal adjacent counterparts. The numbers 1-5 refer to the MSI markers, NR-21, BAT26, BAT25, NR-24, and MONO27, respectively. The arrows indicate the presence of instability. (**B**) Immunohistostaining documenting the absence of MLH1 or MSH2 in tumors having mutant DATE. A representative case that is MSI-stable, MMR proficient, harboring wild-type DATE (30A, 30A) is also presented. (**C**) DATE alteration is detectable in the tumor as compared to adjacent normal tissues, while other markers are intact and exhibit no alterations. Immunostaining corresponding to the same tumor is shown documenting that tumor with unstable DATE is indeed MMR deficient, as it lacks MLH1. (**D**) Representative electropherograms showing the status of MSI markers and DATE of a breast cancer case exhibiting instability and DATE truncation in tumor.

Our data has indicated that DATE mutagenesis occurs in CRC patient tissues and is likely provoked by defective MMR. For ease of further characterization of this phenomenon, we decided to incorporate human CRC cell lines in to our studies. Therefore, next we assessed the MSI and DATE status of human cancer cell lines to verify that genomic instability facilitated by defective MMR correlates with DATE mutagenesis.

We confirmed that human CRC cell lines with known MMR defects exhibit the MSI-H phenotype (**Figure 14A**). Genotyping for DATE revealed that human CRC cell lines with MMR deficiency and MSI have truncated DATE: HCT116 (18A, 21A), HCT-15 (23A), RKO (18A), SW-48 (18A, 23A), LoVo (17A). In contrast, wild-type DATE was observed in the two MSS and MMR proficient cell lines, HT-29 (30A) and SW-620 (30A) (**Figure 14B**). These findings were verified through DNA sequencing (**Figure 14C**). We also assessed the MSI status of other human cell cancer lines that we discovered to have truncated DATE; namely C33A, a cervical carcinoma cells line which has DATE with 12A and Jurkat cells, a lymphoma cell line, which has a DATE with 16A. Our data on DATE and MSI in human cell lines are summarized in **Table 3**.

Cell line	NR-21	BAT-26	BAT-25	NR-24	MONO-27	DATE	Cancer Type	MSI-status	MMR status
HCT116	94	103	118	122	143	18A, 21A	Colon	MSI-H	Deficient
RKO	87	104	114	125	140	18A	Colon	MSI-H	Deficient
LOVO	90,96	104	115,124	127, 131	146	17A	Colon	MSI-H	Deficient
SW-48	92	102	115	128	140,145	18A,23A	Colon	MSI-H	Deficient
SW620	101	115	124	131	152	30A	Colon	MSS	Proficient
HT-29	101	115	125	132	152	30A	Colon	MSS	Proficient
C33A	93	104	112,118	123	143	14A	Cervical	MSI-H	Deficient
HELA	101	114	124	131	153	30A	Cervical	MSS	Proficient
JURKAT	94	109	119	129	147	16A	Leukemia	MSI-H	Deficient

Table 3: Human cancer cell lines known to have MMR deficiency and MSI also exhibit DATE instability

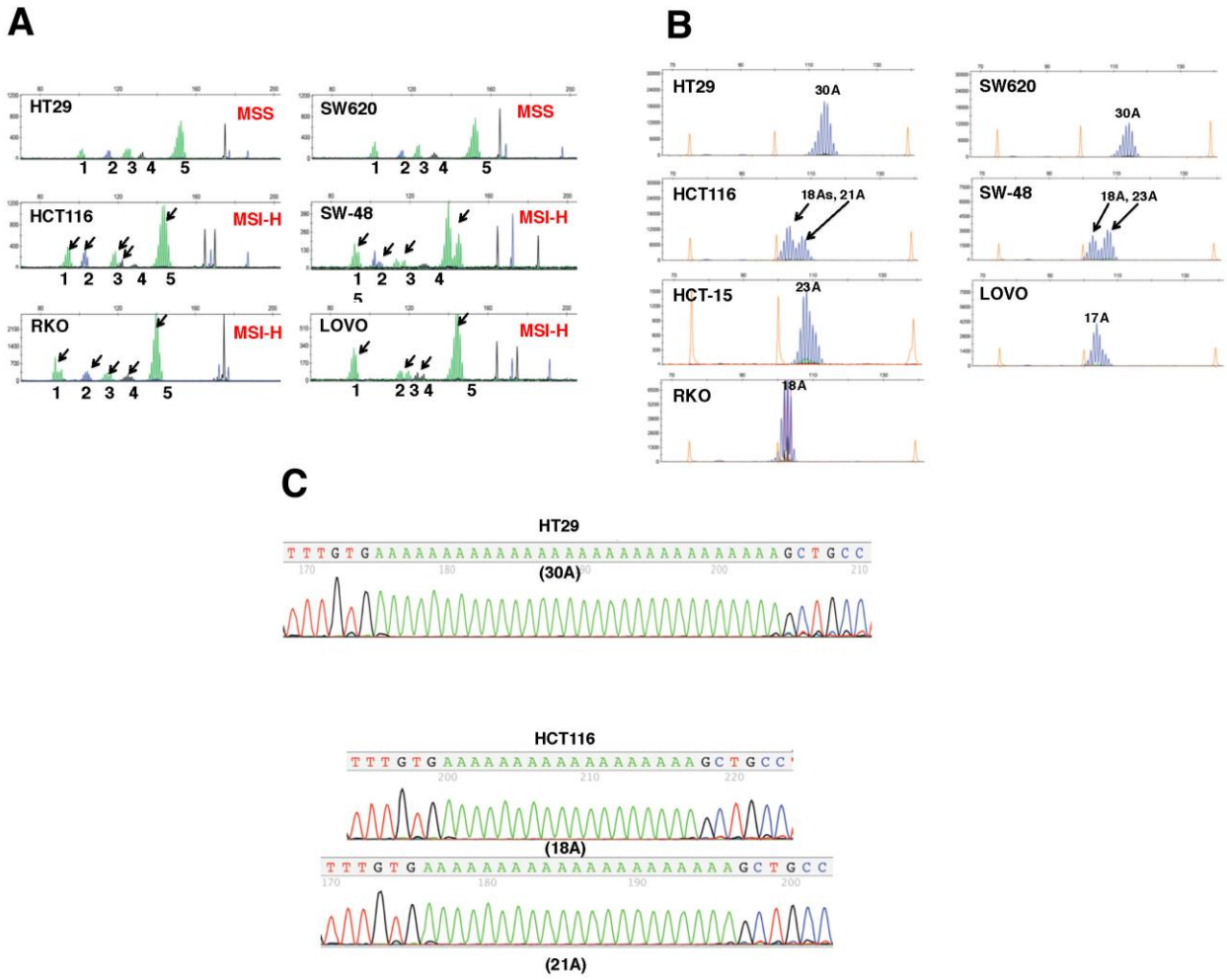

Figure 14. Human CRC cell lines with microsatellite instability harbor mutant DATE
(**A**) Representative electropherograms depicting the status of MSI markers in six human CRC cell lines. MSI status was assessed using a MSI marker panel where numbers 1-7 refer to NR-21, BAT26, BAT25, NR-24, MONO27, BAT-26, and Penta-D, respectively. The arrows indicate the presence of instability at a given marker. Samples were classified as MSI-H if at least two of the five markers showed instability, MSI-L, if one marker showed instability, and MSS, in the absence of instability. (**B**) Representative electropherograms depicting the status of DATE in the human CRC cell lines shown in (**A**), indicating that cell lines with MSI-H phenotype exhibit unstable DATE. (**C**) Representative DNA sequencing results confirming the length of the wild-type DATE in HT-29 (30A) and mutant DATE in HCT116 (18A).

2.5 DISCUSSION

Tumorigenesis has long been described as a multistep process in which sequential activation of proto-oncogenes and inactivation of tumor suppressors occur within a genetically unstable background. This view of cancer as being the result of sequential abnormal molecular events, initially led researchers to imagine that overarching, rather than targeted therapies are likely to have the greatest therapeutic potential. However, it is now becoming clear that the oncogenic phenotype is far more complex than the simple sum of all abnormal molecular events. Instead, we are now understanding that alterations in oncogenes and tumor suppressors that are capable of regulating multiple cellular signaling networks can serve as the single-handed "drivers" of cancer [110]. HGF-Met is one such "driver" which induces complex signaling systems, leading to cell growth, proliferation, escape from cell death, and tumorigenesis. We uncovered that the *HGF* promoter undergoes deletion mutagenesis in human CRC. Our work showed that *HGF* promoter mutation significantly associates with the incidence of CRC, suggesting that it aids in the onset and/or the maintenance of the malignant phenotype. Our data indicating that DATE truncation occurs in both human breast and CRC also suggested that *HGF* promoter mutagenesis may be a ubiquitous phenomenon that is worth investigating in other malignancies as well.

Nucleotide repeat microsatellite regions such as DATE are prone to polymerase slippage during DNA replication and are therefore particularly susceptible to the accumulation of mutations. When a mismatch occurs in a coding region of a key regulatory gene in a MMR deficient background, it can provide an abnormal growth advantage and eventually promote carcinogenesis [76]. Accumulation of genomic and epigenetic alterations facilitated by the loss of genomic stability has long been known contribute to tumorigenesis of sporadic and familial forms

of CRC. MSI caused by aberrant CpG methylation of *MLH1*, or point mutations in *MLH1, MSH2* or other MMR pathway genes, are noted to play crucial roles in the onset and progression of approximately 20% of sporadic CRC [82]. In our studies, we noted that DATE consisted of a poly-A-tract resembling a microsatellite region. Further investigations revealed that DATE mutagenesis is a manifestation of underlying MSI that occurs as a result of defective MMR. Given that HGF is a highly important regulator of multiple cellular processes, occurrence of DATE truncation in a MMR deficient background is expected to provide a significant growth advantage to those cells harboring the mutation (Data supporting this view is shown in Section 3).

Single sequence repeats of poly-A tracts are abundant throughout the human genome and constitute parts of the coding sequence or regulatory elements of many genes [111,112]. The hyper-mutability of poly-A tracts in CRC was highlighted in a study of germline mutations at the polymorphic *BAT-40* locus, which indicated that mutations in *BAT-40* occur in 7% of MMR deficient CRC [113]. In a separate study, a poly-A repeat consisting of 13A located in the 3' untranslated region (3' UTR) of *EGFR* was found to be commonly mutated in MMR deficient, colonic and endometrial carcinomas, leading to increased EGFR mRNA stability [114]. In congruence with these findings, our work further attests to the hyper-mutability of poly-A tracts and their importance in colorectal tumorigenesis. Long poly-A tracts (approximately 30-70A) have previously been documented to act in a transcriptional repressive manner by readily wrapping around histones and effectively reducing accessibility of transcription factors to promoters. In contrast, incorporation of shorter adenosine tracts (i.e.16A, 17A) into nucleosomes were shown to generate a wide and shallow major groove on DNA by disrupting histone-DNA interactions, resulting in increased transcription factor accessibility [111,115,116]. Our earlier investigations in human breast cancer revealed that shortening of DATE causes chromatin

loosening, repressor binding loss, and promotes the binding of transcriptional enhancers, leading to the activation of the *HGF* promoter. In light of these findings and the existing body of results regarding the importance of poly-A tracts in transcriptional regulation, we expect that DATE mutagenesis in CRC would result in similar promoter dysregulation and activation of *HGF* transcription. Our findings regarding the consequences of DATE mutation in CRC, (discussed in detail in Section 3), strongly supports this hypothesis.

Our work also uncovered that DATE is polymorphic in nature, i.e. germline DATE wild-type and truncated variants are observed in the normal population. Interestingly, stratification of our data by ethnicity revealed that the incidence of the truncated DATE variant was greater in the normal tissues of African Americans in comparison to their Caucasian counterparts. Research indicates that African Americans develop CRC at a younger age, have a higher incidence of cancer, show increased mortality and worse prognosis than Caucasians of the same disease stages. Genetic factors are reported, at least in part, to contribute to this observed ethnic discrepancy [117]. It is possible that the significantly increased incidence of DATE truncation observed in the normal tissues of African Americans plays a role in predisposing this population to develop more aggressive forms of CRC at earlier ages and at a greater frequency. While our sample size of African Americans was relatively small, the observed trend may be worth further examination through large-scale studies to conclusively determine whether DATE could be used in the clinical arena as a predictive marker of CRC predisposition and disease prognosis.

Over the last two decades there has been a heavy emphasis on the development of biomarkers to accurately forecast CRC patient prognosis and to refine predictions regarding patient response to therapy. Multiple studies have indicated that the incidence of MSI can be used as prognostic biomarker, predictive of whether patients will respond to adjuvant chemotherapy.

MSI-H patients, particularly those harboring stage II disease, are believed to gain little benefit from 5-FU therapy. Therefore, CRC patients are generally assessed for MSI to determine whether alternative treatment protocols are needed [82]. The incidence of MSI is currently evaluated using a NCI recommended "gold standard" panel consisting of five mononucleotides and two penta-nucleotide repeats [76]. While these markers have shown promise in detecting MSI and in identifying adjuvant therapy resistant CRC subsets, development of more sensitive and reliable biomarkers will increase predictive precision and improve patient outcomes. We found that in human CRC tissues, DATE truncation accurately predicted MMR defects and high-MSI 100% of the time, even in situations where the above mentioned "gold standard" markers failed to detect instability. This data implied that DATE is a highly sensitive indicator of genomic instability. In light of the recent focus on establishing clinically applicable cancer biomarkers, our data encourages the development of DATE as a sensitive marker for the identification of MSI in CRC. Furthermore, our findings warrant the exploration of DATE as a potential predictive marker of CRC patient prognosis and therapeutic response.

3.0 CONSEQUENCES OF *HGF* GENE MUTAGENESIS IN HUMAN CRC

3.1 ABSTRACT

Aberrant *HGF* expression is believed to drive the malignant transformation of several human cancers, but the molecular bases of dysregulated *HGF* expression has largely remained unknown. We discovered that the *HGF* promoter undergoes mutagenesis at an important upstream regulatory region, dubbed DATE, leading to the activation of *HGF* transcription in human CRC cells. In the normal colon epithelium, *HGF* is generally silenced, and paracrine signaling is maintained. *HGF* promoter mutation disrupts this signaling dynamic, resulting in the formation of an HGF-Met autocrine loop in CRC cells. Functional studies, including somatic gene targeting of endogenous wild-type DATE in human CRC cell lines, revealed that DATE mutagenesis is sufficient to activate *HGF* gene transcription. Furthermore, we demonstrated that HGF-Met signaling fosters tumor cell growth by conferring resistance to both forms of cell death, namely apoptosis and necroptosis. We show for the first time that growth factor signaling can directly impact RIPK-1, a key mediator of necroptosis. This is achieved via tyrosine phosphorylation of RIPK-1 by activated Met, resulting in the inhibition of RIPK-1 kinase activity. We also discovered that Met activation causes RIPK-1 recruitment to plasma membrane, polyubiquitination (Lys-48 linked chain) and its subsequent proteasomal degradation. Moreover, we found that aberrant HGF expression in tumor tissues significantly correlates with low RIPK-1 and with poor clinical prognosis. Collectively, our results shed important mechanistic insights into the molecular mechanisms of human colon carcinogenesis, and encourage further study of

HGF-Met as a viable therapeutic target in CRC, especially in those subsets harboring *HGF* gene mutations.

3.2 INTRODUCTION

The HGF-Met signaling axis is a key growth regulator known for its role in inducing complex signaling networks leading to cell survival, growth, and proliferation. When aberrantly activated, however, HGF-Met signaling can promote tumorigenesis [48]. HGF is normally produced by the stromal cell compartment of various tissues and elicits its effects by activating its cell surface receptor, Met, which is highly expressed in epithelial cells [46,49]. *HGF* gene transcription is silenced in normal epithelial cells and its expression in stromal cells is tightly regulated by a variety of signaling molecules, including hormones like estrogen, and cytokines such as TNFα, and IL-1. Although the detailed molecular mechanisms governing cell type specific expression of *HGF* remain obscure to some degree, we now understand that repression of *HGF* transcription in epithelial cells is controlled by multiple cis-acting, upstream regulatory elements, particularly those located within 1 kb upstream from the transcription start site of the *HGF* promoter [57-59,63,65]. We previously showed that the *HGF* gene promoter undergoes mutagenesis (at DATE) in a subset of human breast carcinomas, causing promoter activation. The resultant formation of an HGF-Met autocrine circuit was observed to contribute to breast tumorigenesis. In the present work, we uncovered that DATE is also a target of deletion mutagenesis in human CRC as a result of defective MMR and underlying microsatellite instability. Given the bleak prognosis of human CRC, novel therapeutic targets are direly needed. Our work (shown in Section 2) showed that

DATE mutation significantly associates with the incidence of human CRC, and thus, we hypothesized that it has functional consequences on *HGF* promoter activity, and that harboring truncated DATE promotes cancer cell survival, proliferation, and escape from death.

Genetic alterations in growth regulatory genes such as *HGF* and *MET* are recognized to play a key role in tumorigenesis and the maintenance of the malignant phenotype [49,73]. Mutagenesis of these fundamental regulator genes makes greater contributions towards the onset and the maintenance of cancer, leading to an occurrence known as "oncogenic addiction". This phenomenon refers to the fact that despite harboring multiple molecular alternations, cancer cells may be directly dependent on a single oncogenic pathway for cell survival and proliferation [118]. Targeting these fundamental players has been shown to be extremely beneficial in the treatment of a variety of cancer types [68]. For instance, transient inactivation of transforming oncogenes such as Ras, Myc, BCR-Abl in malignant tumors with complex genetic backgrounds has resulted in the reversal of the transformed phenotype. Clinical examples of successful targeting of oncogenes include neutralizing antibodies against the HER-2 receptor in metastatic breast cancer and the use of Gleevec in chronic myelogenous leukemia and gastrointestinal stromal tumors [110]. Furthermore, in experimentally generated tumors in mice involving multiple genetic events, the intermittent inactivation of the oncogene alone, induced tumor regression [119].

Given the high incidence of DATE mutation observed in CRC, and the well acknowledged role of HGF-Met in cancer, we postulated that the HGF-Met signaling axis serves as an oncogenic driver in a subset of CRC and presents a novel therapeutic target to be utilized in the arena of personalized medicine. We found that DATE mutation is sufficient to activate the *HGF* promoter, and leads to the creation of an HGF-Met autocrine loop in the CRC cells, making them growth factor autonomous. Furthermore, we showed that DATE mutation promotes oncogenic addiction

of CRC cells to HGF-Met signaling, by stimulating cell survival and resistance to cell death. Of note, our results indicated that HGF-Met signaling inhibits necroptosis via phosphorylation-mediated inactivation and down regulation of the key necroptosis initiator protein, RIPK-1. Harboring mutant DATE in tumor was found to correlate with HGF up regulation, RIPK-1 down regulation, significantly larger tumor size, and poor patient prognosis in sporadic human CRC.

3.3 MATERIALS AND METHODS

CRC tissues and cell lines

Archival human colon tumor tissues and paired normal adjacent tissues from 78 patients were obtained. Analysis of DATE status was performed using genomic DNA from human cell lines and tissues. All described cell lines were purchased from American Type Culture Collection (ATCC) and cultured according to the supplier's instructions. (Refer to Section 2.3 for further details regarding these procedures).

HGF and MET mRNA expression analyses

HGF and *MET* mRNA expression were detected by RT-PCR by reverse transcribing total RNA using AMV Reverse Transcriptase (Promega) and Random Primer (Roche Applied Sciences) in a volume of 40 μl according to standard procedures. The forward primer for *HGF* was from exon 11 (5′-CTCATTCCTTGGGATTATTGCCC), and the reverse primer was from exon 14 (5′-CCTTTATCAATGCTCCGCAG). The reverse transcriptase product was used to carry out PCR analysis for 40 cycles using the ABI Prism 7000 Sequence Detection System with

reagents and primers specific to human *HGF* supplied by Applied Biosystems. Diethylpyrocarbonate-treated water was also included as a negative control for contamination.

Transient transfections and luciferase assays

Tumor-derived human *HGF* promoter regions (1.1 kb; –1037 to +56) containing wild-type or DATE of different sizes (30A, 29A, 28A, 27A, 26 A, 25A, and 20A were cloned by PCR and sequence verified) were inserted upstream of the luciferase reporter gene in the pGL2-basic vector (Promega lengths: pHGF30A-Luc, pHGF29A-Luc, pHGF28A-Luc, pHGF27A-Luc, pHGF26A-Luc, pHGF25A-Luc, and pHGF20A-Luc. Human CRC cell lines and fibroblast (NIH-3T3) cells were seeded in 96-well culture plates (3×10^4 cells/well) and cultured for 24 hours before transfection. The renilla luciferase–encoding plasmid, pRL-TK, (Promega Corp) and pHGF-Luc plasmids were transiently co-transfected into cells using Lipofectamine 2000 (Invitrogen). Expression of renilla luciferase activity was used as an internal standard for transfection efficiency. The promoter activities were measured using the Dual Luciferase Reporter Assay System (Promega). After 24 hours, transfected cells were collected and luciferase activity was measured according to manufactures instructions using the luminometer function of Synergy HT Multi-Mode Microplate Reader (Biotek).

