The Texas Medical Center Library
DigitalCommons@TMC

Dissertations and Theses (Open Access)

MD Anderson UTHealth Houston Graduate School

12-2021

Lgr5 Regulation Of Stat3 Signaling And Drug Resistance In Colorectal Cancer

Tressie Posey

Tressie Alexandra Posey

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations

 Part of the Cancer Biology Commons, Cell Biology Commons, Digestive System Diseases Commons, Medical Cell Biology Commons, and the Therapeutics Commons

Recommended Citation

Posey, Tressie and Posey, Tressie Alexandra, "Lgr5 Regulation Of Stat3 Signaling And Drug Resistance In Colorectal Cancer" (2021). *Dissertations and Theses (Open Access)*. 1151.
https://digitalcommons.library.tmc.edu/utgsbs_dissertations/1151

This Thesis (MS) is brought to you for free and open access by the MD Anderson UTHealth Houston Graduate School at DigitalCommons@TMC. It has been accepted for inclusion in Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact digcommons@library.tmc.edu.

LGR5 Regulation of STAT3 Signaling and Drug Resistance in Colorectal Cancer

by

Tressie Alexandra Capri Posey B.S.

APPROVED:

_____
Kendra Carmon, Ph.D.
Advisory Professor

_____
Qingyun (Jim) Liu, Ph.D.

_____
Ali Azhdarinia, Ph.D.

_____
Eduardo Vilar-Sanchez, M.D. Ph.D.

_____
Venkata Lokesh Battula, Ph.D.

APPROVED:

_____
Dean, The University of Texas
MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

LGR5 Regulation of STAT3 Signaling and Drug Resistance in Colorectal Cancer

A

Thesis

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science

by

Tressie Alexandra Capri Posey B.S.
Houston, Texas

December 2021

Acknowledgements

I want to first thank those in my committee, Dr. Liu, Dr. Azhdarnia, Dr. Evilar-Sanchez, and Dr. Battula who have provided their time and guidance throughout my master's degree. This project would not be what it is without their insightful feedback. I also want to extend a special thanks to Dr. Park who was willing to act as a committee member last minute and without whom I would not have been able to defend this year. I also want to thank the academic faculty who had to work on their feet during the COVID-19 pandemic to keep our class afloat through what could only be describe as unprecedented. I also want to thank my partner Grant for his patients, love, and ability to cook when I definitely can't and our two, very large, cats Tim and Penny for their lap cuddles during my late-night studying.

When I started working in Dr. Carmon's lab there were only three of us, and a medical student Treena, I couldn't have predicted how quickly our lab would have grown in what seems like such a short time. But now I can't imagine what it would be without my other lab mates. I want to thank Joan, Liezl, Zhengdong, and Sheng for being a part of such a warm and caring group. I am truly going to miss the birthdays, the holiday celebrations and decorating the lab with streamers and window decals. I want to thank Joan for her friendship and for every time she put my media away when I left it out. I can't express my appreciation for the time we spent together. And finally, I want to thank my advisor Dr. Kendra Carmon for not only her support and guidance but for all of the times we have laughed together these past couple of years. I could not have asked for a more understanding, caring mentor and friend. I would not have made it this far without her help; I will truly miss working with her after graduation.

Yours truly – sus.

LGR5 Regulation of STAT3 Signaling and Drug Resistance in Colorectal Cancer

Tressie Alexandra Capri Posey B.S.

Advisory Professor: Kendra Carmon, Ph.D.