Construction of gene targeting vectors

Genomic DNA from human C33A cell lines (which has the shortest DATE that we have observed [DATE with 12A] and highly expresses *HGF* gene) was used as a template for generating the left and right homology arms for gene targeting by PCR. The left homology arm (LHA) is 1.14 kb. LHA forward primer is called P1 and contains a NotI site. The reverse primer

is named P2, and contains the linker A sequence. The right homology arm of 0.8 kb consists of the forward primer, P3, which contains a linker B sequence, and the reverse primer is P4, which contains a NotI site **(Table 4 and Figure 17A).** The two arms were subcloned into the PCR 2.1 vector in order to verify that the sequence containing the mutated DATE of 12A. The two arms were then amplified by PCR. The forward primer, PN1, and the reverse primer, PN2, were used for amplifying LHA. The forward primer, PN3, and the reverse primer, PN4, were used for amplifying RHA. Two enzyme sites were introduced into these primers as specified in **Table 4**. The pNeDaKO fragment was generated by PCR using primers PN5 and PN6. The pNeDaKO-Neo vector (2.2 kb) was used as a template, which contained Neo gene driven by phosphoglycerate kinase (PGK) promoter, a zeomycin resistance gene (Zeo) driven by EM7 prokaryotic promoter, loxp sites, and the linkers A and B. The LHA, RHA and pNeDaKO fragments were re-subcloned into pAAV-MCS and the target vector, pAAV-Neo-HGF, was generated as depicted in **Figure 17A**.

Name	Sequence
P1:	5’-ATACATACGCGGCCGCATGGACAATGACTGTTTCTTGGAC-3’
P2:	5’-GCTCCAGCTTTTGTTCCCTTTAGAAGGAATAGGGAAGGTTAGCAGGAG-3’
P3:	5’-CGCCCTATAGTGAGTCGTATTACTTTCTGAACTCAGTATGTAGTATAGG-3’
P4:	5’-ATACATACGCGGCCGCATTTGTTCCCACTCTTTTATTCCC-3’
PN1:	5’- ATACATACGCGGCCGCATGGACAATGACTGTTTC-3’
PN2:	5’-GGAATTCCAAGGAATAGGGAAGGTTAGCAGGAG-3’
PN3:	5’- GGAATTCCTTTCTGAACTCAGTATGTAGTATAGG-3’
PN4:	5’- GGGGTACCCCATACATACGCGGCCGCATTTGTTC-3’
PN5:	5’-GGAATTCCACCGCGGATAACTTC-3’
PN6:	5’-GGAATTCCACTCACTATAGGGCG-3’
FP:	5’-ATTTCCGGTGAAAGTCAGTCC-3’
RP:	5’-CTGACTAGGGGAGGAGTAGAAG-3’

Table 4: **Primer sequences used in somatic gene targeting of wild-type DATE**
Primer sequences used in the Construction of pAAV-Neo-HGF targeting vector for somatic gene targeting to replace the endogenous wild-type DATE with a truncated DATE in the HT-29 CRC cell line. Underlined sequences represent restriction enzyme sites or linker sequences.

Somatic gene targeting in human CRC HT-29 cells

The rAAV targeting vector was packaged by mixing pAAV-Neo-mHGF with pAAV-RC and pHelper plasmids from AAV Helper-Free System (Stratagene), and transfected into HEK 293 cells (ATCC) using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. HT-29 cells were grown in a 100 cm^2 flask until 60-80% confluence was reached, washed with HBS, and 500 ul of HEK 293- rAAV lysate and 4.5 ml of the appropriate growth media was added to the flask. The virus was allowed to infect the cells at 37°C for 2-3 hours, following which, the cells were allowed to grow in normal media for 48 hours. The cells were harvested by trypsinization and distributed into twelve 96- well plates with media containing G418 (0.5 mg/ml, Invitrogen). Genomic DNA was extracted from colonies grown in 96-well plates, and the targeting events were screened by PCR using the forward primer (FP) that was situated outside of the LHA, and the reverse primer that was situated in PGK region. The DATE mutation was verified by PCR using primers situated in *HGF* promoter region. The presence of truncated and

wild-type DATE fragment was detected on 15% denaturing polyacrylamide gel containing 8M urea and verified by DNA sequencing.

Preparation of protein extracts and Immunoblotting

Whole cells protein lysates were prepared from cells plated into 6-well plates and treated for various time points, by scraping cells into RIPA buffer containing protease and phosphatase inhibitors. Protein extracts from tissues were prepared by homogenizing 100 mg-500 mg of human colorectal tumors or normal adjacent control tissues in RIPA buffer containing protease and phosphatase inhibitors (100 μL/100 mg). Cell lysates were cleared by centrifuging at 11,000 *g* for 20 minutes and protein concentrations were determined using a BCA assay. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the cleared protein lysates, and proteins were transferred to nitrocellulose membranes. Following blocking for one hour at room temperature in 5% BSA (for phospho-proteins) or 5% milk diluted in Tris-Buffered Saline with 0.1% Tween 20 (TBST), membranes were incubated with primary antibodies diluted in 5% BSA specific to human HGF (AF-294-NA and AB-294-NA) at 1:1000 dilution (R&D Systems), Met (C-28) at 1:250 dilution, Ubiquitin at 1:1000 dilution (Santa Cruz Biotechnology Inc.), RIPK-1 at 1:1000 dilution (Sigma Aldrich), HMGB1 at 1:1000 dilution (Abcam), Actin at 1:4000 dilution (Millipore), phosphorylated Met Tyr 1234/1235, phosphorylated-Akt, total Akt, phosphorylated Erk, total Erk, PARP, K63-linkage Specific Polyubiquitin, and K48-linkage Specific Polyubiquitin (all at 1:1000, Cell Signaling Inc.) overnight at 4°C. After washing with TBST, membranes were incubated with secondary antibodies for two hours at room temperature, and proteins were visualized using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Immunohistochemistry and immunofluorescence

Colorectal cancer tissue sections were deparaffinized in xylene, dehydrated in ethanol, and incubated with polyclonal antibodies specific to human HGF (AF-294-NA) at 1:50 dilution (R&D Systems), Met at 1:100 dilution (C-28; Santa Cruz Biotechnology Inc), and RIPK-1 at 1:500 dilution (Sigma Aldrich) overnight at 4°C. Non-immune IgG primary antibody was used as a negative control. Tissues were then incubated with HRP-conjugated secondary antibody for 30 minutes at room temperature. ABC kit from Vector Laboratories was used to develop the antibody staining. Relative percent area and percent intensity of immunostaining were then quantified using Image J software (National Institutes of Health) in accordance with the recommended guidelines. In summary, threshold analysis was performed on the images by first assessing the total area staining, followed by the area of tissue that is positive for a particular stain. These two values were then used to determine the percent area and intensity of tissue staining. At least three high quality images (obtained using light microscopy) of random fields totaling more than 1,000 cells were quantified per each patient case. The percentage of total area stained and the relative intensity of staining were determined and expressed as bar graphs. HGF and RIPK-1 staining was classified into two groups of low and high by using the median value as a "cutoff", according to the methods previously described in [120]. For immunofluorescence staining for HGF, Met and RIPK-1, cells were grown on coverslips and fixed with methanol, then incubated with primary antibody (HGF antibody from R&D), Met (c-28 Santa Cruz and L6E7, Cell Signaling technology and RIPK-1 also from Cell Signaling technology) at 1:50-100 dilution overnight at 4°C. Cells were washed, and then incubated with biotinylated secondary antibody (Vector Laboratories), indocarbocyanine Cy2 (goat, green 1:100), or Cy3 (rabbit, red 1:200) for

30 minutes at room temperature. Slides were rinsed with PBST and the nuclei were stained with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories).

Cell growth/viability assays

Human CRC cell lines were plated at 5,000 cells/well in 96-well plates and allowed to grow for 24 hours. For drug treatment experiments, cells were the serum starved overnight and treated with 0-20 μM SU11274 (Sigma Aldrich) or 50 μM Necrostatin (Sigma Aldrich) where indicated. All drugs were prepared by dissolving the powder form in DMSO; therefore DMSO was used as the negative control. For HGF neutralizing antibody (R&D Systems) treatment, cells were serum starved overnight and treated with 10 ng/mL of neutralizing antibody or control normal goat IgG diluted in sterile PBS. For active HGF ligand treatment, cells plated at 5,000 cells/well were allowed to grow for 24 hours and serum starved for 48 hours in order to remove the growth and survival effects induced by serum. Cells were then treated with lyophilized active recombinant HGF (R&D Systems) at 50 ng/mL diluted in sterile PBS. Cell growth and viability were measured using MTT (Sigma Aldrich) assays at the indicated time-points post-treatment. For MTT assays, the MTT reagent, Thiazolyl Blue Tetrazolium Bromide (Sigma Aldrich) was dissolved in sterile PBS at a concentration of 1 mg/mL. Media was removed from 96-well plates and 100 μL of MTT solution was added to each well. Cells were allowed to incubate in MTT reagent for 1 hour at 37°C and the blue product in cells was solubilized by adding 100 μL 40 mM HCL in isopropanol. The absorbance was quantified using Synergy HT Multi-Mode Microplate Reader at a wavelength of 590 nm with a reference filter of 620 nm. Results were averaged between three replicates.

In-Situ Caspase-3 assay

To assess Caspase 3/7 mediated apoptosis in live cells, CellEvent® Caspase-3 Detection Reagent (Invitrogen) was diluted in media, and added to cells in 96-well plates at a concentration of 8 μM, simultaneously with SU11274. Plates were incubated at 37°C for varying periods of time. The fluorescence emission of the bound dye was read using a microplate reader at excitation/emission maxima of 502/530. Apoptotic cells were visualized using a fluorescent microscope.

Determination of RIPK-1 tyrosine phosphorylation site.

Recombinant pure active human RIPK-1 (Creative Biomart) was incubated with pure active human Met kinase (Invitrogen) in kinase buffer for 30 minutes, samples were subjected to SDS-PAGE, and portions of the gel were analyzed by western blot, whereas the remaining portion of the gel was subjected to Coomassie blue staining. The band corresponding to RIPK-1 was cut out and sent to the Proteomic Facility of Ohio State University (Web Address: www.ccic.ohio-state.edu/MS) without revealing the identity of the sample. They correctly identified it as human RIPK-1 and determined that Tyr384 is phosphorylated.

3.4 RESULTS

3.4.1 DATE mutation reactivates the silenced *HGF* promoter

Long poly-A tracts are documented to contribute to transcription repression while shortening of these regions disrupt histone-DNA interactions, increase transcription factor accessibility, and facilitate promoter activation [111-113,115,116]. Our data up to this point have shown that that the DATE poly-A tract undergoes deletion mutagenesis in human CRC as a result of defective MMR, and that DATE mutation significantly associates with the incidence of cancer. Therefore, our next aim was to examine the functional consequences of DATE truncation on *HGF* promoter activity in human CRC. We subcloned 1.1 kb *hHGF* promoter fragments harboring DATE with different numbers of adenosines (As) deleted, upstream of a luciferase reporter plasmid, and transfected these vectors into human CRC cell lines. Shortening of DATE (20A, 25A) resulted in a significant increase in *HGF* promoter activity as compared to wild-type DATE (30A), suggesting that truncation (loss of deoxyadenosines) relieves the repressor function of DATE on the *HGF* promoter (**Figure 15A**). We carried out the same experiment in normal fibroblasts to confirm that truncation of DATE results in loss of its repressor function in normal cells (**Figure 15B**). Additional fine mapping studies showed that shortening DATE by even by one base (i.e. DATE with 29A) significantly increases promoter activity in HCT116, RKO and LOVO cell lines when compared to the activity of wild-type construct (**Figure 15C and Table 5**). However, given that in human tissues, detecting the deletion of a single nucleotide through genotyping is difficult, we designated the loss of at least 2As as the threshold for DATE truncation in order to eliminate false positives. In order to test if DATE can influence promoter activity out of the context of *HGF* promoter, we engineered heterologous promoter constructs having DATE

with 14A or 30A placed upstream of the SV40 basal promoter driving the luciferase reporter gene. We noted that the heterologous construct consisting of truncated DATE was not capable of significantly suppressing SV40 driven luciferase reporter activity in comparison to the wild-type construct, further indicating that truncation results in the loss of its natural repressive function (**Figure 15D**).

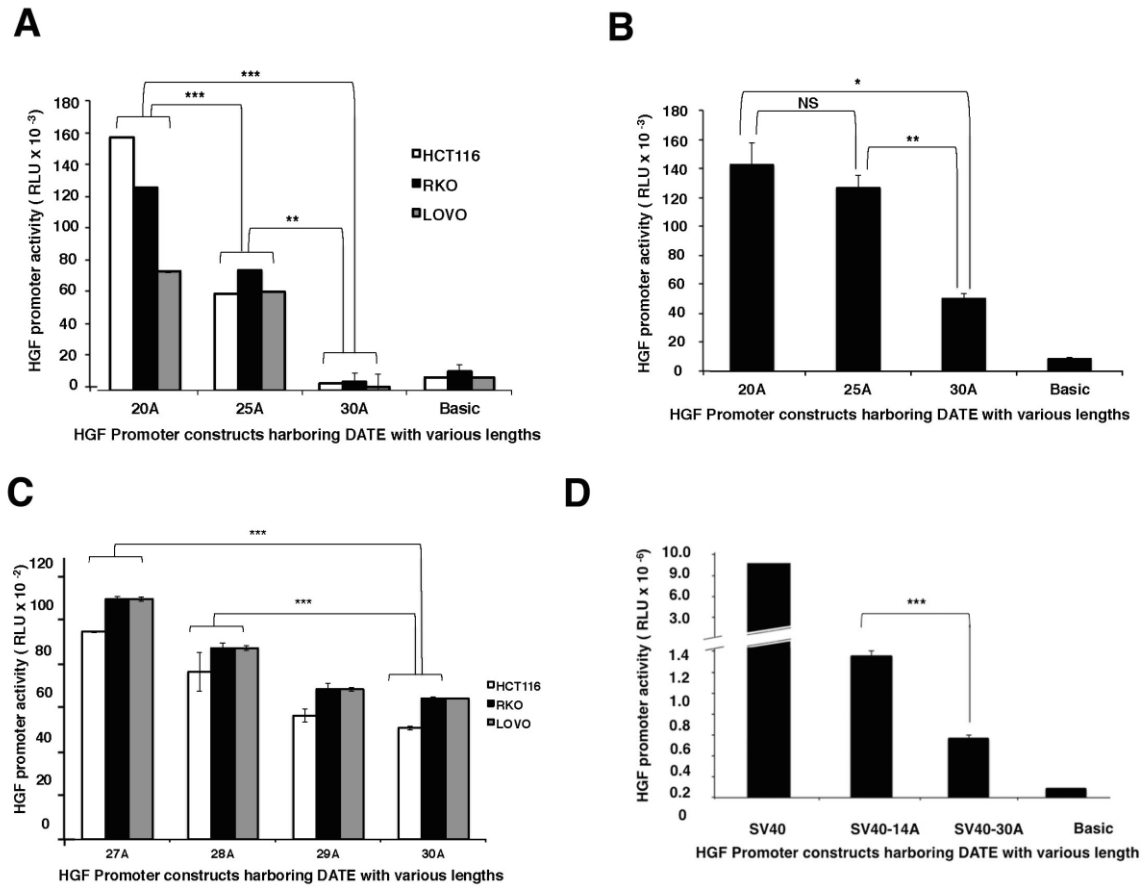

Figure 15. DATE truncation causes reactivation of the silenced *HGF* promoter
(**A**) Assessment of *HGF* Promoter activity in human CRC cell lines and (**B**) normal fibroblast cell lines transiently transfected with luciferase reporter constructs driven by human *HGF* promoter region (1.1 kb) harboring wild-type (30A) or shortened DATE. (**C**) Additional fine mapping studies (using luciferase reporter assay) carried out to determine the threshold of DATE shortening necessary to activate *HGF* promoter activity (**D**) Shortened and wild-type DATE were incorporated into a heterologous SV40 driven promoter to assess whether DATE can affect promoter activity out of the context of the *HGF* promoter. Bars are mean $\pm$ SD for two experiments. Significant differences between the groups corresponding to $p < 0.05$, 0.01, 0.001, and no difference are depicted by *, **, ***, and NS, respectively.

Cell line	20A	25A	26A	27A	28A	29A	30A
HCT116	+	+	-	-	-	-	-
SW620	+	+	-	-	-	-	-
RKO	+	+	+	+	+	-	-
LOVO	+	+	+	+	+	+	-
HCT-15	+	+	+	+	+	+	-

Table 5: Luciferase reporter assays are used to demonstrate that DATE truncation causes HGF promoter activation in CRC cells
DATE constructs of varying lengths were used to drive the luciferase reporter. The + sign denotes significant increases in promoter activity in comparison to the 30A, wild-type construct. While the loss of even one deoxyadenosine resulted in significantly increased promoter activity in some CRC cell lines, given that detecting the deletion of a single nucleotide is difficult in human tissues, we designated the loss of at least 2A as the threshold for DATE truncation.

3.4.2 DATE truncation leads to autocrine HGF-Met signaling in human CRC cells

To determine whether DATE truncation affects the endogenous HGF expression in CRC cells, we analyzed human CRC cell lines that have wild-type or truncated DATE, for *HGF* and *MET* mRNA and protein expression. We discovered that cell lines harboring wild-type DATE (HT-29 and SW620) do not express *HGF* mRNA or protein, while those cell lines with truncated DATE (HCT116, HCT-15, RKO, SW-48, LOVO) express robust levels of *HGF* mRNA and protein. All CRC cell lines, regardless of DATE status, expressed abundant *MET* mRNA and protein (**Figure 16A-C**). Next, standard doses of pure recombinant HGF protein was run on a western blot and signal strengths (% intensity of staining) were measured using the Image J software program (**Figure 16D**). The signal strengths of these standardized controls were then compared to the densitometric signals of HGF protein from cell lysates, in order to approximate the HGF protein amount expressed by the CRC cell lines. Using this method, CRC cell lines harboring truncated DATE were estimated to express approximately 50-70 ng of HGF protein

(**Figure 16E**). Immmunoblotting for Met phosphorylation showed that Met was in an activated state in CRC cells having mutant DATE and expressing HGF, indicating that an autocrine HGF-Met signaling loop is operational in these cells (**Figure 16C and F**).

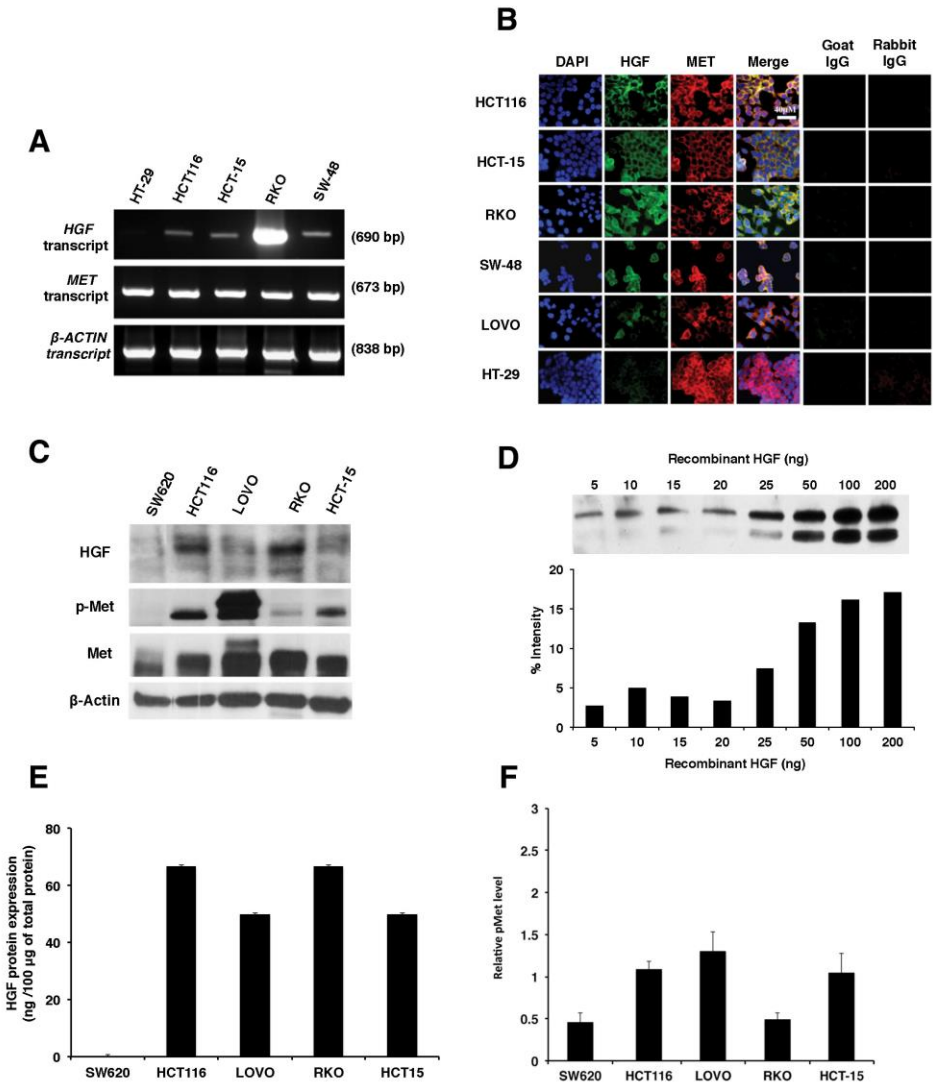

Figure 16. DATE truncation leads to HGF production and autocrine HGF-Met signaling in human CRC cells

(**A, B**) RT-PCR results indicating the presence of *HGF* transcript in DATE mutant CRC cell lines. (**B**) Detection of HGF and Met protein expression in human CRC cell lines using immunofluorescence. (**C**) Detection of HGF, Met, and phosphorylated Met (Tyr 1234/1235) expression in the human CRC cell lines using western blotting. (**D**) Top panel: A dose response of pure recombinant human HGF protein in western blot. Both pro-HGF and mature alpha chain of HGF are shown. Bottom panel: Quantification of HGF protein signal described above. Bar graphs indicate HGF protein expression measured as percent intensity using Image J software. (**E**) Quantification of HGF protein expression (ng/ 100 µg of total protein) in the human CRC cell lines using the standardized controls from (**D**). (**F**) Western blotting analysis of phosphorylated-Met in human CRC cell lines quantified using densitometry and normalized to Met. Bars are mean ± SD.