The greatest difficulty in treating colorectal cancer (CRC) is the development of drug resistance which leads to relapse after treatment and progression to metastasis. Cancer stem cells (CSCs) are believed to drive relapse because of their capacity to self-renew, acquire resistance mechanisms, and differentiate promoting tumor growth and heterogeneity. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), is a bona-fide marker of CSCs and has been considered a viable target for CSC specific therapeutic development. While we showed targeting LGR5 with antibody-drug conjugates (ADCs) led to tumor regression in CRC xenografts, once treatment was ceased the tumor ultimately relapsed, possibly due to the reemergence of LGR5+ CSCs. Recent studies have shown that LGR5+ CSCs undergo plasticity converting from an LGR5+ state to a more chemo-resistant LGR5- state which can lead to relapse. Recently, we reported that loss of LGR5 expression, through shRNA knockdown (KD) or CRISPR knockout (KO) in multiple CRC cell lines, increased both chemotherapy resistance and proliferation, however the complete mechanisms by which this occurs is unknown. Through comprehensive western analysis, we discovered that loss of LGR5 expression increased phosphorylation of STAT3(Y705) and MET(Y1234/1235). Interestingly, we also found that LGR5+ CRC cells treated with irinotecan or LGR5-targeted ADCs, converted these cells to an LGR5- state with increased MET/STAT3 activation. LGR5 overexpression in a CRC cell line which doesn't endogenously express LGR5, resulted in decreased phosphorylation of MET/STAT3 and enhanced sensitivity to chemotherapy. We next showed that LGR5- CRC cells are dependent on MET/STAT3 signaling for their acquired

drug resistance and blockade of this pathway through chemical inhibition can be used to enhance chemotherapy and ADC efficacy in vitro. Also, combination treatment with STAT3 inhibitor, stattic, and irinotecan in vivo resulted in increased tumor inhibition and survival compared to either monotherapy alone. Our findings suggest that loss of LGR5 expression in CRC cells promotes drug resistance, at least in part, through the activation of MET/STAT3 signaling and thus inhibition of STAT3 activity may restore drug sensitivity of LGR5- CRC cells. Co-targeting LGR5+ CSCs along with molecular mechanisms that drive drug resistance and cancer cell plasticity may be essential for a more effective CRC treatment.

Table of Contents

List of Figures

List of Abbreviations

ADC – Antibody-Drug Conjugate

APC – Adenomatous Polyposis Coli

CBC – Crypto Basal Columnar

CRC – Colorectal Cancer

CSC – Cancer Stem Cells

LGR5 – Leucine-rich Repeat Containing G Protein-Coupled Receptor 5

ECD – Extracellular Domain

EGFR – Epidermal Growth Factor Receptor

FAP – Familial Adenomatous Polyposis

FOLFOX – Folinic acid, 5-Fluorouracil, Oxaliplatin

FOLFIRI - Folinic acid, 5-Fluorouracil, Irinotecan

FOFOXFIRI - Folinic acid, 5-Fluorouracil, Oxaliplatin, Irinotecan

5-FU - 5-Fluorouracil

FZD – Frizzled

GPCR – G Protein Coupled Receptors

Gp130 – Glycoprotein 130

HGF – Hepatocyte Growth Factor

IL-6R – Interleukin-6 Receptor

IQGAP1 – IQ Motif Containing GTPase Activating Protein 1

JAK – Janus Kinase

LPR5/6 – Low-density Lipoprotein Receptor-related Protein 5/6

mAbs – Monoclonal Antibodies

MAPK – Mitogen Activated Protein Kinase

MET – Mesenchymal Epithelial Transition/HG Receptor

MSI – Microsatellite Instability

MSS – Microsatellite Stability

RAC1 – Ras-related C3 Botulinum Toxin Substrate 1

RNF43 – Ring Finger protein 43

RSPO - R-Spondin

STAT3 – Signal Transducer and activator of Transcription 3

WNT – Wingless/Integrated

VEGF – Vascular Endothelial Growth Factor

ZNFR3 – Zinc and Ring Finger 3

CHAPTER 1: INTRODUCTION

1.1 Colorectal Cancer (CRC) Disease Impact

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and third most common cause of cancer-related death globally, accounting for 10% and 9.4% respectively[1]. In the United States alone, as of 2019 around 1.6 million men and women are currently living with a history of CRC, an additional 150,000 diagnosed, and an estimated 50,000 patients succumbed to the disease in 2020[2]. With increasing incidence rates of CRC, the global burden is expected to increase 60% over the next decade, culminating in more than 2.2 million new cases and an estimated 1.1 million deaths by 2030[3].

Diagnosing CRC early is critical, as patients with early-stage diagnoses have an overall 5-year survival rate of up to 90%. However, the majority of CRC patients diagnosed with advanced or later stage CRC only have an estimated 5-year survival rate between 10%-14%[1,2]. While CRC surveillance through colonoscopy screening traditionally began at the age of 50, the growing incidence rate among patients between the ages of 20-49 recently prompted the U.S. Preventative Services Task Force to lower the age of initial screening to 45 in an effort to improve outcomes through early detection[4–6].

As late diagnosis is associated with advanced stages of CRC and poor prognosis, the delay in non-urgent medical procedures due to the COVID-19 pandemic has many physicians, patient advocates, and CRC researchers concerned. According to an early estimate, CRC screenings have dropped by up to 86%; this delay in regular CRC screenings, especially in medically underserved communities, could have a significant downstream impact on patient outcomes[7]. While it cannot be determined how long it will take to return to pre-pandemic

screening levels or how great of an impact on public health this may cause, an increase in the diagnosis of more advanced CRCs can be expected in time[8].

1.2 CRC Progression and Treatment

While the majority of CRCs arise through sporadic appearance (70-80%), a small portion is due to inherited genetic risk factors such as familial adenomatous polyposis (FAP), Lynch Syndrome, or MYH-gene associated polyposis[9]. Once initiated, CRC develops through a gradual, stepwise accumulation of genetic alterations which transform normal colonic mucosa, to a polyp, which is followed by tumor progression, and eventually metastatic disease (Fig. 1)[10]. This initial adenoma is most associated with mutations of Adenomatous Polyposis Coli (*APC*), which can be inherited, as is seen in FAP, or acquired through somatic/sporadic occurrence[11]. *APC* mutations lead to deregulation of the canonical Wnt/β-catenin signaling pathway, resulting in enhanced transcription of target genes which causes an increase in cell growth and proliferation[11,12]. After initiation through *APC* mutation, subsequent mutations such *KRAS* and *BRAF* which act as oncogenic drivers continue to promote cellular expansion of the tumor[13]. Eventually, mutations in *PI3KCA*, *SMAD4*, *TP53*, and other genes can arise, generating malignant carcinoma behavior and leading to invasion and metastasis[10,14]. CRC can also be classified into two major groups, microsatellite instability (MSI) where CRC cells are hypermutated due to defective mismatch repair which accounts for ~16% of cases, or non-hypermutated microsatellite stable (MSS) which account for ~84% of cases. These factors can have implication on adjuvant treatment decisions[15].