Next we asked whether DATE truncation by itself was sufficient to cause activation of the endogenous *HGF* gene. To assess this, we replaced the endogenous wild-type DATE in HT-29 CRC cells with a truncated version (DATE with 12A, which is the shortest DATE we have noted to exist in tumor cells) using a somatic gene targeting approach employing the recombinant adeno-associated viruses (rAAV) vector for gene replacement. The gene targeting protocol is summarized in **Figure 17A and C: top panel**. We verified that the targeting event replaced the endogenous DATE with the truncated form by sequencing the DATE region (**Figure 17B**). HT-29 clones, harboring the truncated DATE variant showed activation of the *HGF* gene, as indicated by expression of *HGF* transcript (**Figure 17C, bottom panel**). Notably, we observed that in some of these clones, truncated DATE reverted back to the wild-type DATE and *HGF* expression ceased over-time as cells were passaged (see clone #4 in **Figure 17C bottom panel** that is indicated by an asterisk). This is reasonable since HT-29 cells are MMR proficient and hence repaired the truncated DATE to its wild-type form. Together these results indicated DATE truncation is sufficient to activate *HGF* gene in human CRC cells.

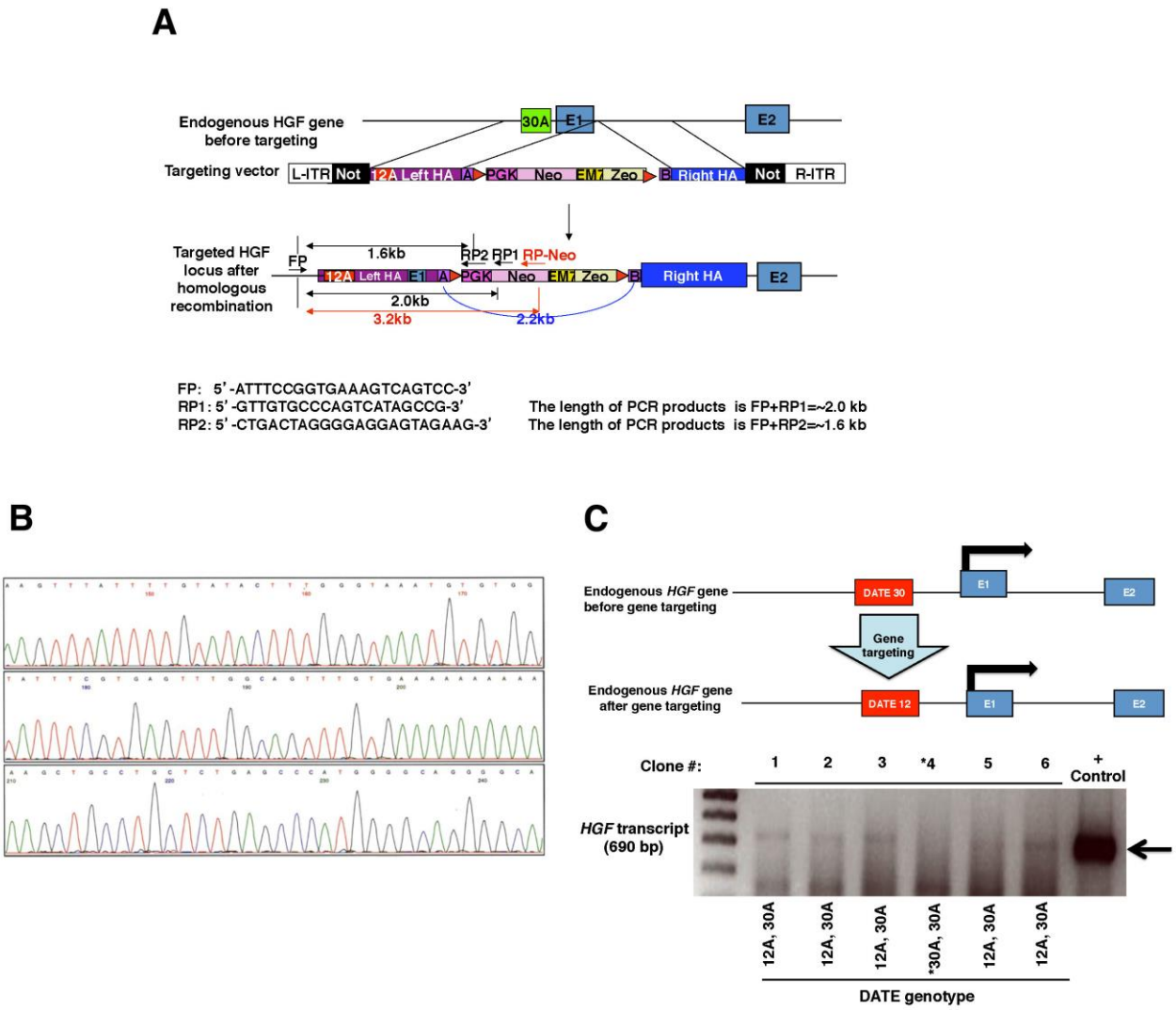

Figure 17. DATE truncation is sufficient to activate endogenous *HGF* gene expression
(**A**) Schematic representation of DATE replacement by homologous gene targeting approach showing the targeting vector and primers used to verify the targeting event. E1 and E2 exons 1 and 2, respectively. (**B**) DNA sequencing data confirming that wild-type-DATE was replaced with truncated DATE following somatic gene targeting of DATE wild-type HT-29 cells. Note that the incorporation of 12A is underlined in the sequence. (**C**) Top panel: Replacement of endogenous DATE with the mutant version (12A). Bottom panel: *HGF* transcript is observed in the targeted clones that incorporated the mutant DATE construct. RNA from the DATE mutant cervical carcinoma cell line, C33A (indicated by arrow), was analyzed as a positive control.

Our data so far indicated that DATE truncation is sufficient to activate the *HGF* promoter and leads to autocrine HGF-Met signaling in CRC cell lines. We next wanted to verify that this phenomenon was clinically relevant in human CRC patients. Therefore, we assessed the status

of HGF and Met protein expression in DATE wild-type and DATE mutant human CRC tissues and corresponding adjacent normal tissues. Using IHC for HGF, we found that tumors with mutant DATE have robust HGF expression, whereas those with wild-type DATE showed nearly undetectable levels of HGF (**Figure 18A**). Adjacent normal epithelium (harboring wild-type DATE) of both DATE mutant and DATE wild-type tumors showed low/undetectable HGF expression (**Figure 18A**). The percent area and the intensity of HGF staining in the tumor tissues (as detected by IHC) were quantified using Image J analysis software. DATE mutant tissues were observed to have significantly increased HGF protein abundance in comparison to the tumors harboring wild-type DATE (**Figure 18B**). These findings were also confirmed by western blotting (**Figure 18C**) and densitometric analysis (**Figure 18D**). Additionally, we verified that human CRC tissues harboring mutant DATE and high HGF expression, are deficient in MMR proteins using IHC for MLH1 and MHS2 (**Figure 18E)**.

We previously noted that DATE is polymorphic in nature, and that normal human tissues can harbor truncated DATE variants (**Figure 12B**). We questioned whether normal adjacent colorectal tissues harboring mutant DATE exhibit increased HGF expression in comparison to those with wild-type DATE. We assessed the normal adjacent tissues of CRC patients using western blotting, and found that individuals with truncated DATE variant in their normal tissues showed significantly increased HGF expression, (particularly an increase in activated HGF β chain) in comparison to those harboring wild-type DATE (**Figure 18F and G**). These data suggested that similar to the HGF up-regulation in colorectal tumor tissues mediated by DATE mutagenesis, possessing the truncated DATE polymorphism could lead to increased overall HGF expression in normal human tissues as well.

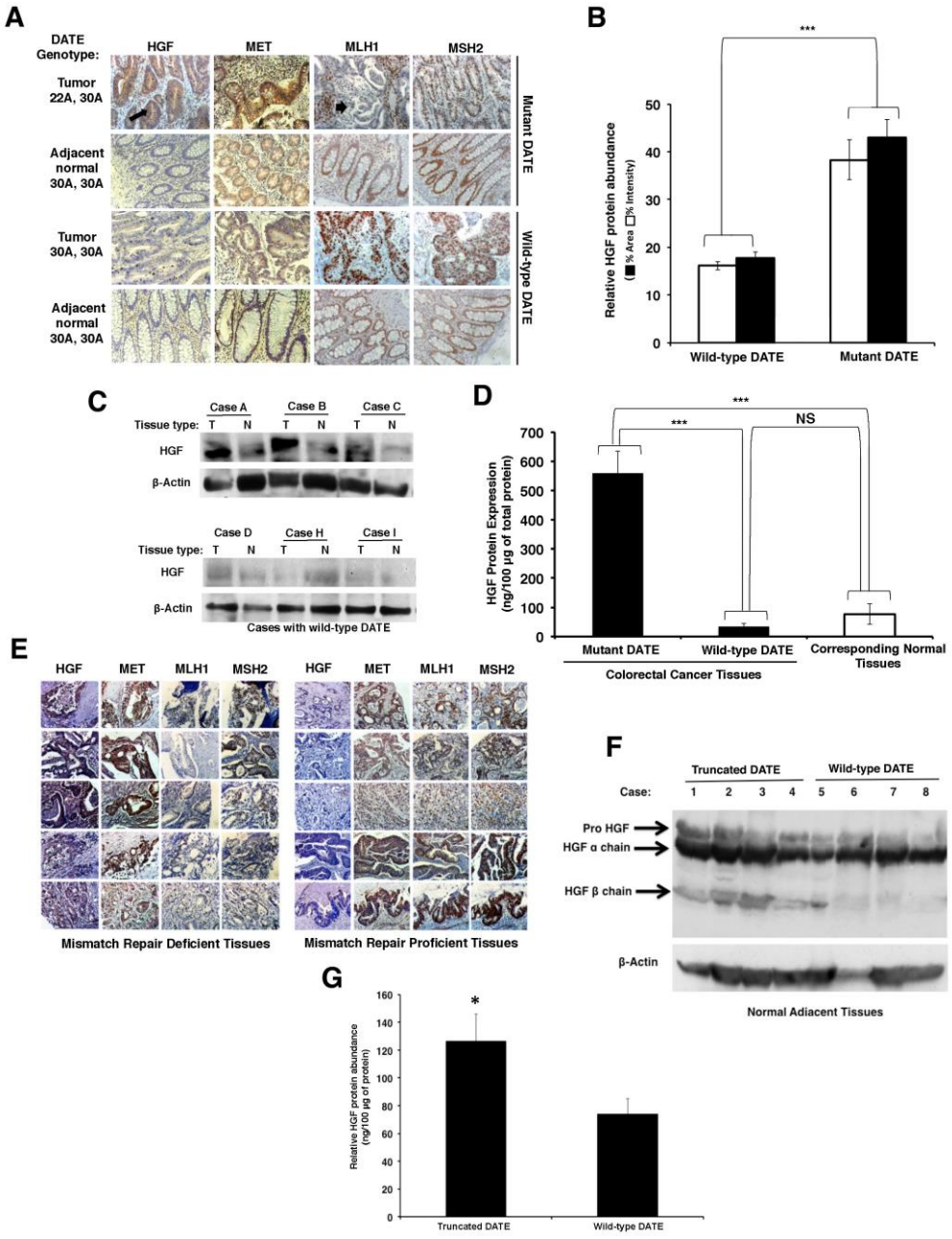

Figure 18. DATE truncation correlates with HGF up-regulation in human CRC tissues
Representative IHC for HGF, MET and the MMR proteins, MLH1 and MSH2, in DATE wild-type and DATE mutant human CRC and their corresponding adjacent normal tissues. The arrow and the arrowhead shows increased HGF protein abundance and deficient MMR protein expression in DATE mutant tumors. (**B**) Quantification of HGF protein abundance from (**A**). (**C, D**) Western blot and densitometric quantification of HGF protein abundance in CRC tissues. (**E**) Representative IHC of HGF and MET protein abundance in MMR proficient and MMR deficient human colorectal tumor using tissue microarray platform. (**F,G**) HGF expression in normal adjacent tissues with truncated and wild-type DATE, assessed by western blot, and quantified using densitometry. Bars are mean $\pm$ SE. Significant differences between the groups corresponding to $p < 0.05$ and $p < 0.001$ are depicted by * and *** respectively.

3.4.3 CRC cells with truncated DATE have operational HGF-Met signaling and are addicted to HGF for cell viability

Our next goal was to investigate the functional consequences of aberrant HGF expression in CRC cells. We hypothesized that HGF producing CRC cells have an "oncogenic addiction" to HGF, and therefore rely on this growth-signaling axis for proliferation and survival. To test this possibility, we took a two-pronged approach that involved both blocking and activating the HGF-Met signaling pathway, and assessing the outcome at the cellular and molecular levels. To block HGF-Met signaling, we treated human CRC cell lines that had DATE mutation and produce HGF, with a HGF neutralizing antibody or an IgG control antibody for 24 hours. We observed that activation of Met and its downstream signaling mediators, Akt and Erk, were dampened in response to HGF neutralization in cells harboring DATE mutations, resulting in reduced cell growth and viability, as detected by MTT cell viability assays (**Figure 19A–C**). In complementary studies, we tested whether the HGF-Met signaling in CRC cell lines mentioned above was indeed operational by stimulating serum-deprived cells with recombinant human HGF for 24 hours. We found that HGF treatment activated Met and its key downstream signaling effectors Akt and Erk (**Figure 19D**), and led to increased cell growth particularly in DATE mutant CRC cells (**Figure**. **19E and F**).

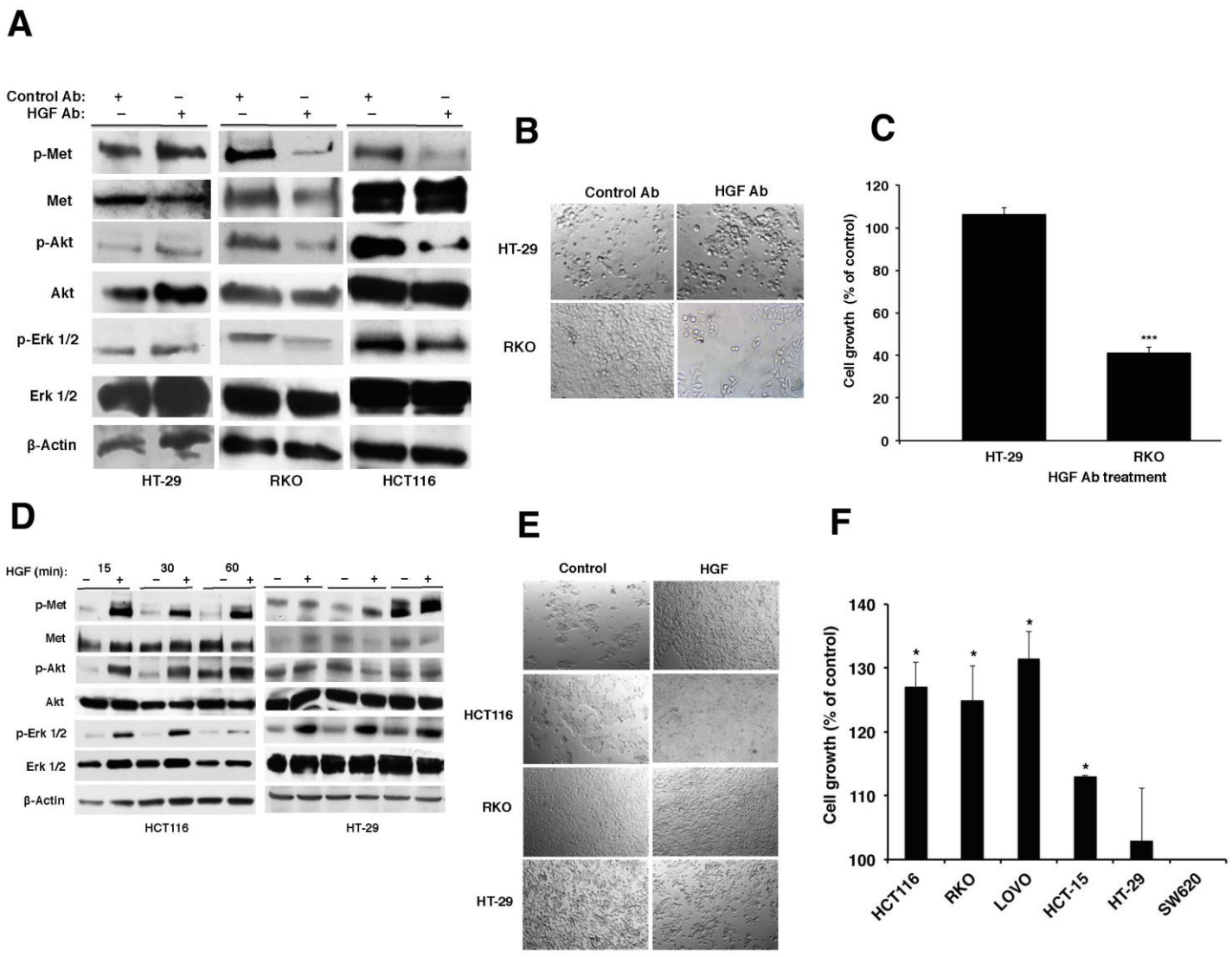

Figure 19. DATE mutant CRC cells are addicted to HGF for cell growth

(**A-C**) Neutralizing of HGF using HGF antibody for 24 hours blocks HGF signaling and inhibits cell growth. (**A**) Western blotting showing that HGF neutralization inhibits Met activation and signaling. (**B**) Representative phase contrast microscopy images of CRC cells treated with HGF antibody (**C**) The impact of HGF antibody on cell growth and viability. (**D-F**) Human CRC cell lines with mutant DATE have an operational HGF-Met signaling pathway as determined by gain of function studies using exogenous HGF. (**D**) Human CRC cells incubated with recombinant human HGF for 24 hours and assessed for Met activation and signaling by western blot. (**E**) Representative phase contrast microscopy images of CRC cells treated with exogenous recombinant human HGF. (**F**) The impact of HGF treatment on cell growth and viability. Bars are mean $\pm$ SE. Significant differences between the groups corresponding to $p < 0.05, 0.01, 0.001$ are depicted by *, **, ***, respectively.

The results of the above described studies involving treatment of CRC cells with recombinant HGF or HGF neutralizing antibodies suggest that DATE mutant cell lines have a functional HGF-Met signaling system, and depend on this signaling axis for growth. To test this further, we inhibited the Met receptor with the highly specific, small molecule inhibitor of Met kinase called SU11274 [121] and determined its effects on Met activation and cell growth behavior. Treatment with increasing concentrations SU11274 resulted in complete inhibition of Met activation and marked reduction of activation of its downstream signaling effectors Akt and Erk in DATE mutant HGF producing cells like HCT116 and RKO, and HCT-15 cell lines (**Figure 20A and B**). SU11274 treated cells exhibited phenotypic characteristics of cell death such as detached, rounded and wrinkled cells (**Figure 20C**) and had significantly lower cell growth and viability (**Figure 20D**). Assessment of markers of apoptosis such as Caspase-3 (**Figure 20E**) and PARP cleavage (**Figure 20A**) showed only marginal or no alteration of these factors suggesting that inhibition of Met may cause cell death by an alternate pathway.