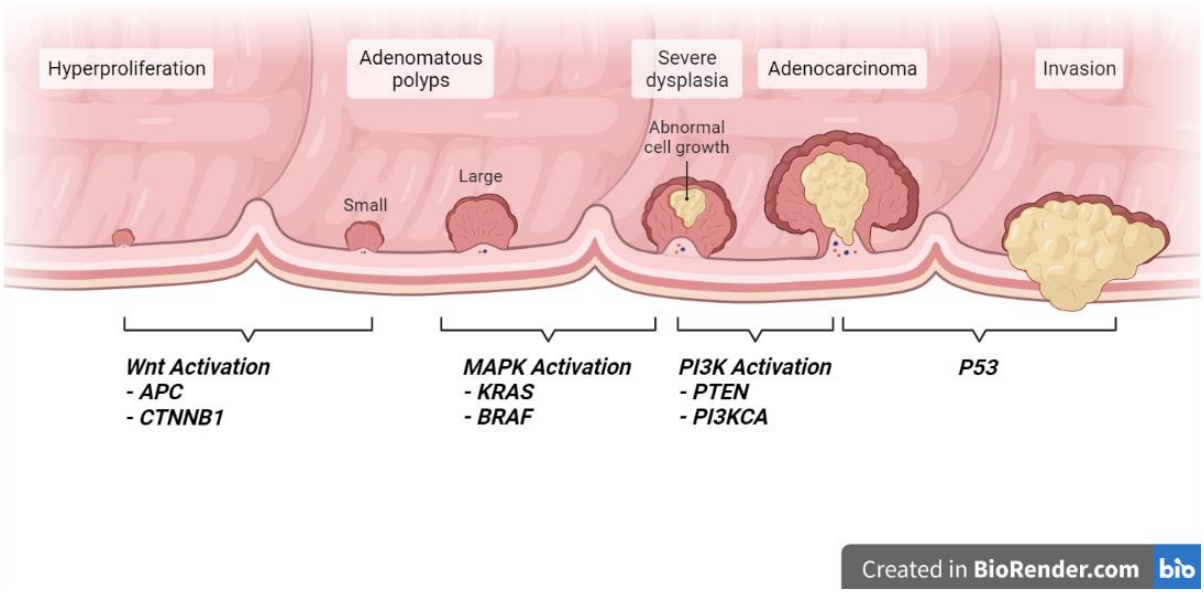


Figure 1: Adenoma to Carcinoma Sequence. Colorectal cancer progresses initially through the formation of a benign adenoma brought on through sporadic or inherited oncogenic mutations, primarily loss of functional APC. As the polyp develops, cells acquire aberrant mutations such as KRAS, SMAD4, PTEN, and p53, gradually increasing in malignancy and developing into an adenocarcinoma, followed by metastasis. (Adapted from "Benign and Malignant Colon Cancer", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates)

CRC staging is classified by location and depth of invasion (T stage), presentation in lymph nodes (N stage), and the presence of distal metastasis (M stage); staging is then used for therapeutic decisions[16]. Based on the American Joint Committee on Cancer (AJCC): stage 1, CRC has not grown or invaded the inner mucosa layer of the colon nor has spread to the neighboring lymph nodes; in stage 2, the tumor has grown into the outermost layer but may or may not have grown through the mucosa layer; in stage 3, the cancer has grown through the mucosa layer and has spread to nearby lymph nodes; and at stage 4, the cancer has spread to at least one distant organ or other parts of the abdominal cavity[17]. The CRC adenoma-carcinoma sequence can take between 10-15 years before neoplastic transformation leads to the progression of invasive carcinoma[18].

For stage 1-2 CRC, patients undergo surgical resection of the cancer and nearby lymph nodes. Depending on how advanced the disease is, or if the tumor is obstructing function, surgery is performed laparoscopically or through complete colonic resection. Surgery may also be followed by adjuvant chemotherapy, however its use at such an early stage is considered controversial[19,20]. In more advanced stage 2 or stage 3 CRC, surgery is still the primary form of treatment, but it is performed with pre- and/or post-operative chemotherapy to reduce incidences of local recurrence[21,22]. Conventional chemotherapy is based on 5-fluorouracil (5-FU), given as a singular therapy or in combination with other chemotherapeutics[23,24]. 5-FU is a variation of the metabolite uracil with a fluorine substitution for hydrogen at the carbon-5 position. Due to the tumor's preferential uptake of uracil for rapid generation of RNA, 5-FU functions as an inhibitor of RNA transcription[25]. In combination, 5-FU is administered with folinic acid (leucovorin, FOL) and either oxaliplatin (FOLFOX), which forms DNA adducts through its platinum complex, or irinotecan (FOLFIRI), which inhibits topoisomerase 1 halting replication and transcription, or both (FOLFOXFIRI)[26,27]. Because of the metastatic nature invading of both lymph nodes and distant organs, stage 4 CRC is often considered incurable with an average overall 5-year survival of 10-14%[1,2,28]. Stage 4 is treated with 5-FU combination therapies (FOLFOX/FOLFIRI/FOLFOXFIRI), and more recently with the inclusion of monoclonal antibodies (mAbs), such as Bevacizumab that directly targets vascular endothelial growth factor (VEGF) and Cetuximab that targets epidermal growth factor receptor (EGFR), which extend overall survival[29,30].