The standard chemotherapy administered in human CRC is 5-FU, and cells exhibiting MSI-H phenotype are believed to be largely resistant to this treatment modality [82]. Given that our data up to this point indicates that HGF-Met pathway plays a crucial role in human CRC cell survival, we asked whether inhibiting the HGF-Met signaling pathway could enhance human CRC cell killing by 5-FU treatment. Our results showed that while at low doses, single SU11274 or 5-FU therapy had minimal effects on cell viability, combination therapy significantly enhanced cell death in the DATE mutant cell lines: MTT assays indicated significant loss of cell viability (**Figure 20F**) and phase contrast microscopy images showed notable phenotypic characteristics of cell death (**Figure 20G**) in the DATE mutant cell lines following 5-FU and SU11274 combination treatment. Furthermore, dose response studies showed that, the concentration of 5-

FU required to inhibit 50% of CRC viability (IC-50) was strikingly reduced when CRC cells were co-treated with SU11274, suggesting that Met inhibition may help increase the efficacy of 5-FU treatment in human CRC, particularly in MMR deficient patient subsets **(Table 6).**

CRC cell line	IC-50 for 5-FU single treatment (μM)	IC-50 for 5-FU in combination with SU11274 (μM)
HCT116	142.04	106.55
LOVO	223.13	85.54
HCT-15	554.26	357.32
RKO	121.05	85.54

Table 6: IC-50 of 5-Fluorouracil in CRC cells, alone and in combination with the Met inhibitor, SU11274 (20 μM)

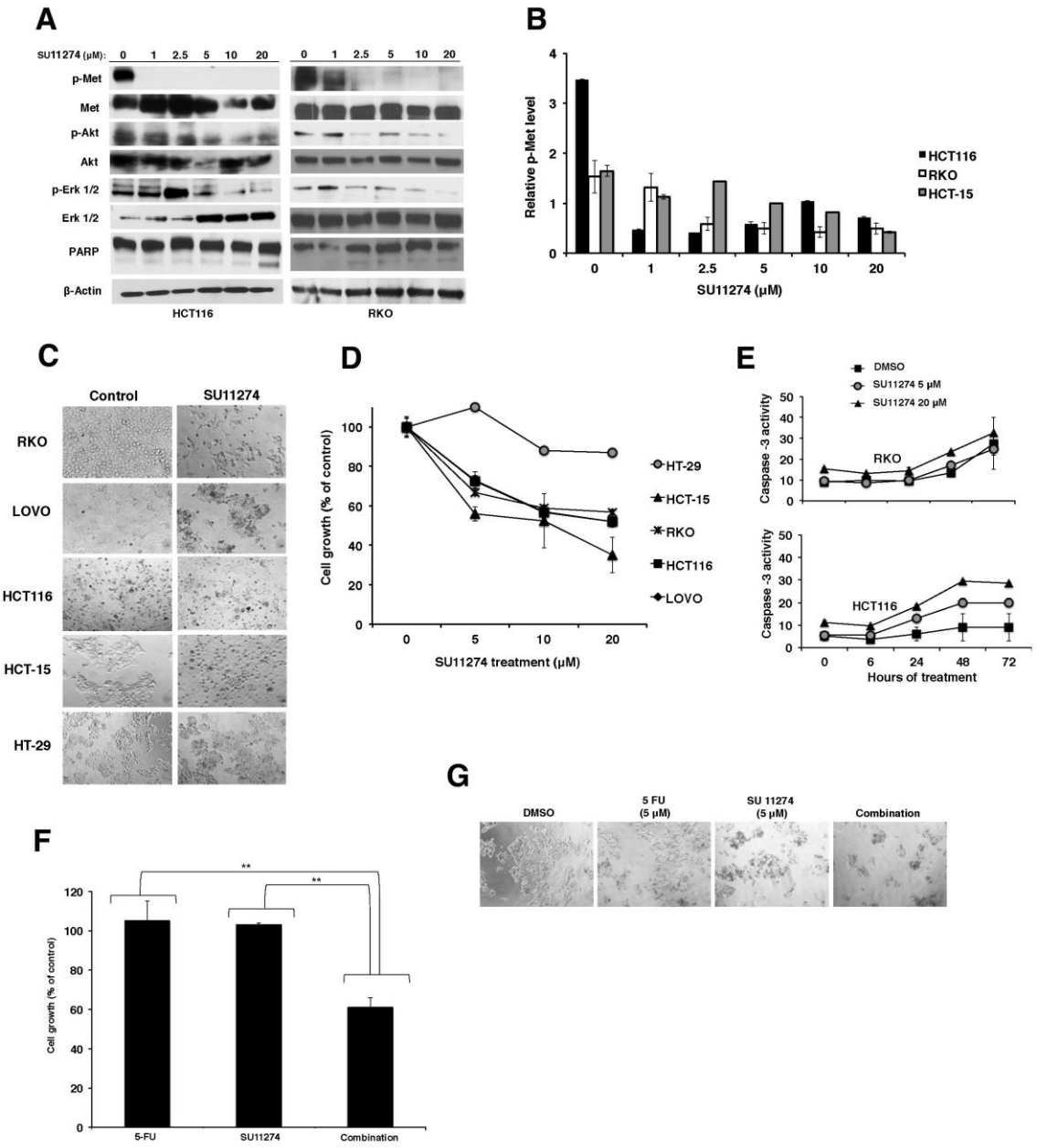

Figure 20. Inhibition of HGF-Met signaling causes significant reduction in CRC cell viability

(**A**) Representative western blot of CRC cells treated with various doses of Met inhibitor, SU12274, and assessed for indicators of Met activation and signaling. (**B**) Quantification of Met inhibition by increasing doses of SU11274. (**C**) Phase contrast microscopy of SU11274 treated CRC cells (**D**) Cell viability of CRC cells following 24 hours of SU11274 treatment. (**E**) Caspase activity used to assess apoptosis following Met inhibition. (**F**) Cell viability assays of CRC treated with 5-FU alone and in combination with SU11274 for 24 hours. (**G**) Phase contrast microscopy of CRC cells treated with 5-FU alone and in combination with SU11274. Bars are mean $\pm$ SE. Significant differences between the groups corresponding to $p < 0.01$ is depicted by **.

3.4.4 Inhibition of autocrine HGF-Met signaling promotes induction of necroptosis in CRC

Necroptosis is now understood to be an actively regulated, alternative pathway of cell death induced by a variety of stimuli. The release of HMBG1 into the media is a well documented biochemical indicator of necroptosis [122]. Thus we analyzed SU11274 treated cells for this marker and discovered that Met inhibition indeed resulted in a dose-dependent increase in the HMGB1 release in response to SU11274 (**Figure 21A**). On the other hand, treatment with hydrogen peroxide, a well-known inducer of cell death through necroptosis, resulted in HMGB1 release that was totally abrogated when co-treated with exogenous HGF (**Figure 21A**). These data suggested to us that HGF-Met signaling axis has an anti-necrotic function. This notion was further supported by experiments in which we inhibited HGF-Met signaling by SU11274 and in the presence or absence of RIPK-1 inhibitor Necrostatin (Nec-1, a specific, allosteric inhibitor of RIPK-1 known to prevent necroptosis) [123]. The data showed that Nec-1 treatment significantly rescued cell death induced by Met inhibition (**Figure 21B**). These results led us to postulate that HGF-Met axis may modulate the induction of necroptosis thorough RIPK-1. Moreover, DATE wild-type, HGF non-expressing cells like HT-29, were found to be highly sensitive to TNF-induced necrosis, whereas DATE mutant, HGF producing cells like HCT116, were resistant to TNFα but could be sensitized to it by blocking HGF-Met signaling with SU11274, further suggesting that HGF plays an anti-necrotic role (**Figure 21C**).

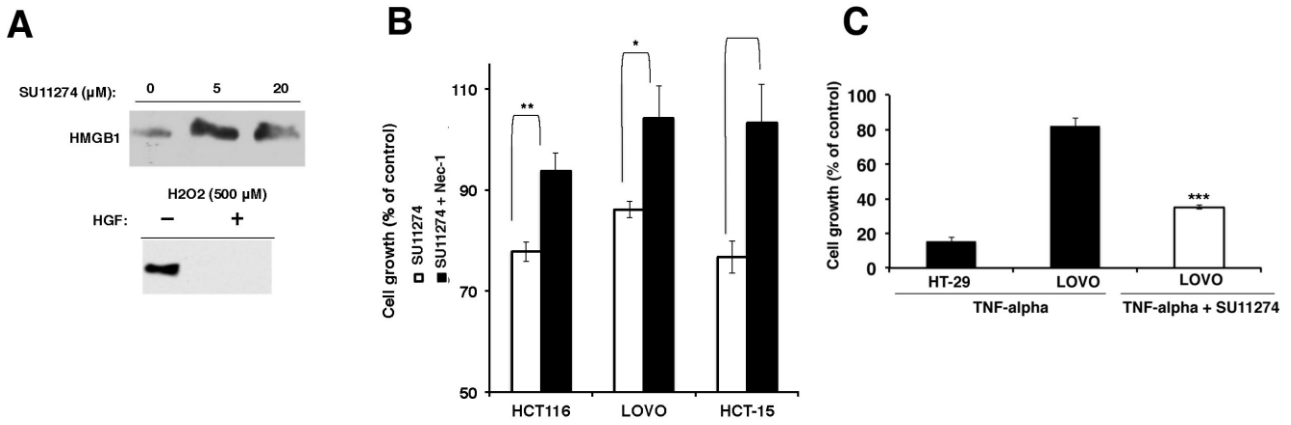

Figure 21. Inhibition of Met causes necroptosis in CRC cells
(**A**) Human CRC cells were treated with the Met inhibitor for 24 hours, and assessed for the release of the necroptosis indicator, HMGB1, into the serum. Impact of HGF treatment on H202 mediated release of HMGB1 was also assessed by western blot. (**B**) Necrostatin, an inhibitor of RIPK-1 was used to rescue the growth inhibition induced by treatment with the Met inhibitor in human CRC cells. (**C**) Human CRC cells were treated with SU11274 and TNFα to determine whether DATE mutant cells can be sensitized to TNFα induced necroptosis following inhibition of Met. Bars are mean $\pm$ SE. Significant differences between the groups corresponding to $p < 0.05$, $p < 0.01$, and $p < 0.001$ are depicted by *, **.***, respectively.

Given that RIPK-1 is the key player of cell death induction by necroptosis [97,99,124] we asked whether HGF-Met has an impact on RIPK-1. We discovered that inhibition of Met by SU11274 induced a marked up regulation of RIPK-1 in a dose and time dependent manner (**Figure 22A**). On the other hand, activation of Met by exogenous HGF resulted in reduction of RIPK-1 in CRC cell lines and in primary cultures of rat hepatocytes (which are well known targets of HGF) (**Figure 22B**). The down regulation of RIPK-1 was highly specific to HGF treatment, and could not be observed when CRC cells were treated with the pan-kinase inhibitor, Staurosporine, or the agonistic anti-Fas antibody, CH11 (**Figure 22C**). We also investigated the ability of HGF to induce rapid RIPK-1 down-regulation in intact cells using immunofluorescence microscopy (**Figure 22D**). Under these conditions, TNFα was also able to achieve marginal RIPK-1 down modulation (**Figure 22D**), which was confirmed by western blot.

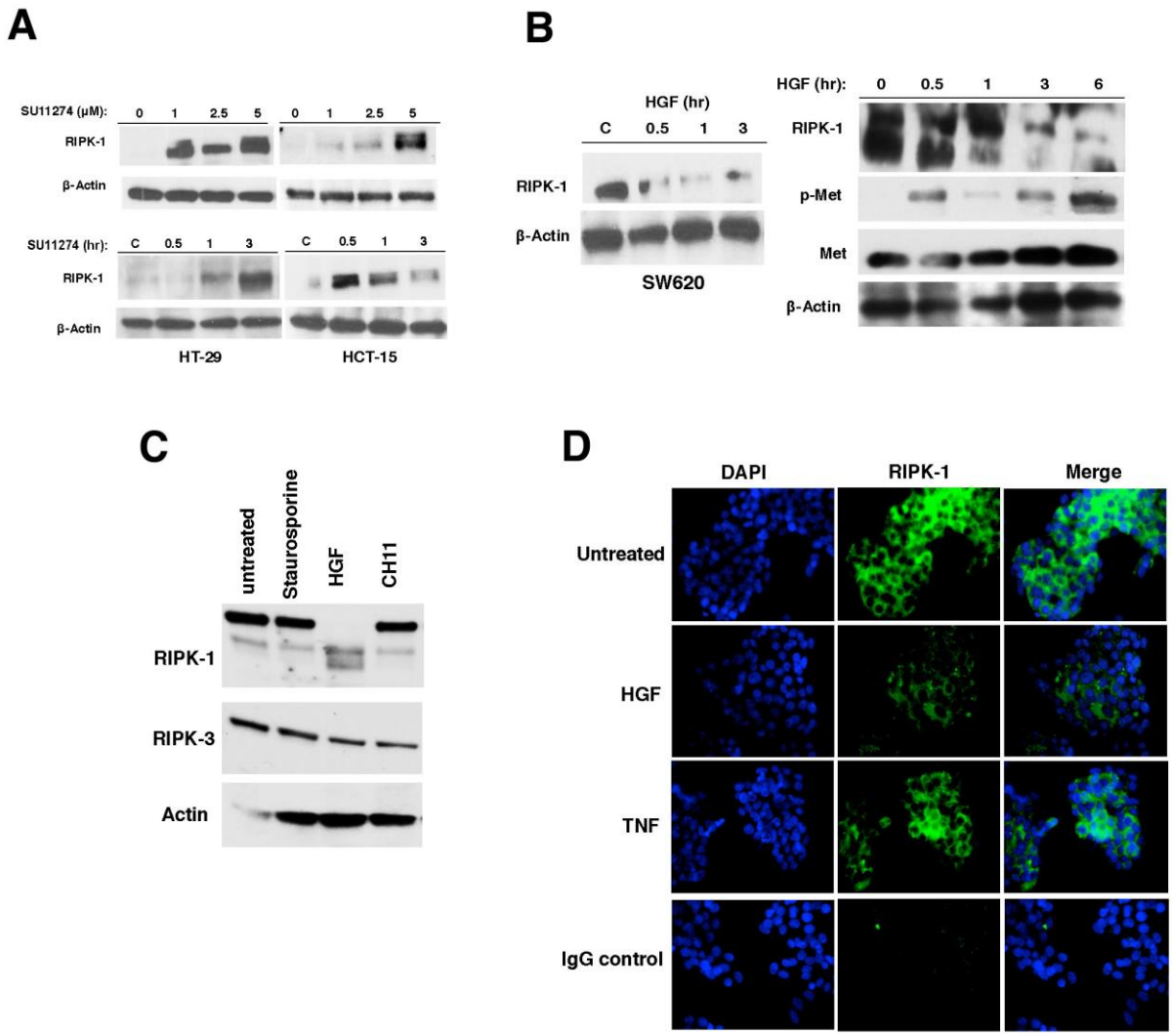

Figure 22. Active HGF-Met signaling down regulates RIPK-1 in CRC cells and primary rat hepatocytes

(**A**) CRC cells treated with Met inhibitor for 6 hours caused up-regulation of RIPK-1, a key mediator of necroptosis. (**B**) Conversely HGF treatment down regulates RIPK-1. (**C**) Following treated of CRC cells with a variety of compounds for 6 hours, down regulation of RIPK-was noted to be specific to HGF. (**D**) Hep-G2 cells were treated with HGF or TNF for 30 minutes and processed for IF. HGF mediated down regulation of RIPK-1 was observed in intact cells, using immunofluorescence microscopy.

Encouraged by these data, we administered recombinant HGF at concentrations known to activate Met systemically to mice as we previously described [125], and analyzed the livers for modulation of RIPK-1. We observed a significant down regulation in RIPK-1 (within two hours) in HGF-treated mice as compared to the saline treated control mice, confirming our *in-vitro* data that HGF is capable of modulating RIPK-1 protein levels (**Figure 23**).

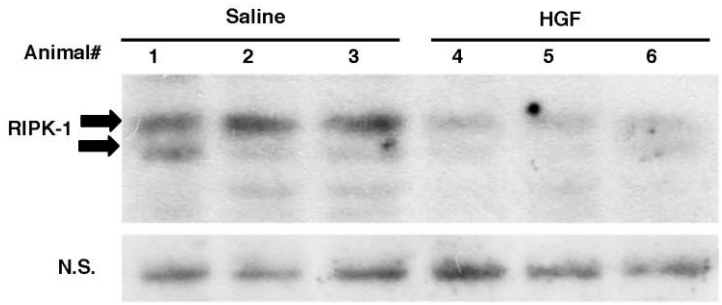

Figure 23. HGF injection causes down regulation of RIPK-1 in vivo
A total of 6 mice (db/db) were injected systemically with saline (N=3) or HGF (N=3, 0.5 ug/gr body weight) and their livers were harvested two hours later and analyzed for RIPK-1 expression by western blot. N.S. is a non-specific band, with Mw of 80 kDa depicted for equal loading. RIPK-1 down regulation was observed in the HGF treated cohort.

We next examined human CRC tissues for RIPK-1 and HGF expression and found that those tumors with truncated DATE and upregulated HGF expression also have a significantly reduced levels of RIPK-1 protein as compared with DATE wild-type CRC tissues with low HGF expression. Tissues with high HGF and low RIPK-1 also exhibited fewer necrotic regions (areas where cells are lacking membranes and harbor heavy immune infiltration) (**Figure 24A-C**). Linear regression analysis revealed a strong negative correlation between HGF and RIPK-1 protein levels: high HGF protein abundance significantly associated with lower levels of RIPK-1 protein (**Figure 24D, $p < 0.01$**).

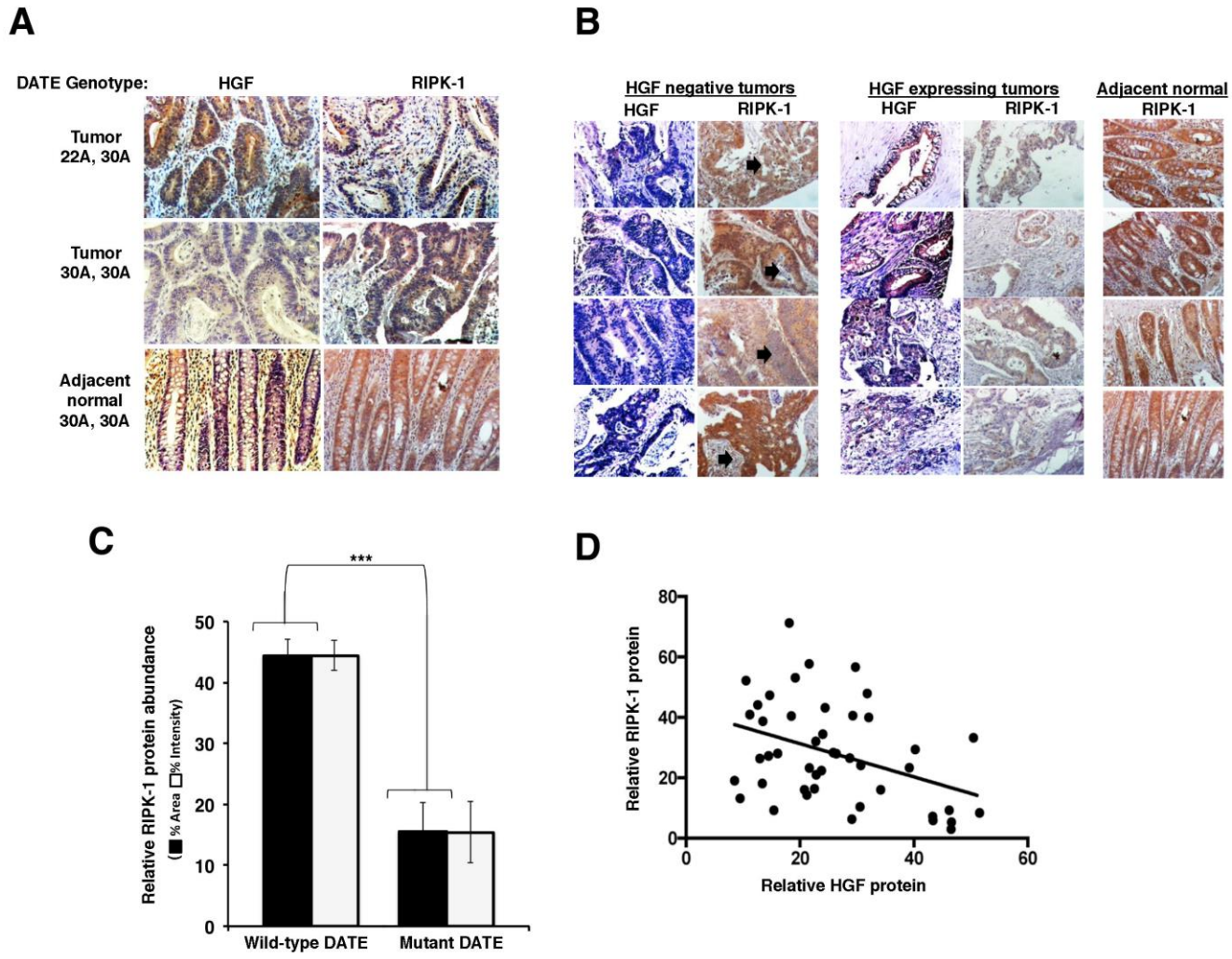

Figure 24. HGF expression correlates with down regulation of RIPK-1 in human CRC patient tissues

(A) DATE mutant and wild-type human CRC tissues were stained for HGF and RIPK-1. DATE mutant CRC tissues showed high HGF expression, as depicted by the representative immunohistostaining images. (**B**) Tumors with high HGF had low RIPK-1, and exhibited fewer signs of necrosis. Necrotic regions (indicated by arrowheads, depict regions with cells lacking cell membranes and having heave immune infiltration) are present in HGF negative tumors (**C**) HGF and RIPK-1 protein abundance in the immunohistochemically stained CRC tissue sections were quantified using Image Analysis Software. (**D**) Linear regression analysis of the above-described data indicated that HGF and RIPK-1 protein expression showed a significantly negative correlation. Bars are mean $\pm$ SEM for n= 40. Significant differences between the groups corresponding to $p < 0.001$ is depicted by ***.

3.4.5 Autocrine HGF-Met signaling promotes escape from necroptosis via direct post translational modification and modulation of RIPK-1

The above data prompted us to decipher the underlying mechanisms of HGF-Met mediated RIPK-1 down regulation and escape from necroptosis. It is reported that RIPK-1 is highly regulated post-translationally. Given that our intitial studies indicated that down-regulation is not at the mRNA level, we hypothesized RIPK-1 down-regulation may occur at the protein level. It is well-known that upon stimulation by cytokines like TNFα, RIPK-1 is recruited from the cytoplasm to the plasma membrane and polyubiquitinated (Lys63-linked chain). This RIPK-1 is incorporated into a signalosome that leads to NF-κB activation and is then degradaed via the proteosome to terminate signaling [97,99,102,124,126]. To determine whether HGF-Met axis induces RIPK-1 recruitment to the plasma membrane, we treated CRC cells with HGF, fractionated cell lysates to plasma membrane (M) and cytosolic portions (C) and assessed RIPK-1 levels. We found that greater than 90% of the RIPK-1 pool resides in the cytosolic fraction in unstimulated cells. HGF treatment induced marked recruitment of RIPK-1 to the plasma membrane within one hour of treatment. Quantification of the data showed that HGF treatment induced significant recruitment of RIPK-1 to plasma membrane which reached more than 60% and was accompanied by a proportional reduction of RIPK-1 abundance in the cytoplasm (**Figure 25A and B**). Further exploration of the time course of RIPK-1 recruitment to the plasma membrane by HGF revealed that it occurs rapidly within 10 minutes of HGF stimulation which was concomitant with Met activation (**Figure 25C**). Met was mainly found in the plasma membrane fraction whereas cIAP1 was largely cytosolic (**Figure 25C**). In these experiments TNFα was used as a positive control as it is a well known inducer of RIPK-1 recruitment to the plasma membrane [102]. It is important to note that HGF-induced RIPK-1 recruitment to plasma

membrane could be enhanced when HGF treatment was done in the presence of proteasome inhibitor MG-132 (**Figure 25C**), suggesting that recruited RIPK-1 is rapidly degraded by the proteasome.