1.3 Challenges and Drug Resistance

The most prevalent obstacles in treating CRC is therapeutic resistance and relapse. In fact, 30-40% of total patients develop recurrent disease, ranging from 5% in stage 1, to 60% in stage 4, with around 70% of all recurrent disease observed within 2 years post-treatment[2,3]. Recurrent disease often presents as distant metastasis occurring in the liver and lungs and with increased malignancy[31,32]. This is due to nearly half of all metastatic CRC developing resistance to 5-FU based chemotherapies, like FOLFIRI and FOLFOX, through multiple drug resistant mechanisms[33,34]. Chemotherapy resistance has led to the inclusion of therapeutic mAbs (i.e., Bevacizumab and Cetuximab) as treatment options to improve response rate[35,36]. Unfortunately, the potential benefit of targeted therapy is only limited to a few months, as the tumor can develop secondary resistance mechanisms to evade direct targeting of EGFR and VEGF[2,37,38]. Furthermore, cetuximab has only shown to have potential benefit in the fraction of CRC patients with wild-type *KRAS* and *BRAF*[39]. In addition, checkpoint inhibitor immunotherapies (i.e., anti-CTLA4/PD1/PDL1), which have been proven to be effective against other types of cancers, have only shown to be effective in a subset of microsatellite instability-high (MSI-H)/mismatch repair deficient metastatic CRC patients (~16%), whereas the remainder are microsatellite stable (MSS)[39,40]. In consequence, it is critical to develop new therapeutics to improve the treatment of late-stage CRC.

1.4 Colorectal Cancer Stem Cells

Drug resistance in CRC is closely related to its immense heterogeneity at both the intertumoral and intratumoral levels. Tumors can be composed of a variety of different molecularly defined subgroups which can determine therapeutic response[41]. Cancer stem cells (CSCs) are a small sub-population within the tumor architecture that exhibit pluripotent stem cell characteristics, able to self-renew and give rise to the different cancer cells which make-up the tumor. CSCs are also believed to drive drug resistance, tumor initiation, growth, and likely play a role in relapse and metastasis (Fig. 2)[42–44].