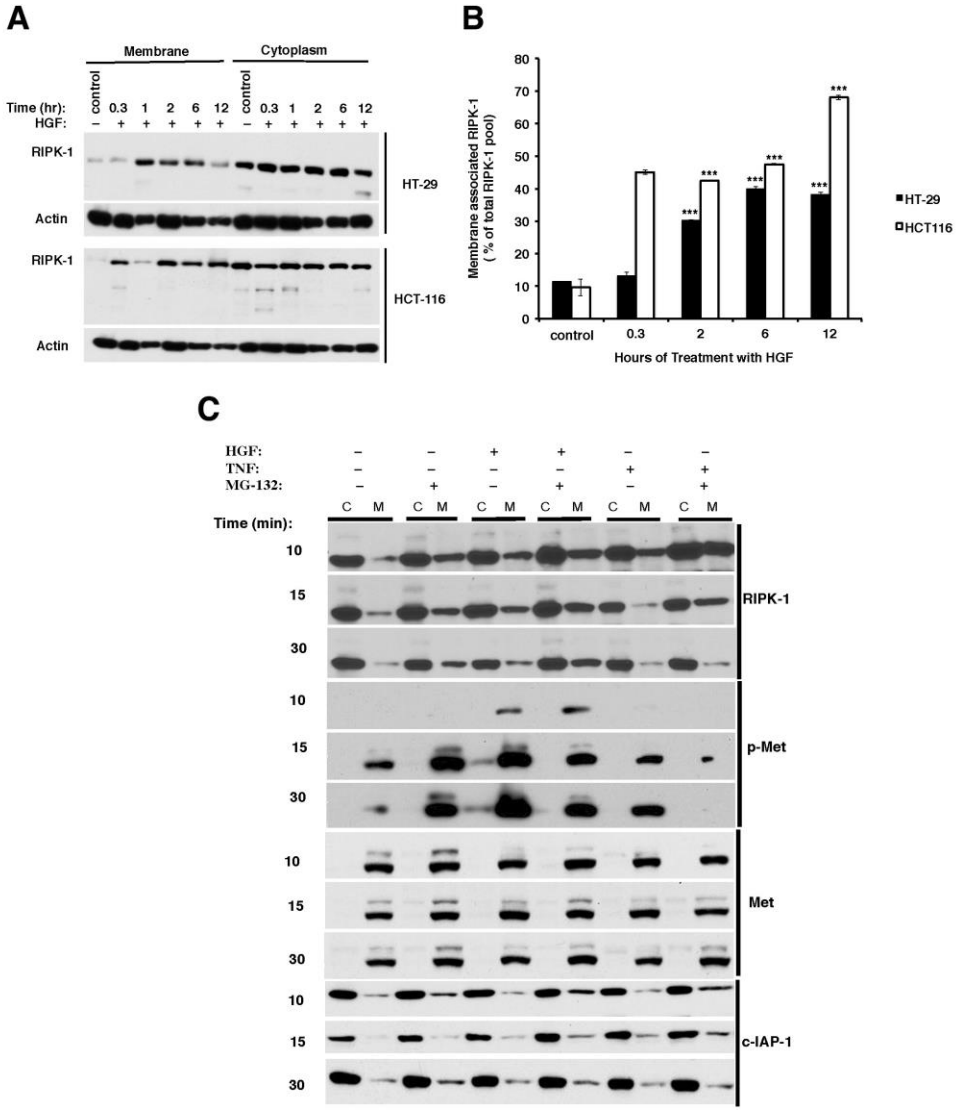

Figure 25. HGF-Met signaling causes rapid recruitment of RIPK-1 to the plasma membrane (**A**) CRC cells were pre-treated with MG-132 for one hour and then stimulated with or without HGF as indicated and assessed for RIPK-1 by western blot. (**B**) Quantification of the data by densitometric analysis. (**C**) Time course of RIPK-1 recruitment to plasma membrane by HGF. CRC cells were pre-treated with or without MG-132 for one hour and then stimulated with or without HGF or TNFα as indicated. C denotes cytosolic fraction and M membrane. RIPK-1 level was assessed by western and the blots were then sequentially, re-probed for the indicated proteins. Bars are mean $\pm$ SD. Significant differences between the groups corresponding to $p < 0.001$ is depicted by ***.

The ability of HGF to impact RIPK-1 at the cellular level was investigated using immunofluorescence microscopy in intact cells . **Figure 26** depicts that in unstimulated cells RIPK-1 [105] shows a highly diffused cytoplasmic pattern of distribution whereas Met shows plasma membrane localization. Stimulation with HGF (but not TNF) provoked a pronounced redistribution of RIPK-1 to the plasma membrane within 20 minutes of treatment. Interestingly, both Met and RIPK-1 formed highly punctate patterns of staining (indicated by the arrowheads in **Figure 26**) which is a hallmark of Met clustering and internalization.

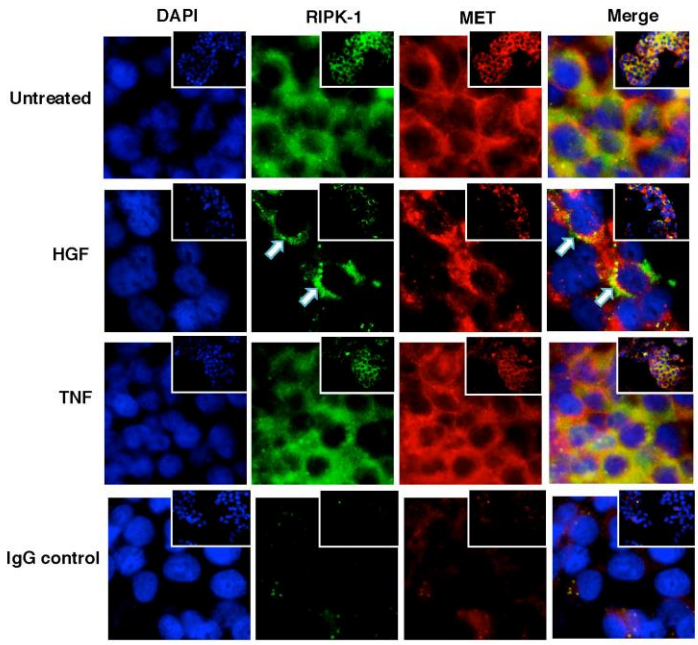

Figure 26. HGF-Met signaling results in down regulation of RIPK-1 in vivo
Hep-G2 cells were treated with or without HGF or TNFα and assessed for RIPK-1 using immunofluorescence microscopy. Rabbit and mouse IgG were used as negative controls for staining. The images indicate that HGF-Met axis impacts RIPK-1 cellular distribution using RIPK-1 and Met specific antibodies as indicated. Arrowheads indicate that HGF causes robust redistribution of RIPK-1 to the membrane. (note the punctate staining).

Membrane associated RIPK-1 exhibited a ladder/smear pattern of migration in the presence of the proteasome inhibitor, MG132, which is a hallmark of polyubiquitinylation (**Figure 27A**). Polyubiquitinated RIPK-1 is proposed to induce some cell survival genes via

activating p65 subunit of NF-κB [97,99,103,124]. Notably, we observed that down-regulation of RIPK-1 by HGF did not affect the level of phosphorylated p65, an indicator of active NF-κB mediated cell survival signaling (**Figure 27B**). To directly assess whether RIPK-1 is indeed ubiquitinated by HGF treatment, we immunoprecipitated RIPK-1 from CRC cell lysates and immunoblotted with total and linkage specific (Lys48 and Lys63) ubiquitin antibodies. Untreated or MG132 treated cells did not exhibit RIPK-1 ubiquitination. However, cells treated with the combination of HGF and MG132 showed robust RIPK-1 ubiquitination which was mainly of the Lys48-linked chain type, indicating that it is destined for degradation by the proteasome machinary. Of note, we found that Met and RIPK-1 form a complex following HGF stimulation, since Met was immunoprecipitated with RIPK-1. Interestingly, this was observed only in samples treated with HGF and MG132 (**Figure 27C**) suggesting that Met-RIPK-1 complex is very unstable.

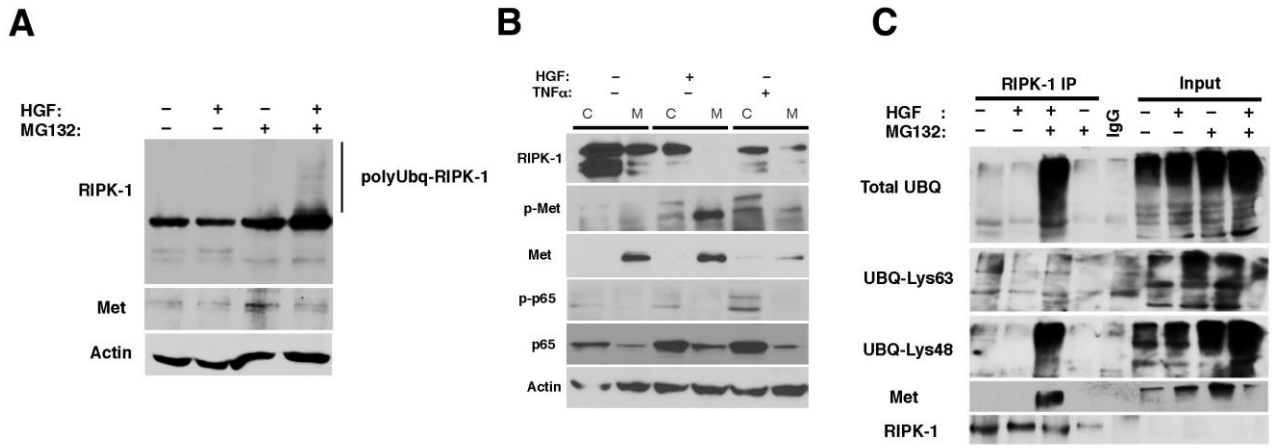

Figure 27. HGF induces polyubiquitination of RIPK-1
(**A**) Serum deprived CRC cells were treated with or without HGF in the presence or absence of MG-132 for three hours. Membrane fraction was prepared and assessed for RIPK-1 by western blot. (**B**) HepG2 cells were treated with or without HGF or TNFα for 30 minutes and assessed by western blotting. Down regulation of RIPK-1 by HGF was found to not affect phospho-p65 levels. (**C**) CRC cells were treated with or without HGF in the presence of absence of MG-132 and their lysates were subjected to IP with RIPK-1 antibody, and western blotting was performed as indicated to detect ubiquitination of RIPK-1 and its association with Met.

It is known that RIPK-1 is polyubiquitinated by E3 ligases like TRAF-2. We therefore hypothesized that RIPK-1 polyubiquitinylation induced by HGF may be carried out by TRAF-2. We uncovered that like RIPK-1, TRAF-2 was indeed recruited to the plasma membrane with HGF treatment in Hep-G2 cells (**Figure 28A**). Blotting for Met was used to distinguish the membrane fraction and C-IAP was used to identify the cytoplasmic fration, as well as to confirm equal protein loading. We also noted that HGF treatment induced a western blot banding pattern that resembled ubiquitination of TRAF-2 (**Figure 28B**). Immunoprecipitation of TRAF-2 from the membrane lysates and western blotting for RIPK-1 indicated that HGF also induced association of TRAF-2 to RIPK-1 and that TRAF-2 polyubiquitination (likely self-induced) was primarily of the Lys48-linked type (**Figure 28C**).

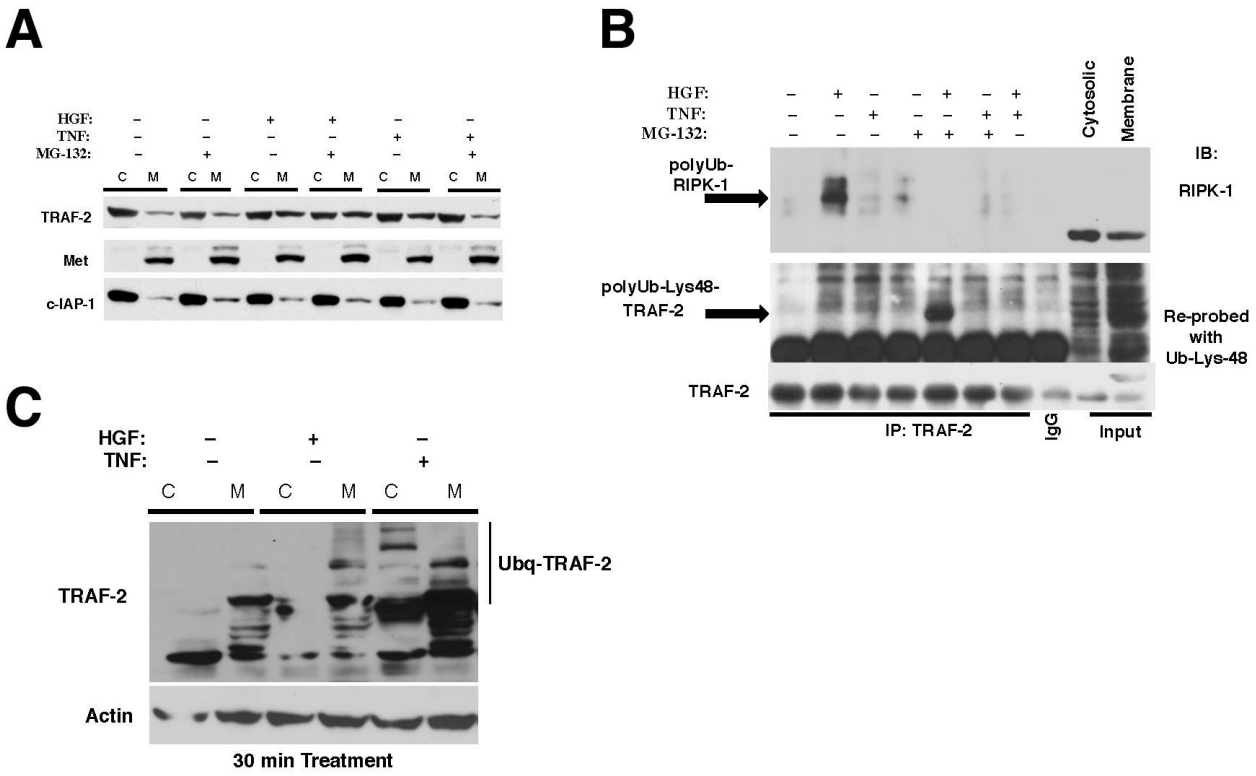

Figure 28. HGF stimulation causes the recruitment of TRAF-2 to plasma membrane and its association with RIPK-1

(**A**) Serum deprived HepG-2 cells were pre-treated MG-132 for one hour and then with HGF or TNFα for 30 minutes and the lysates were subjected to western blot to detect TRAF-2 cellular localization. (**B**) Serum deprived HepG-2 cells were treated with or without HGF for 30 minutes and their cytosolic (C) and membrane (M) fractions were subjected to western blotting for TRAF-2. It was noted that HGF induces polyubiquitination of plasma membrane associated TRAF-2. (**C**) Lysates from (A) were IP with TRAF-2 antibody and western blotted for RIPK-1 to detect association. Polyubiquitin antibodies were used to detect ubiquitination of TRAF-2.

Given the fact that Met is a tyrosine kinase and RIPK-1 could be recruited to the plasma membrane by Met activation and the finding that Met and RIPK-1 associated with each other, we asked if Met can tyrosine phosphorylate RIPK-1 hence targeting it for ubiquitination. Phospho-tyrosine sites in target proteins are well-known to act as binding sites for E3 ligases leading to ubiquitinylation of the target proteins. Indeed, Met itself once activated by HGF, autophosphorylates on tyrosine residue 1003 which then recruits E3 ligase Cbl leading to

ubiquitinylation and degradation of Met and its associated proteins via proteasome [127]. Thus we performed immunoprecipitation and western blot studies using RIPK-1 and phosphotyrosine antibodies to test if RIPK-1 becomes tyrosine phosphorylated by Met. Remarkably, we discovered that HGF induced tyrosine phosphorylation of RIPK-1 (**Figure 29A)**. Time course experiments revealed that HGF stimulation causes rapid and transient tyrosine phosphorylation of RIPK-1 within 30 minutes (immunoprecipitation with phosphotyrosine antibody pY20 and immunoblotting with RIPK-1 **Figure 29B**. Collectively, these data suggested that RIPK-1 is most likely directly tyrosine phosphorylated by Met. To test this further, we incubated pure recombinant active human Met kinase (His-tgged, 55 kDa) with pure recombinant active RIPK-1 (GST-tagged full-length RIPK-1, 110 kDa) in kinase buffer and assessed for RIPK-1 tyrosine phosphorylation. Incubation of Met with RIPK-1 resulted in robust tyrosine phosphorylation of RIPK-1 (**Figure 29C**). Proteomic analyses using MS/MS MALDI-TOF identified that RIPK-1 became phosphorylated by Met at Tyr384 residue. The sequence of this site is KLQDEANYHL and is located in the intermediate domain of RIPK-1 near K377, which is the major site of RIPK-1 ubiquitination [97,100,124]. We found that this Tyr site and the motif (KLQDEANYHL) are highly conserved in various organisms from humans down to at least Xenopus indicating its functional importance. We subsequently asked whether tyrosine phosphorylation of RIPK-1 has functional consequences. It is known that RIPK-1 activates itself through the autophosphorylation of the serine residues within its kinase domain [99]. Notably, we found that tyrosine phosphorylation of RIPK-1 by Met resulted in marked reduction of RIPK-1 activity, as indicatated by the signifciant reduction in serine phosphorylation of RIPK-1 (**Figure 29C and D**).

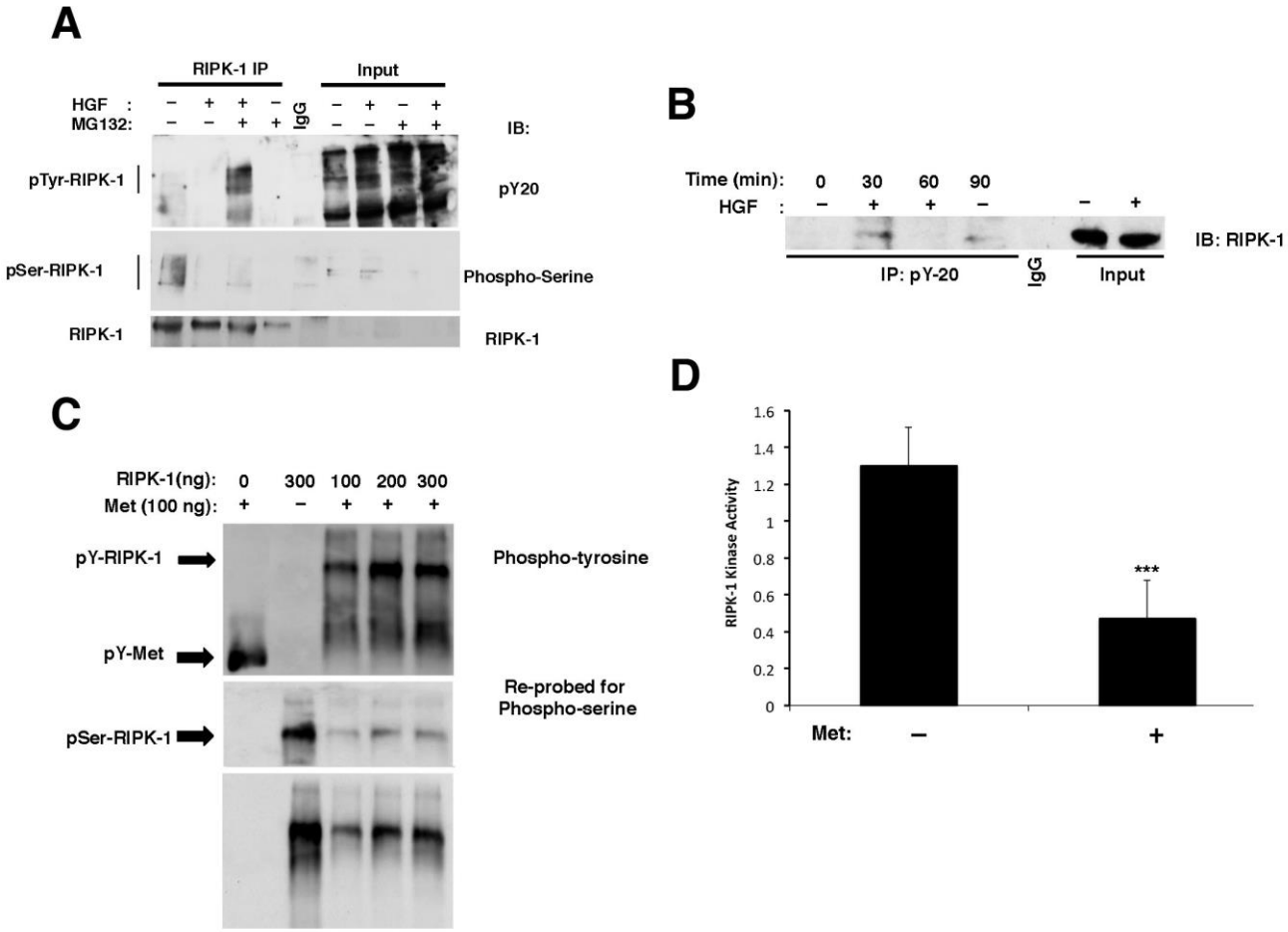

Figure 29. Met directly tyrosine phosphorylates RIPK-1 decreasing its autocatalytic activity as indicated by the reduction in RIPK-1 serine phosphorylation

(**A**) CRC cells were treated with or without HGF in the presence or absence of MG-132, and their membrane fractions were subjected to IP with RIPK-1 antibody and western blotting with phosphotyrosine and phosphoserine antibodies. (**B**) Serum deprived CRC cells were treated with or without HGF and the lysates were subjected to IP with a phosphotyrosine antibody (pY20) and then western blotted for RIPK-1. (**C**) An in-vitro kinase assay was performed by incubating pure recombinant human Met Kinase with varying amounts of pure recombinant human RIPK-1 in kinase buffer for 30 minutes. The samples then subjected to western blotting, to detect tyrosine and serine phosphorylation of RIPK-1. (**D**) Quantification of RIPK-1 serine autophosphorylation from (**C**) indicating loss of RIPK-1 kinase activity following incubation with Met kinase. Bars are mean $\pm$ SD. Significant differences between the groups corresponding to $p < 0.001$ is depicted by ***.

In order to determine whether the ability to phosphorylate RIPK-1 is limited to Met, we performed an *in-vitro* kinase assay using recombinant human RIPK-1 and several different human pure recombinant tyrosine kinases (EGFR, INSR,SRC). We found that all of the tested tyrosine

kinases were capable of phosphorylating RIPK-1 as indicated by western blotting of the kinase assay samples with the pY-20 phosphotyrosine antibody (**Figure 30**). We noted that incubation of RIPK-1 with INSR showed particularily robust tyrosine phosphorylation of RIPK-1, while weaker phosphoryation was observed following incubation with SRC. Additionally, although EGFR appears to phosphorylate RIPK-1 (as indicated by the stronger western blot stain observed when RIPK-1 was incubated with EGFR), due to the fact that EGFR runs at a molecular weight similar to that of RIPK-1, no concrete conclusions regarding its impact on RIPK-1 could be made.

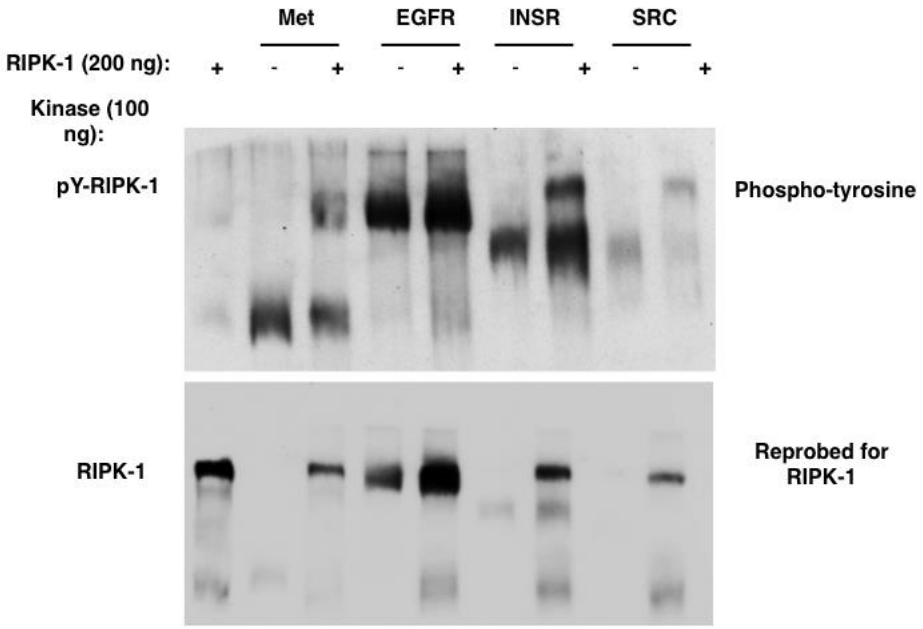

Figure 30. RIPK-1 can be phosphorylated by multiple tyrosine kinases
An in-vitro kinase assay was performed by incubating multiple human pure recombinant tyrosine kinases (Met, EGFR, INSR, and SRC) with pure recombinant human RIPK-1 in kinase assay buffer for 30 minutes. The samples were then subjected to western blotting using phosphotyrosine antibodies to detect phosphorylation of RIPK-1.

3.4.6 Autocrine HGF-Met signaling associates with negative patient prognosis in CRC

We next asked whether DATE mutagenesis in CRC impacted patient prognosis. We found that DATE mutant tumors were significantly larger as compared to DATE wild-type tumors (average tumor size of 7.27 cm vs. 4. 96 cm (**$p < 0.01$**). High HGF protein abundance in tumors (as observed in DATE mutant tumors) also significantly associated with advanced tumor stages (**Figure 31A, $p < 0.001$**). Kaplan-Mier survival analysis indicated that patients with mutant DATE and low RIPK-1 protein abundance exhibited significantly lower 5- year survival in comparison to those with wild-type DATE and high levels of RIPK-1 protein (P= 0.0003, and 0.001, respectively, (**Figure 31B and C**).

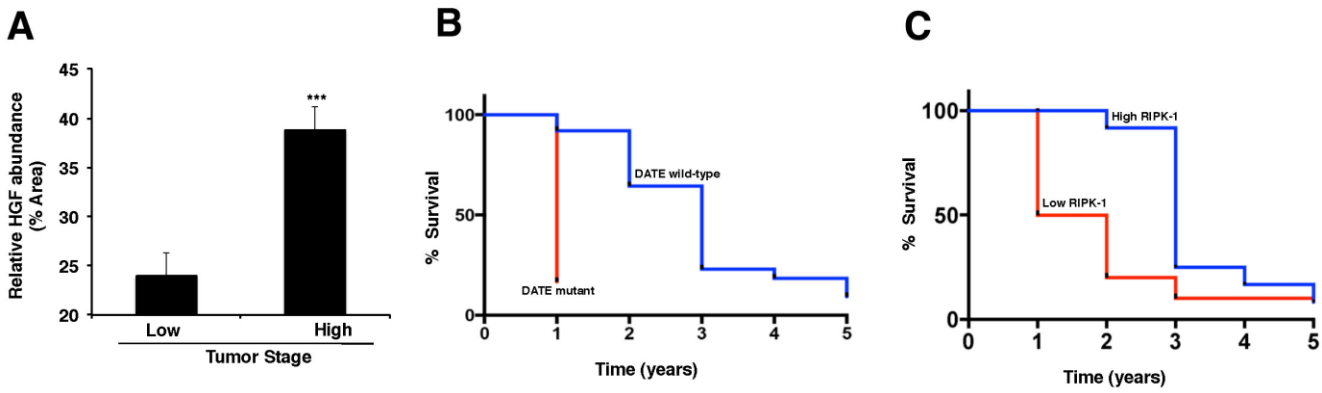

Figure 31. DATE mutation and low RIPK-1 levels associate with poor patient prognosis
(**A**) Quantification of RIPK-1 and HGF protein abundance were assessed by IHC and quantified using Image Analysis Software. Tumors were classified as low (TMN stage I and II) or high (TMN stage IV) stage using the pathology reports of the samples. Tumors with high HGF expression displayed advanced tumor stages overall, while those with low HGF expression exhibited low tumor stages. Bars are mean $\pm$ SEM for n= 40. (**B, C**) Kaplan-Mier survival analysis was performed to determine the association of (**B**) DATE mutation and (**C**) RIPK-1 levels with 5-year CRC patient survival. Significant differences between the groups corresponding to $p < 0.001$ is depicted by ***.

3.5 DISCUSSION

CRC is the third leading form of cancer related death worldwide, and despite the therapeutic advances pioneered over the last decade that have led to significant increases in patient survival, nearly 50% of patients who undergo surgery, die of metastatic disease. Given its bleak prognosis, novel therapeutic targets and innovative strategies are needed to improve the future outlook of CRC [128]. Our findings indicate that HGF-Met signaling pathway is aberrantly activated in a significant subset of human sporadic CRC, and that HGF-Met signaling axis presents a potential therapeutic target for the personalized treatment of CRC.

Deregulated growth factor signaling is a cardinal features of cancer cells [73]. For instance, gene overexpression of HGF/Met, and ligand independent activation of Met, among other mechanisms, are believed to play crucial roles in non-small cell lung carcinoma (NSCLC), gastric, ovarian, pancreatic, thyroid, breast, head and neck, colon and kidney cancers [48]. While the role of aberrant HGF-Met signaling in promoting un-regulated cell survival and growth is well documented, the detailed molecular mechanisms involved are yet to be fully understood. Our work here elucidates the causes and the consequences of aberrant HGF production and autocrine HGF-Met signaling in CRC.

It is well known that both chromosomal and microsatellite instability promote colorectal tumorigenesis by leading to the accumulation of mutations in tumor suppressors and oncogenic driver genes [73,129]. Our initial investigations (Data shown in Section 2) revealed that defective MMR mediated MSI is the underlying cause of a *HGF* gene promoter mutation (interchangeably referred to as DATE truncation). Furthermore, DATE mutagenesis significantly associated with the incidence of CRC, suggesting that it plays roles in the onset and/or maintenance of the malignant phenotype. Our studies regarding the consequences of DATE mutation in cancer (Data

shown in Section 3) indicated that shortening of DATE is sufficient to promote transcriptional activation of the otherwise silenced *HGF* gene in colonic epithelial cells. In other words, truncation of DATE relieves its repressive function, leading to dysregulated, autonomous HGF production in epithelial cells, which are normally subject to paracrine signaling via HGF that is produced by the stromal cells. We noted that autocrine production of HGF causes activation of the oncogenic driver kinase, Met, and confers a selective growth advantage by stimulating cell proliferation, survival, and escape from necroptotic cell death. These findings are further discussed below.

HGF-Met signaling is known to promote cell growth by inhibiting apoptosis and by inducing cell proliferation via activation of PI3K/Akt/mTOR/p70S6K and MAPK/ERK [49]. Concurring with these reports, the treatment of DATE mutant CRC cell lines with HGF neutralizing antibody or a small molecule Met kinase inhibitor, resulted in significantly reduced activation of Met and its key downstream effectors, leading to reduced cell growth and viability, while stimulation with HGF led to the converse results. These findings suggested that DATE mutant CRC cells exhibit an "oncogenic addiction" to HGF-Met signaling.

Programmed necrosis, dubbed necroptosis, is initiated by death receptor signaling (such as tumor necrosis factor receptor 1 and FAS) and requires the kinase activity of RIPK-1, which autophosphorylates itself and subsequently RIP-3, leading to the formation of the necrosome [97]. Our understanding of the role of growth factor signaling in preventing necroptosis is lacking, and the mechanisms involved are yet to be discerned. Our report here shows for the first time that RIPK-1 is directly regulated by HGF-Met, and will serve as a pioneering milestone in further exploration of this area. We discovered that in human CRC, HGF-Met protects against necroptosis by down regulating RIPK-1. Interestingly, we also discovered that RIPK-1 down

regulation in response to HGF also occurs in primary hepatocyte cultures and in the livers mice injected with HGF, indicating that modulation of necroptosis by HGF-Met signaling is not limited to CRC and cell culture. We showed here that following HGF stimulation, RIPK-1 is recruited to the membrane where it then becomes tyrosine phosphorylated on residue 384 and subsequently polyubiquitinated likely by TRAF-2. RIPK-1 is a known substrate of TRAF-2 [130] and E3 ubiquitin ligases such as TRAF-2 are known to self-regulate through polyubiquitin modification [131]. Interestingly, we found that following HGF stimulation TRAF-2 becomes polyubiquitinated (Lys48-linked) at the plasma membrane. According to the current models, RIPK-1 is believed to participate in inducing cells survival, inflammation, and death by forming different protein complexes [97,99,103,124]. Complex I is believed to form rapidly at the plasma membrane upon stimulation with pro-inflammatory stimuli like TNFα and contains TNFR-1, TRADD, RIPK-1, TRAF-2, and cIAP1/2 [102]. In this complex, RIPK-1 becomes polyubiquitinated (Lys63-linked chain type), and acts as a docking site for recruitment and activation of the components of the NF-κB pathway. RIPK-1 then undergoes process of ubiquitin editing by A20 and Cyld and its polyubiquitin moiety switches to Lys48-linked chain kind, which targets it to proteasomal degradation to terminate NF-κB induction [97,99,103,124]

Under some stress conditions, this activated RIPK-1 undergoes dissociation and forms complex IIA, which contains active RIPK-1, FADD, and Caspase-8, which can induce apoptosis. Alternatively, complex IIB, which is composed of RIPK-1 and RIP-3, can form and provoke necroptosis (especially when apoptotic Caspases are inhibited) [102]. Interestingly, we previously reported that Met also prohibits apoptosis by sequestering Fas [132,133] and also by directly inhibiting Caspase-3 by the virtue of harboring a novel decoy like capsase-3 site, which consists of DNADDEVDT motif, located at Met's c-terminal end. This site has two tandem Caspase-3

cleavage sites that entrap and inhibit the active site of Caspase-3 during the cleavage process [134]. It should be noted that modulation of RIPK-1 by HGF treatment did not dampen phosphorylation of NF-κB p65 subunit, indicating NF-κB activation was not affected. Regardless, RIPK-1 was recently discovered to play a non-essential role in NF-κB signaling as determined by experiments using RIPK-1 deficient MEFs [130]. Moreover, we showed that tyrosine phosphorylation of RIPK-1 by Met, inhibited the kinase activity of RIPK-1. Notably, detailed structure-function studies have shown that the kinase activity of RIPK-1 is not required for NF-κB activation (RIPK-1 merely acts as scaffold and docking site to activate NF-κB), but is essential for induction of necrosis [97,99,124,130]. Based on these outcomes, we believe that HGF-Met axis down-regulates RIPK-1 and prevents the onset of necroptosis, without influencing pro-survival NF-κB signaling, thus, creating an ideal molecular environment for cancer cell survival and proliferation.

Our findings also have important clinical implications since we found that HGF overexpression in the tumor correlated with reduced levels of RIPK-1, larger tumor size, high tumor stage and poor patient survival. We showed that HGF expressing tumor cells have operational HGF-Met autocrine circuit and that HGF down regulates RIPK-1. On the other hand, blocking Met signaling by small molecule Met inhibitor like SU11274, dramatically up regulated RIPK-1 and induced necroptosis. Our results suggest that necroptosis is a major mechanism of cancer cell death that may be inhibited via the activation of HGF-Met signaling. Our proposed model for *HGF* gene mutation and its molecular consequences are shown in **Figure 32**.

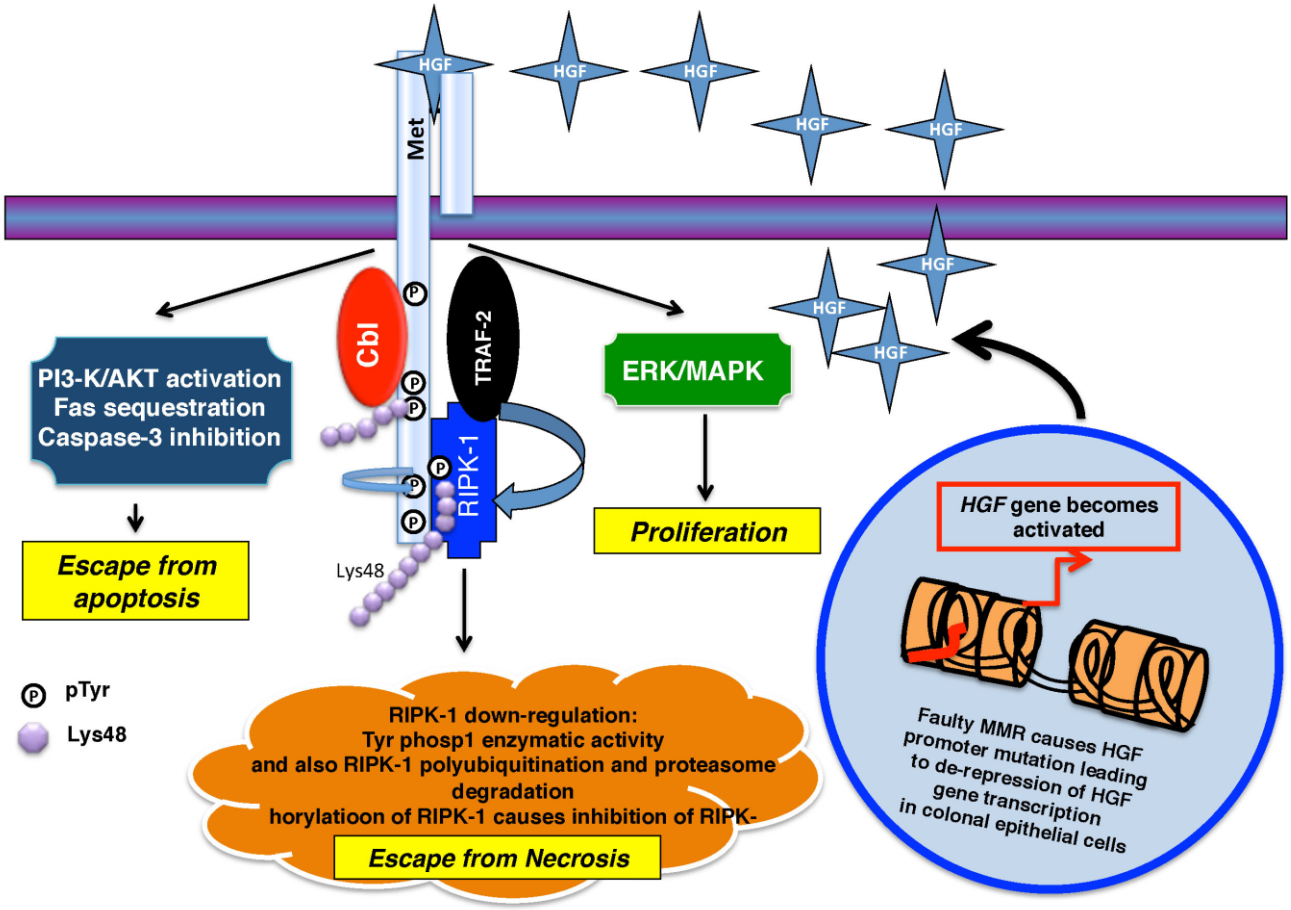

Figure 32. Model of the causes and consequences of *HGF* gene mutation in human CRC

The clinical benefits of inducing necroptosis in cancer have been recently documented in multiple studies. For instance, radiation treatment was noted to induce RIPK-1 dependent necroptosis in anaplastic thyroid and adrenocortical cancer cells, and promote tumor reduction [135]. Our findings suggest that inhibition of HGF-Met signaling can induce RIPK-1 mediated necroptosis and thus presents a viable therapeutic strategy in the treatment of human CRC. There are a number of Met kinase inhibitors and anti-HGF therapies currently in phase I and II clinical trials for the treatment of lung, renal, gastric, brain and colorectal cancer [48,68]. The targetable

nature of HGF-Met signaling in CRC was recently demonstrated using a selective small molecule Met kinase inhibitor, ARQ 197 (Tivantinib). In-vitro assays using ARQ 197 showed a dose and a time-dependent decrease in the proliferative capacity of CRC cell lines, and ARQ 197 injection in mice bearing CRC cell line xenografts caused inhibition of tumor growth [136]. In phase I clinical trials, Tivantinib was well tolerated and showed encouraging anti-tumor activity in patients with metastatic CRC in-conjunction with the chemotherapeutics, Irinotecan and cetuximab [71]. MMR deficiency and MSI is documented to play a fundamental role in a number of other malignant conditions beside hereditary non-polyposis colorectal cancer (HNPCC Lynch syndrome and its associated cancers), such as cancers of the endometrium, stomach, pancreas-biliary tract, small intestine, urothelium, ovary and glioblastomas [76,82,108,109]. Our work encourages further studies to develop modalities by which HGF-Met can be efficiently targeted in tumors for the treatment of CRC.

We previously showed (In Section 2) that DATE is polymorphic in nature and that the truncated DATE variant occurs in the general population at an overall frequency of about 8%. Here, we uncovered that normal colonic tissues of subjects with truncated DATE variant have higher HGF expression than those with wild-type DATE. We speculate that having the truncated DATE (hence hyperactive *HGF* promoter variant) is a double-edged sword; it can provide robust HGF expression and promote efficient tissue regeneration and repair, but it also can promote tumorigenesis if sufficient genetic/epigenetic lesions in key oncogenes and tumor suppressor genes accumulate. We suspect that harboring truncated DATE polymorphism and high HGF production in normal tissues contribute to earlier malignant transformation and more aggressive tumor growth in patients. Our work uncovered that the incidence of truncated DATE in normal tissues is significantly higher within the African American population. Interestingly, a recent

study of dialysis patients found significant up-regulation of HGF in the serum of African-American patients, in comparison to the Caucasian counterparts [137]. This observation, combined with our data, suggests that the increased incidence of polymorphic DATE and the resultant autocrine HGF production by normal cells may underlie the up-regulated serum HGF levels observed in African Africans. Although our sample size was relatively small, given the strong oncogenic properties of active HGF-Met signaling, these findings also imply that the elevated incidence of truncated DATE polymorphism in normal tissues may be partly responsible for the early tumor onset and the worsened prognosis observed within the African American CRC patient cohort. Future large-scale population studies are needed to determine whether individuals with truncated DATE variant are predisposed to CRC.

Our work identified autocrine activation of HGF-Met signaling as a potential therapeutic target in human CRC. Our model for the manner in which autocrine HGF production in cells provides a selective growth advantage and likely contributes to colon carcinogenesis is shown in **Figure 33**. The findings of this study encourage further clinical investigations to establish routine DATE testing and anti-HGF/Met therapy for the treatment of CRC patients harboring DATE mutations. Our report also provides impetus for investigations of the dynamic modulation of RIPK-1, not only by HGF-Met, but also potentially by other tyrosine kinase growth factor receptors. Finally, given the importance of RIPK-1 in promoting inflammation, and its ability to be modulated by HGF, we suggest additional studies to determine whether HGF can suppress inflammation both within and outside the context of cancer.

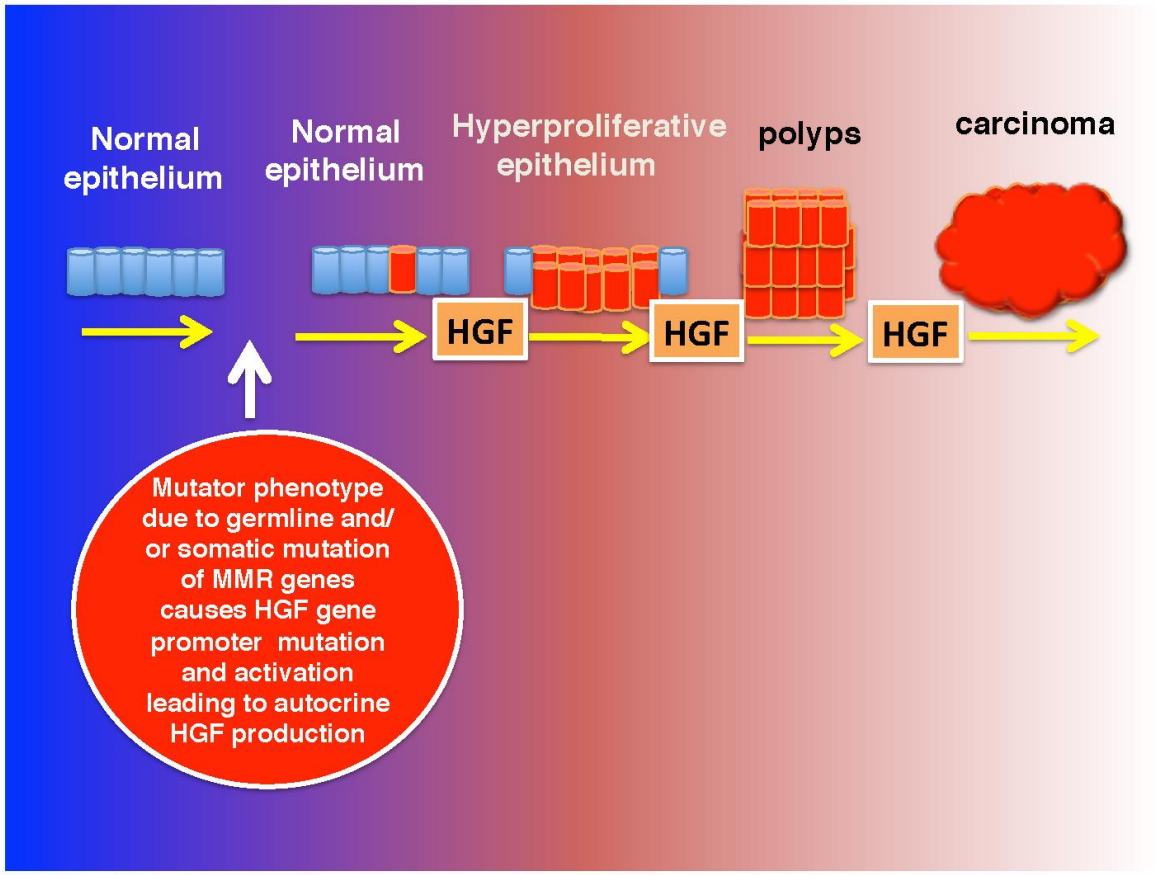

Figure 33. Proposed model outlining the role of autocrine HGF production in colon tumorigenesis

Proposed model showing that mutator phenotype due to germline (i.e. HNPCC) and/or somatic mutation of MMR genes causes *HGF* gene promoter mutation and activation, leading to HGF autocrine production in colonic epithelial cells (shown in red). Autocrine HGF in turn confers a selective growth advantage, and in corporation with other deregulated essential players, (i.e. oncogenes and tumor suppressors genes), drives colon tumor promotion.

4.0 IMPLICATIONS AND FUTURE DIRECTIONS

Mortality from CRC accounts for a significant portion of cancer related deaths worldwide. Current treatment methods include local endoscopic resection, extensive surgery (may include lymph node resection), as well as adjuvant and non-adjuvant chemotherapy. While the treatment strategies and the degree of therapeutic success differ depending on the stage of diagnosis, the overall bleak prognosis of metastatic and recurring CRC highlight the tremendous need for novel therapeutic targets and approaches. Given the well-known tumorigenic role played by the HGF-Met signaling pathway in a number of malignancies, we aimed to identify whether *HGF* undergoes mutagenesis and plays an oncogenic role in human CRC.

Our work is the first to indicate that a subset of CRC patients harboring *HGF* promoter mutations may benefit from inhibition of the HGF-Met signaling axis. We were also the first to demonstrate that genomic instability provoked by faulty DNA mismatch repair leads to *HGF* gene promoter mutation and the oncogenic addiction of human CRC cells to autocrine HGF-Met signaling. We uncovered that the subset of CRC patients harboring *HGF* promoter mutations present considerably larger tumors and significantly reduced 5-year survival. These findings suggest that DATE status has prognostic significance in human CRC. The well tolerated Met inhibitor, Tivantinib, is currently being tested in phase II clinical trials for the treatment of human CRC [71]. While the Tivantinib study aims to assess the overall patient response to anti-Met therapy, our data suggests that maximal therapeutic benefit may be achieved in those harboring truncated DATE. Although additional studies are needed to confirm the clinical relevance of DATE, our findings imply that stratification of CRC cases by DATE status may be useful in

predicting who will benefit from targeted Met therapy, and aid in the development more effective and personalized treatment plans.

Escape from controlled cell death is a hallmark of cancer cells [73]. Studies of molecular mechanisms of human CRC have revealed that defective cell death signaling is fundamental to the onset and progression of CRC. To date, resistance to apoptosis has been identified as the major cause of therapeutic failure and disease recurrence, and thus, nearly all non-invasive therapeutic approaches have aimed to promote CRC cell apoptosis [138]. However, given the recent findings indicating that escape from alternate forms of controlled death (such as necroptosis) play crucial roles in maintaining the malignant phenotype, our current understanding of the landscape of cell death in cancer is rapidly changing. Our investigations described here, as well as a number of other recent studies, imply that evasion of necroptosis significantly contributes to colorectal carcinogenesis and therefore presents a novel therapeutic target [138]. Our results are of particular significance, as they demonstrate for the first time that receptor growth factor signaling can inhibit necroptosis via direct post-translational modification and proteasome-mediated down regulation of a critical necroptosis executioner protein, RIPK-1. We also showed that down regulation RIPK-1 via HGF-Met signaling associates with poor CRC patient prognosis, suggesting that escape from necroptosis makes significant contributions to tumor progression. Our findings encourage further investigations into the role of RIPK-1 in cancer and whether growth factor signaling and/or RIPK-1 present viable therapeutic targets in the treatment of colorectal and other human cancers.

While it may be beneficial to induce necroptosis in cancer to achieve tumor reduction, disease situations exist where it is advantageous to prevent the onset of necroptosis. For instance, excessive necroptotic cell death in the intestinal epithelium is observed in chronic inflammatory diseases such as Crohn's disease and ulcerative colitis. Terminal ileum biopsies from patients

suffering from Crohn's disease show necroptotic cell death at the crypt base. It's been speculated that Paneth cell abnormalities and the constitutive expression/activation of RIPK1 and RIP-3, contribute to the elevated necroptosis observed in the ileum and the pathogenesis of Crohn's disease [139]. Our novel findings indicating that activation of HGF-Met signaling down-regulates RIPK-1 may be applicable in the management of Crohn's disease and ulcerative colitis. We propose further investigations to determine whether the treatment of Crohn's disease patients with HGF could inhibit excessive intestinal necroptosis and provide a viable therapeutic strategy to manage inflammatory bowel disease.

The difficulty in developing safe and effective cancer therapies lies not in the inability to identify chemicals capable of eliminating cancer cells, but in ensuring that the drug concentrations at which cancer cells are eradicated cause minimal harm to the normal tissues of the patient. Most chemotherapeutic agents were historically identified based on their in vitro capabilities to eliminate rapidly dividing cells. As such, these agents generally kill rapidly dividing normal cells including the bone-marrow hematopoietic precursors and gastrointestinal mucosal epithelial cells, and cause irreversible injury to certain non-rapidly dividing normal tissues as well. Furthermore, using higher concentrations of chemotherapeutics can result in off-target effects that lead to increased toxicity and unforeseen biological effects. In order to improve these narrow therapeutic windows and eliminate cancer cells with minimal patient toxicity, combination therapy may be utilized [140]. Synthetic lethality traditionally refers to a situation where a single mutation in either of two genes is compatible with survival, but simultaneous mutations in both genes induce cell death [141]. This model can also be extended to define lethal interactions between two drugs, such as that observed with the use of targeted EGFR inhibitors and cytotoxic agents. For instance, combining traditional chemotherapeutics such as 5-FU and Cisplatin with molecularly targeted

therapies such as the EGFR inhibitor, Cetuximab, has resulted in significant prognostic improvements in metastatic colorectal and head and neck cancer patients. In this case, Cetuximab targets EGFR overexpression on the cancer cells, while 5-FU therapy inhibits DNA replication, leading to tumor cell death and improved therapeutic responses in patients receiving the combination therapy in comparison to those receiving single treatments [142]. Similarly, our work indicated that inhibition of Met in combination with other treatment modalities, such as TNFα and 5-FU results in increased tumor cell death. TNFα has been approved for the treatment of melanoma and soft-tissue sarcomas in Europe, and 5-FU is used as the standard therapy in CRC. Despite their remarkable anticancer activities, both agents pose significant toxicity to the patient. Our data suggests that using low doses of TNFα/5-FU treatments in combination with HGF-Met inhibitors, may allow for tumor reduction with minimal patient toxicity.

While our work sheds new light on the causes and consequences of *HGF* gene mutation in CRC, it also strongly highlights the need for future experimentation to answer the many questions raised by our findings. All our studies on the cell killing abilities of HGF-Met inhibition were largely based on *in-vitro* data. Therefore, we propose the use of mouse xenografts using human CRC cell lines to verify the anti-tumor activity of small molecule Met inhibitors and anti-HGF neutralizing antibodies, *in vivo*, alone and in combination with other cytotoxic agents. We also uncovered that DATE is polymorphic in nature and that presence of the truncated DATE variant in adjacent normal tissues associates with increased HGF production. Given the well-known tumorigenic properties of deregulated growth factor signaling, we postulate that harboring the truncated DATE variant in normal tissues may predispose individuals to develop CRC. To assess this, we propose the screening of normal individuals for DATE polymorphisms and performing 10-20 year prospective studies to evaluate the relationship between truncated DATE

and risk of CRC. Our findings also indicated that DATE mutagenesis in tumor associated with larger tumor size and worsened patient prognosis. Given these findings, we recommend extending the study of DATE to larger patient cohorts to determine its value as a diagnostic and prognostic marker in human CRC. Additionally, our data indicated that DATE mutation is the result of underlying MMR defects and DATE status was a highly sensitive indicator of genomic instability, as it was capable of detecting MSI with more precision than the current gold-standard markers of instability. These discoveries suggested that DATE mutagenesis is likely an early event in the process of CRC tumorigenesis. To determine the point at which DATE truncation occurs during the tumorigenic process, early and late stage colorectal adenomas could be assessed for DATE status. If DATE mutagenesis and autocrine HGF production are found to be early events during the tumorigenic process, additional clinical studies could be performed to determine whether using HGF-Met inhibition as front-line therapy for early stage CRC is advantageous in improving overall patient prognosis. Conclusively, our findings invite further exploration of the role of aberrant HGF-Met signaling in CRC, and encourage the development of DATE as a novel biomarker to guide the personalized treatment of human CRC.

BIBLIOGRAPHY

1 Haggar, F. A. & Boushey, R. P. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clin Colon Rectal Surg* **22**, 191-197, doi:10.1055/s-0029-1242458 (2009).

2 Parkin, D. M., Bray, F., Ferlay, J. & Pisani, P. Global cancer statistics, 2002. *CA Cancer J Clin* **55**, 74-108 (2005).

3 Siegel, R., Naishadham, D. & Jemal, A. Cancer statistics, 2013. *CA Cancer J Clin* **63**, 11-30, doi:10.3322/caac.21166 (2013).

4 Janout, V. & Kollarova, H. Epidemiology of colorectal cancer. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* **145**, 5-10 (2001).

5 Boyle, P. & Langman, J. S. ABC of colorectal cancer: Epidemiology. *Bmj* **321**, 805-808 (2000).

6 Hayat, M. J., Howlader, N., Reichman, M. E. & Edwards, B. K. Cancer statistics, trends, and multiple primary cancer analyses from the Surveillance, Epidemiology, and End Results (SEER) Program. *Oncologist* **12**, 20-37, doi:10.1634/theoncologist.12-1-20 (2007).

7 Labianca, R., Beretta, G. D., Mosconi, S., Milesi, L. & Pessi, M. A. Colorectal cancer: screening. *Ann Oncol* **16 Suppl 2**, ii127-132, doi:10.1093/annonc/mdi730 (2005).

8 O'Connell, J. B., Maggard, M. A., Livingston, E. H. & Yo, C. K. Colorectal cancer in the young. *Am J Surg* **187**, 343-348, doi:10.1016/j.amjsurg.2003.12.020 (2004).

9 de Jong, A. E. *et al.* Prevalence of adenomas among young individuals at average risk for colorectal cancer. *Am J Gastroenterol* **100**, 139-143, doi:10.1111/j.1572-0241.2005.41000.x (2005).

10 Boardman, L. A. *et al.* Colorectal cancer risks in relatives of young-onset cases: is risk the same across all first-degree relatives? *Clin Gastroenterol Hepatol* **5**, 1195-1198, doi:10.1016/j.cgh.2007.06.001 (2007).

11 Al-Sukhni, W., Aronson, M. & Gallinger, S. Hereditary colorectal cancer syndromes: familial adenomatous polyposis and lynch syndrome. *Surg Clin North Am* **88**, 819-844, vii, doi:10.1016/j.suc.2008.04.012 (2008).

12 Papadopoulos, N. *et al.* Mutation of a mutL homolog in hereditary colon cancer. *Science* **263**, 1625-1629 (1994).

13 Johnson, I. T. & Lund, E. K. Review article: nutrition, obesity and colorectal cancer. *Aliment Pharmacol Ther* **26**, 161-181, doi:10.1111/j.1365-2036.2007.03371.x (2007).

14 Larsson, S. C. & Wolk, A. Meat consumption and risk of colorectal cancer: a meta-analysis of prospective studies. *Int J Cancer* **119**, 2657-2664, doi:10.1002/ijc.22170 (2006).

15 Zisman, A. L., Nickolov, A., Brand, R. E., Gorchow, A. & Roy, H. K. Associations between the age at diagnosis and location of colorectal cancer and the use of alcohol and tobacco: implications for screening. *Arch Intern Med* **166**, 629-634, doi:10.1001/archinte.166.6.629 (2006).

16 Compton, C. C. & Greene, F. L. The staging of colorectal cancer: 2004 and beyond. *CA: a cancer journal for clinicians* **54**, 295-308 (2004).

17 D'Angelica, M. *et al.* Patterns of initial recurrence in completely resected gastric adenocarcinoma. *Ann Surg* **240**, 808-816, doi:00000658-200411000-00012 [pii] (2004).
18 Goldberg, R. M. *et al.* Surgery for recurrent colon cancer: strategies for identifying resectable recurrence and success rates after resection. Eastern Cooperative Oncology Group, the North Central Cancer Treatment Group, and the Southwest Oncology Group. *Ann Intern Med* **129**, 27-35 (1998).
19 Negrini, S., Gorgoulis, V. G. & Halazonetis, T. D. Genomic instability--an evolving hallmark of cancer. *Nature reviews. Molecular cell biology* **11**, 220-228, doi:10.1038/nrm2858 (2010).
20 Grady, W. M. Genomic instability and colon cancer. *Cancer Metastasis Rev* **23**, 11-27 (2004).
21 Lievre, A., Blons, H. & Laurent-Puig, P. Oncogenic mutations as predictive factors in colorectal cancer. *Oncogene* **29**, 3033-3043, doi:10.1038/onc.2010.89 (2010).
22 Segditsas, S. & Tomlinson, I. Colorectal cancer and genetic alterations in the Wnt pathway. *Oncogene* **25**, 7531-7537, doi:10.1038/sj.onc.1210059 (2006).
23 Markman, B., Javier Ramos, F., Capdevila, J. & Tabernero, J. EGFR and KRAS in colorectal cancer. *Adv Clin Chem* **51**, 71-119 (2010).
24 Kataoka, H., Hamasuna, R., Itoh, H., Kitamura, N. & Koono, M. Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma. *Cancer research* **60**, 6148-6159 (2000).
25 Direnzo, M. F. *et al.* Overexpression and Amplification of the Met/Hgf Receptor Gene during the Progression of Colorectal-Cancer. *Clinical Cancer Research* **1**, 147-154 (1995).
26 Kataoka, H., Itoh, H., Hamasuna, R., Meng, J. Y. & Koono, M. Pericellular activation of hepatocyte growth factor/scatter factor (HGF/SF) in colorectal carcinomas: roles of HGF activator (HGFA) and HGFA inhibitor type 1 (HAI-1). *Hum Cell* **14**, 83-93 (2001).
27 Artale, S. *et al.* Mutations of KRAS and BRAF in primary and matched metastatic sites of colorectal cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **26**, 4217-4219, doi:10.1200/JCO.2008.18.7286 (2008).
28 Barault, L. *et al.* Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers. *International journal of cancer. Journal international du cancer* **122**, 2255-2259, doi:10.1002/ijc.23388 (2008).
29 Lampropoulos, P. *et al.* TGF-beta signalling in colon carcinogenesis. *Cancer Lett* **314**, 1-7, doi:10.1016/j.canlet.2011.09.041 (2012).
30 Ku, J. L. *et al.* Genetic alterations of the TGF-beta signaling pathway in colorectal cancer cell lines: a novel mutation in Smad3 associated with the inactivation of TGF-beta-induced transcriptional activation. *Cancer Lett* **247**, 283-292, doi:10.1016/j.canlet.2006.05.008 (2007).
31 Bellam, N. & Pasche, B. Tgf-beta signaling alterations and colon cancer. *Cancer Treat Res* **155**, 85-103, doi:10.1007/978-1-4419-6033-7_5 (2010).
32 Smith, G. *et al.* Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. *Proc Natl Acad Sci U S A* **99**, 9433-9438, doi:10.1073/pnas.122612899 (2002).
33 Aklilu, M. & Eng, C. The current landscape of locally advanced rectal cancer. *Nat Rev Clin Oncol* **8**, 649-659, doi:10.1038/nrclinonc.2011.118 (2011).

34 Brown, G. Thin section MRI in multidisciplinary pre-operative decision making for patients with rectal cancer. *Br J Radiol* **78 Spec No 2**, S117-127, doi:10.1259/bjr/15128198 (2005).
35 Kim, J. C. *et al.* Preoperative concurrent radiotherapy with capecitabine before total mesorectal excision in locally advanced rectal cancer. *Int J Radiat Oncol Biol Phys* **63**, 346-353, doi:10.1016/j.ijrobp.2005.02.046 (2005).
36 Ng, K. & Schrag, D. Microsatellite instability and adjuvant fluorouracil chemotherapy: a mismatch? *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 3207-3210, doi:10.1200/JCO.2010.28.9314 (2010).
37 Sinicrope, F. A. *et al.* DNA mismatch repair status and colon cancer recurrence and survival in clinical trials of 5-fluorouracil-based adjuvant therapy. *J Natl Cancer Inst* **103**, 863-875, doi:10.1093/jnci/djr153 (2011).
38 Tejpar, S. *et al.* Microsatellite instability (MSI) in stage II and III colon cancer treated with 5FU-LV or 5FU-LV and irinotecan (PETACC 3-EORTC 40993-SAKK 60/00 trial). *J Clin Oncol* **27** (2009).
39 Koopman, M. *et al.* Deficient mismatch repair system in patients with sporadic advanced colorectal cancer. *Brit J Cancer* **100**, 266-273, doi:Doi 10.1038/Sj.Bjc.6604867 (2009).
40 French, A. J. *et al.* Prognostic significance of defective mismatch repair and BRAF V600E in patients with colon cancer. *Clin Cancer Res* **14**, 3408-3415, doi:Doi 10.1158/1078-0432.Ccr-07-1489 (2008).
41 Ribic, C. M. *et al.* Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *New Engl J Med* **349**, 247-257, doi:Doi 10.1056/Nejmoa022289 (2003).
42 Van Schaeybroeck, S., Allen, W. L., Turkington, R. C. & Johnston, P. G. Implementing prognostic and predictive biomarkers in CRC clinical trials. *Nat Rev Clin Oncol* **8**, 222-232, doi:10.1038/nrclinonc.2011.15 (2011).
43 Ogino, S. *et al.* CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* **58**, 90-96, doi:10.1136/gut.2008.155473 (2009).
44 Dahlin, A. M. *et al.* The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. *Clinical cancer research : an official journal of the American Association for Cancer Research* **16**, 1845-1855, doi:10.1158/1078-0432.CCR-09-2594 (2010).
45 Ogino, S. *et al.* KRAS mutation in stage III colon cancer and clinical outcome following intergroup trial CALGB 89803. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**, 7322-7329, doi:10.1158/1078-0432.CCR-09-1570 (2009).
46 Zarnegar, R. & Michalopoulos, G. K. The many faces of hepatocyte growth factor: from hepatopoiesis to hematopoiesis. *J Cell Biol* **129**, 1177-1180 (1995).
47 Nakamura, T. & Mizuno, S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proc Jpn Acad Ser B Phys Biol Sci* **86**, 588-610 (2010).
48 Gherardi, E., Birchmeier, W., Birchmeier, C. & Vande Woude, G. Targeting MET in cancer: rationale and progress. *Nature reviews. Cancer* **12**, 89-103, doi:10.1038/nrc3205 (2012).

49 Trusolino, L., Bertotti, A. & Comoglio, P. M. MET signalling: principles and functions in development, organ regeneration and cancer. *Nature reviews. Molecular cell biology* **11**, 834-848, doi:10.1038/nrm3012 (2010).
50 Birchmeier, C., Birchmeier, W., Gherardi, E. & Vande Woude, G. F. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* **4**, 915-925, doi:10.1038/nrm1261 (2003).
51 Maina, F., Hilton, M. C., Ponzetto, C., Davies, A. M. & Klein, R. Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons. *Genes Dev* **11**, 3341-3350 (1997).
52 Huh, C. G. *et al.* Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 4477-4482, doi:10.1073/pnas.0306068101 (2004).
53 Liu, Y. Hepatocyte growth factor in kidney fibrosis: therapeutic potential and mechanisms of action. *Am J Physiol Renal Physiol* **287**, F7-16, doi:10.1152/ajprenal.00451.2003 (2004).
54 Chmielowiec, J. *et al.* c-Met is essential for wound healing in the skin. *J Cell Biol* **177**, 151-162, doi:10.1083/jcb.200701086 (2007).
55 Sonnenberg, E., Meyer, D., Weidner, K. M. & Birchmeier, C. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J Cell Biol* **123**, 223-235 (1993).
56 Michalopoulos, G. K., Zarnegar, R., Houck, K. & Pediaditakis, P. Hepatopoietins A and B and hepatocyte growth. *Dig Dis Sci* **36**, 681-686 (1991).
57 Bell, A. W., Jiang, J. G., Chen, Q., Liu, Y. & Zarnegar, R. The upstream regulatory regions of the hepatocyte growth factor gene promoter are essential for its expression in transgenic mice. *The Journal of biological chemistry* **273**, 6900-6908 (1998).
58 Jiang, J. G., Bell, A., Liu, Y. & Zarnegar, R. Transcriptional regulation of the hepatocyte growth factor gene by the nuclear receptors chicken ovalbumin upstream promoter transcription factor and estrogen receptor. *The Journal of biological chemistry* **272**, 3928-3934 (1997).
59 Jiang, J. G., Gao, B. & Zarnegar, R. The concerted regulatory functions of the transcription factors nuclear factor-1 and upstream stimulatory factor on a composite element in the promoter of the hepatocyte growth factor gene. *Oncogene* **19**, 2786-2790, doi:10.1038/sj.onc.1203581 (2000).
60 Seol, D. W., Chen, Q. & Zarnegar, R. Transcriptional activation of the hepatocyte growth factor receptor (c-met) gene by its ligand (hepatocyte growth factor) is mediated through AP-1. *Oncogene* **19**, 1132-1137, doi:10.1038/sj.onc.1203404 (2000).
61 Liu, M. L., Mars, W. M., Zarnegar, R. & Michalopoulos, G. K. Collagenase pretreatment and the mitogenic effects of hepatocyte growth factor and transforming growth factor-alpha in adult rat liver. *Hepatology* **19**, 1521-1527 (1994).
62 Liu, M. L., Mars, W. M., Zarnegar, R. & Michalopoulos, G. K. Uptake and distribution of hepatocyte growth factor in normal and regenerating adult rat liver. *Am J Pathol* **144**, 129-140 (1994).
63 Liu, Y., Lin, L. & Zarnegar, R. Modulation of hepatocyte growth factor gene expression by estrogen in mouse ovary. *Molecular and cellular endocrinology* **104**, 173-181 (1994).

64 Zarnegar, R., DeFrances, M. C., Kost, D. P., Lindroos, P. & Michalopoulos, G. K. Expression of hepatocyte growth factor mRNA in regenerating rat liver after partial hepatectomy. *Biochem Biophys Res Commun* **177**, 559-565 (1991).
65 Ma, J. *et al.* Somatic mutation and functional polymorphism of a novel regulatory element in the HGF gene promoter causes its aberrant expression in human breast cancer. *The Journal of clinical investigation* **119**, 478-491, doi:10.1172/JCI36640 (2009).
66 Cecchi, F., Rabe, D. C. & Bottaro, D. P. Targeting the HGF/Met signalling pathway in cancer. *Eur J Cancer* **46**, 1260-1270, doi:10.1016/j.ejca.2010.02.028 (2010).
67 Nakamura, T., Sakai, K. & Matsumoto, K. Hepatocyte growth factor twenty years on: Much more than a growth factor. *J Gastroenterol Hepatol* **26 Suppl 1**, 188-202, doi:10.1111/j.1440-1746.2010.06549.x (2011).
68 Corso, S., Comoglio, P. M. & Giordano, S. Cancer therapy: can the challenge be MET? *Trends in molecular medicine* **11**, 284-292, doi:10.1016/j.molmed.2005.04.005 (2005).
69 Peters, S. & Adjei, A. A. MET: a promising anticancer therapeutic target. *Nat Rev Clin Oncol* **9**, 314-326, doi:10.1038/nrclinonc.2012.71 (2012).
70 Munshi, N. *et al.* ARQ 197, a novel and selective inhibitor of the human c-Met receptor tyrosine kinase with antitumor activity. *Molecular cancer therapeutics* **9**, 1544-1553, doi:10.1158/1535-7163.MCT-09-1173 (2010).
71 Yap, T. A. *et al.* Final results of a pharmacokinetic (PK) and pharmacodynamic (PD) phase I trial of ARQ 197 incorporating dynamic contrast-enhanced (DCE) magnetic resonance imaging (MRI) studies investigating the antiangiogenic activity of selective c-Met inhibition. *Journal of Clinical Oncology* **27** (2009).
72 Eder, J. P., Vande Woude, G. F., Boerner, S. A. & LoRusso, P. M. Novel therapeutic inhibitors of the c-Met signaling pathway in cancer. *Clin Cancer Res* **15**, 2207-2214, doi:1078-0432.CCR-08-1306 [pii]10.1158/1078-0432.CCR-08-1306 (2009).
73 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).
74 Thompson, S. L., Bakhoum, S. F. & Compton, D. A. Mechanisms of chromosomal instability. *Curr Biol* **20**, R285-295, doi:10.1016/j.cub.2010.01.034 (2010).
75 Jefford, C. E. & Irminger-Finger, I. Mechanisms of chromosome instability in cancers. *Crit Rev Oncol Hematol* **59**, 1-14, doi:10.1016/j.critrevonc.2006.02.005 (2006).
76 Atkin, N. B. Microsatellite instability. *Cytogenetics and cell genetics* **92**, 177-181, doi:56898 (2001).
77 Edelmann, W. *et al.* Tumorigenesis in Mlh1 and Mlh1/Apc1638N mutant mice. *Cancer Res* **59**, 1301-1307 (1999).
78 Banno, K. *et al.* Endometrial cancer as a familial tumor: pathology and molecular carcinogenesis (review). *Curr Genomics* **10**, 127-132, doi:10.2174/138920209787847069 (2009).
79 Vasen, H. F. Review article: The Lynch syndrome (hereditary nonpolyposis colorectal cancer). *Aliment Pharmacol Ther* **26 Suppl 2**, 113-126, doi:10.1111/j.1365-2036.2007.03479.x (2007).
80 Jass, J. R. HNPCC and sporadic MSI-H colorectal cancer: a review of the morphological similarities and differences. *Fam Cancer* **3**, 93-100, doi:10.1023/B:FAME.0000039849.86008.b7 (2004).

81 Mitchell, R. J., Farrington, S. M., Dunlop, M. G. & Campbell, H. Mismatch repair genes hMLH1 and hMSH2 and colorectal cancer: a HuGE review. *American journal of epidemiology* **156**, 885-902 (2002).
82 Vilar, E. & Gruber, S. B. Microsatellite instability in colorectal cancer-the stable evidence. *Nature reviews. Clinical oncology* **7**, 153-162, doi:10.1038/nrclinonc.2009.237 (2010).
83 Li, G. M. Mechanisms and functions of DNA mismatch repair. *Cell Res* **18**, 85-98, doi:10.1038/cr.2007.115 (2008).
84 Umar, A. *et al.* Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst* **96**, 261-268 (2004).
85 Loukola, A. *et al.* Microsatellite marker analysis in screening for hereditary nonpolyposis colorectal cancer (HNPCC). *Cancer Res* **61**, 4545-4549 (2001).
86 Kerr, J. F., Wyllie, A. H. & Currie, A. R. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Brit J Cancer* **26**, 239-257 (1972).
87 Cummings, M. C., Winterford, C. M. & Walker, N. I. Apoptosis. *Am J Surg Pathol* **21**, 88-101 (1997).
88 Huerta, S., Goulet, E. J. & Livingston, E. H. Colon cancer and apoptosis. *Am J Surg* **191**, 517-526, doi:10.1016/j.amjsurg.2005.11.009 (2006).
89 Bursch, W. The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death and Differentiation* **8**, 569-581, doi:10.1038/sj.cdd.4400852 (2001).
90 Gozuacik, D. & Kimchi, A. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* **23**, 2891-2906, doi:10.1038/sj.onc.1207521 (2004).
91 Selvakumaran, M., Amaravadi, R. K., Vasilevskaya, I. A. & O'Dwyer, P. J. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 2995-3007, doi:10.1158/1078-0432.CCR-12-1542 (2013).
92 Li, J. *et al.* Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. *Annals of surgical oncology* **16**, 761-771, doi:10.1245/s10434-008-0260-0 (2009).
93 Thyagarajan, A. *et al.* Triterpenes from Ganoderma Lucidum induce autophagy in colon cancer through the inhibition of p38 mitogen-activated kinase (p38 MAPK). *Nutrition and cancer* **62**, 630-640, doi:10.1080/01635580903532390 (2010).
94 Zhao, Y. *et al.* Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity. *Nature cell biology* **12**, 665-675, doi:10.1038/ncb2069 (2010).
95 Xiong, H. Y. *et al.* Autophagic cell death induced by 5-FU in Bax or PUMA deficient human colon cancer cell. *Cancer letters* **288**, 68-74, doi:10.1016/j.canlet.2009.06.039 (2010).
96 Yang, Z. J., Chee, C. E., Huang, S. & Sinicrope, F. A. The role of autophagy in cancer: therapeutic implications. *Molecular cancer therapeutics* **10**, 1533-1541, doi:10.1158/1535-7163.MCT-11-0047 (2011).
97 Vandenabeele, P., Galluzzi, L., Vanden Berghe, T. & Kroemer, G. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nature reviews. Molecular cell biology* **11**, 700-714, doi:10.1038/nrm2970 (2010).
98 Kroemer, G. *et al.* Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* **16**, 3-11, doi:10.1038/cdd.2008.150 (2009).

99 Ofengeim, D. & Yuan, J. Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. *Nature reviews. Molecular cell biology* **14**, 727-736, doi:10.1038/nrm3683 (2013).
100 Li, H., Kobayashi, M., Blonska, M., You, Y. & Lin, X. Ubiquitination of RIP is required for tumor necrosis factor alpha-induced NF-kappaB activation. *The Journal of biological chemistry* **281**, 13636-13643, doi:10.1074/jbc.M600620200 (2006).
101 Festjens, N., Vanden Berghe, T., Cornelis, S. & Vandenabeele, P. RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell death and differentiation* **14**, 400-410, doi:10.1038/sj.cdd.4402085 (2007).
102 Wajant, H. & Scheurich, P. TNFR1-induced activation of the classical NF-kappaB pathway. *The FEBS journal* **278**, 862-876, doi:10.1111/j.1742-4658.2011.08015.x (2011).
103 Wertz, I. E. & Dixit, V. M. Signaling to NF-kappaB: regulation by ubiquitination. *Cold Spring Harbor perspectives in biology* **2**, a003350, doi:10.1101/cshperspect.a003350 (2010).
104 Yuan, J. & Kroemer, G. Alternative cell death mechanisms in development and beyond. *Genes & development* **24**, 2592-2602, doi:10.1101/gad.1984410 (2010).
105 Green, D. R., Oberst, A., Dillon, C. P., Weinlich, R. & Salvesen, G. S. RIPK-dependent necrosis and its regulation by caspases: a mystery in five acts. *Molecular cell* **44**, 9-16, doi:10.1016/j.molcel.2011.09.003 (2011).
106 Kreuz, S. *et al.* NFkappaB activation by Fas is mediated through FADD, caspase-8, and RIP and is inhibited by FLIP. *The Journal of cell biology* **166**, 369-380, doi:10.1083/jcb.200401036 (2004).
107 Moriwaki, K. & Chan, F. K. RIP3: a molecular switch for necrosis and inflammation. *Genes Dev* **27**, 1640-1649, doi:10.1101/gad.223321.113 (2013).
108 Mitchell, R. J., Farrington, S. M., Dunlop, M. G. & Campbell, H. Mismatch repair genes hMLH1 and hMSH2 and colorectal cancer: a HuGE review. *Am J Epidemiol* **156**, 885-902 (2002).
109 Seitz, S. *et al.* Identification of microsatellite instability and mismatch repair gene mutations in breast cancer cell lines. *Genes Chromosomes & Cancer* **37**, 29-35, doi:Doi 10.1002/Gcc.10196 (2003).
110 Felsher, D. W. Cancer revoked: oncogenes as therapeutic targets. *Nature reviews. Cancer* **3**, 375-380, doi:10.1038/nrc1070 (2003).
111 Fox, K. R. Wrapping of genomic polydA.polydT tracts around nucleosome core particles. *Nucleic acids research* **20**, 1235-1242 (1992).
112 Raveh-Sadka, T. *et al.* Manipulating nucleosome disfavoring sequences allows fine-tune regulation of gene expression in yeast. *Nature genetics* **44**, 743-750, doi:10.1038/ng.2305 (2012).
113 Bacon, A. L., Dunlop, M. G. & Farrington, S. M. Hypermutability at a poly(A/T) tract in the human germline. *Nucleic acids research* **29**, 4405-4413 (2001).
114 Yuan, Z. *et al.* An A13 repeat within the 3'-untranslated region of epidermal growth factor receptor (EGFR) is frequently mutated in microsatellite instability colon cancers and is associated with increased EGFR expression. *Cancer research* **69**, 7811-7818, doi:10.1158/0008-5472.CAN-09-0986 (2009).
115 Iyer, V. & Struhl, K. Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. *The EMBO journal* **14**, 2570-2579 (1995).

116 Reardon, B. J., Winters, R. S., Gordon, D. & Winter, E. A peptide motif that recognizes A.T tracts in DNA. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 11327-11331 (1993).
117 Agrawal, S. *et al.* Colorectal cancer in African Americans. *The American journal of gastroenterology* **100**, 515-523; discussion 514, doi:10.1111/j.1572-0241.2005.41829.x (2005).
118 Weinstein, I. B. & Joe, A. Oncogene addiction. *Cancer research* **68**, 3077-3080; discussion 3080, doi:10.1158/0008-5472.CAN-07-3293 (2008).
119 Pelengaris, S., Khan, M. & Evan, G. I. Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. *Cell* **109**, 321-334 (2002).
120 Siegfried, J. M. *et al.* Association of immunoreactive hepatocyte growth factor with poor survival in resectable non-small cell lung cancer. *Cancer Res* **57**, 433-439 (1997).
121 Wang, X. *et al.* Potent and selective inhibitors of the Met [hepatocyte growth factor/scatter factor (HGF/SF) receptor] tyrosine kinase block HGF/SF-induced tumor cell growth and invasion. *Molecular cancer therapeutics* **2**, 1085-1092 (2003).
122 Raucci, A., Palumbo, R. & Bianchi, M. E. HMGB1: a signal of necrosis. *Autoimmunity* **40**, 285-289, doi:10.1080/08916930701356978 (2007).
123 Degterev, A. *et al.* Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nature Chemical Biology* **4**, 313-321, doi:Doi 10.1038/Nchembio.83 (2008).
124 Vandenabeele, P., Declercq, W., Van Herreweghe, F. & Vanden Berghe, T. The Role of the Kinases RIP1 and RIP3 in TNF-Induced Necrosis. *Science signaling* **3**, doi:ARTN re4 DOI 10.1126/scisignal.3115re4 (2010).
125 Fafalios, A. *et al.* A hepatocyte growth factor receptor (Met)-insulin receptor hybrid governs hepatic glucose metabolism. *Nature medicine* **17**, 1577-1584, doi:10.1038/nm.2531 (2011).
126 Kulathu, Y. & Komander, D. Atypical ubiquitylation - the unexplored world of polyubiquitin beyond Lys48 and Lys63 linkages. *Nature reviews. Molecular cell biology* **13**, 508-523, doi:10.1038/nrm3394 (2012).
127 Mohapatra, B. *et al.* Protein tyrosine kinase regulation by ubiquitination: critical roles of Cbl-family ubiquitin ligases. *Biochimica et biophysica acta* **1833**, 122-139, doi:10.1016/j.bbamcr.2012.10.010 (2013).
128 Jemal, A., Siegel, R., Xu, J. & Ward, E. Cancer statistics, 2010. *CA: a cancer journal for clinicians* **60**, 277-300, doi:10.3322/caac.20073 (2010).
129 Grady, W. M. & Carethers, J. M. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* **135**, 1079-1099, doi:10.1053/j.gastro.2008.07.076 (2008).
130 Wong, W. W. L. *et al.* RIPK1 is not essential for TNFR1-induced activation of NF-kappa B. *Cell Death and Differentiation* **17**, 482-487, doi:Doi 10.1038/Cdd.2009.178 (2010).
131 de Bie, P. & Ciechanover, A. Ubiquitination of E3 ligases: self-regulation of the ubiquitin system via proteolytic and non-proteolytic mechanisms. *Cell Death and Differentiation* **18**, 1393-1402, doi:10.1038/cdd.2011.16 (2011).
132 Wang, X. *et al.* A mechanism of cell survival: sequestration of Fas by the HGF receptor Met. *Molecular cell* **9**, 411-421 (2002).
133 Zou, C. *et al.* Lack of Fas antagonism by Met in human fatty liver disease. *Nature medicine* **13**, 1078-1085, doi:10.1038/nm1625 (2007).

134 Ma, J. *et al.* A novel death defying domain in met entraps the active site of caspase-3 and blocks apoptosis in hepatocytes. *Hepatology*, doi:10.1002/hep.26769 (2013).
135 Nehs, M. A. *et al.* Necroptosis is a novel mechanism of radiation-induced cell death in anaplastic thyroid and adrenocortical cancers. *Surgery* **150**, 1032-1039, doi:10.1016/j.surg.2011.09.012 (2011).
136 Munshi, N. *et al.* ARQ 197, a Novel and Selective Inhibitor of the Human c-Met Receptor Tyrosine Kinase with Antitumor Activity. *Molecular Cancer Therapeutics* **9**, 1544-1553, doi:Doi 10.1158/1535-7163.Mct-09-1173 (2010).
137 Lohr, J. W., Lee, T. P., Farooqui, M. & Mookerjee, B. K. Increased levels of serum hepatocyte growth factor in patients with end-stage renal disease. *J Med* **31**, 131-141 (2000).
138 Koehler, B. C., Jager, D. & Schulze-Bergkamen, H. Targeting cell death signaling in colorectal cancer: Current strategies and future perspectives. *World journal of gastroenterology : WJG* **20**, 1923-1934, doi:10.3748/wjg.v20.i8.1923 (2014).
139 Gunther, C., Neumann, H., Neurath, M. F. & Becker, C. Apoptosis, necrosis and necroptosis: cell death regulation in the intestinal epithelium. *Gut* **62**, 1062-1071, doi:10.1136/gutjnl-2011-301364 (2013).
140 Li, F., Zhao, C. & Wang, L. Molecular-targeted agents combination therapy for cancer: developments and potentials. *International journal of cancer. Journal international du cancer* **134**, 1257-1269, doi:10.1002/ijc.28261 (2014).
141 Kaelin, W. G., Jr. The concept of synthetic lethality in the context of anticancer therapy. *Nature reviews. Cancer* **5**, 689-698, doi:10.1038/nrc1691 (2005).
142 Raoul, J. L. *et al.* Cetuximab in combination with irinotecan/5-fluorouracil/folinic acid (FOLFIRI) in the initial treatment of metastatic colorectal cancer: a multicentre two-part phase I/II study. *BMC cancer* **9**, 112, doi:10.1186/1471-2407-9-112 (2009).