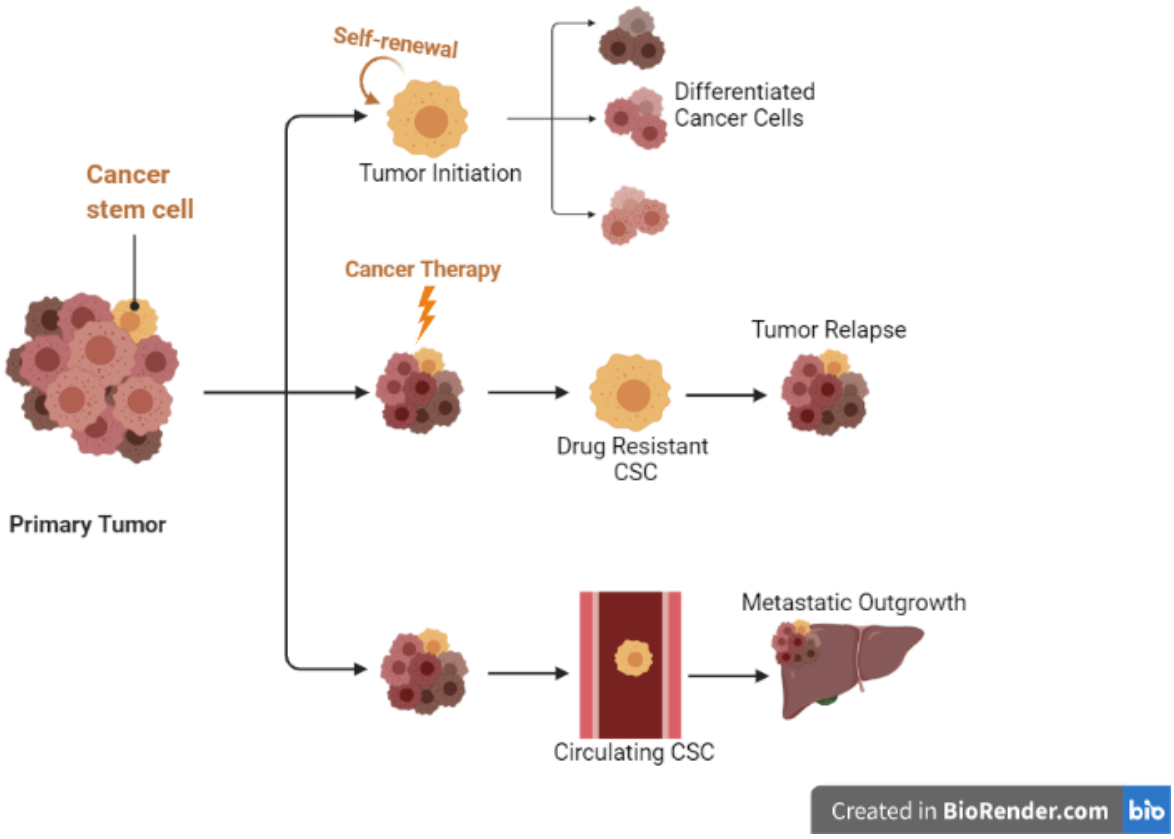


Figure 2: The Role of Cancer Stem Cells. Cancer stem cells (CSC) have the capability to self-renew and generate differentiated daughter cells to initiate and maintain tumor outgrowth. CSCs are also drug resistant to standard cancer therapies such as radiation and chemotherapy (5-FU and irinotecan). CSCs also comprise a portion of circulating cancer cells which can initiate metastatic outgrowth. (Adapted from "Stochastic vs Cancer Stem Cell Models", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.)

The traditional theory of how CSC arise depicts oncogenic mutations occuring in normal adult stem cells resulting in tumor initiation. This theory presuming CSCs are the only population with tumorigenic potential, however this linear progression has been directly countered by the observation of mature cells de-differentiating into a stem-like state in order to recapitulate the CSC population[42,45,46]. Once present, CSCs are capable of asymmetrically dividing to produce identical daughter cells and differentiated tumor cells which are required to maintain tumor growth[45]. CSCs and their progeny can contain a surprising amount of genetic variability; they have been also shown to exhibit high expression of multiple drug resistant proteins, proteins involved in DNA repair, and upregulation of signaling pathways which promotes genetic variability leading to increased tumor heterogeneity[47]. CSC genetic variability works in deadly combination with their slow cycling nature, evading standard chemotherapies which tend to target rapidly proliferating cells[46]. This has led many researchers to investigate possible CSC targeting therapies to eliminate the tumorigenic potential at its source.

1.5 LGR5 as a Marker of Normal and Cancer Stem Cells

Leucine-rich repeat containing G protein-coupled receptor (LGR5) has been validated as a marker for normal adult intestinal stem cells, expressed by the cycling crypt basal columnar (CBC) stem cells restricted to the bottom of crypts in the gastrointestinal tract[48,49]. These LGR5+ stem cells, asymmetrically divide and differentiate into all colonic epithelium lineages and replenish the transit-amplifying region every 4-5 days[49,50]. LGR5 has also been validated as a stem cell marker for normal adult stem cells within the liver, skin, stomach, and ovarian epithelium[51–54].

LGR5 is a bona fide marker for colorectal CSCs and is highly expressed in approximately 60-70% of CRC [55–58]. High LGR5 expression has been observed in every step of the adenoma to carcinoma progression of CRC, including distant metastasis[59,60]. Despite its high expression in CRC, there have been conflicting reports on its prognostic value. While some correlation has been found with LGR5 expression and tumor progression, advanced stage, and unfavorable prognosis; conflicting evidence have shown no true correlation with tumor aggressiveness[61–63].

1.6 LGR5 Function in Wnt Signaling and Interaction with IQGAP1

LGR5 is a seven transmembrane protein in the Rhodopsin-like family of G protein coupled receptors (GPCR) with a large extracellular domain (ECD) that contains 17 leucine-rich repeat sequences[64]. LGR5 has been established as a receptor for R-Spondin ligands, (RSPO1-4), and shown to interact with the Wnt receptors Frizzled (Fzd) and Low-density lipoprotein receptor-related protein 5/6 (LRP5/6). These proteins work concomitantly to modulate Wnt/β-catenin signaling to dysregulate the destruction complex which is composed of APC, Disheveled, Axin, CLIα, and GSK-3β, preventing this complex from marking β-catenin for degradation (Fig. 3)[65–69]. Along with being a modulator of the Wnt pathway, LGR5 is also a target gene for β-catenin transcription[70]. However, the complete mechanism and its role in modulating Wnt/β-catenin, or other signaling pathways remains somewhat unclear.

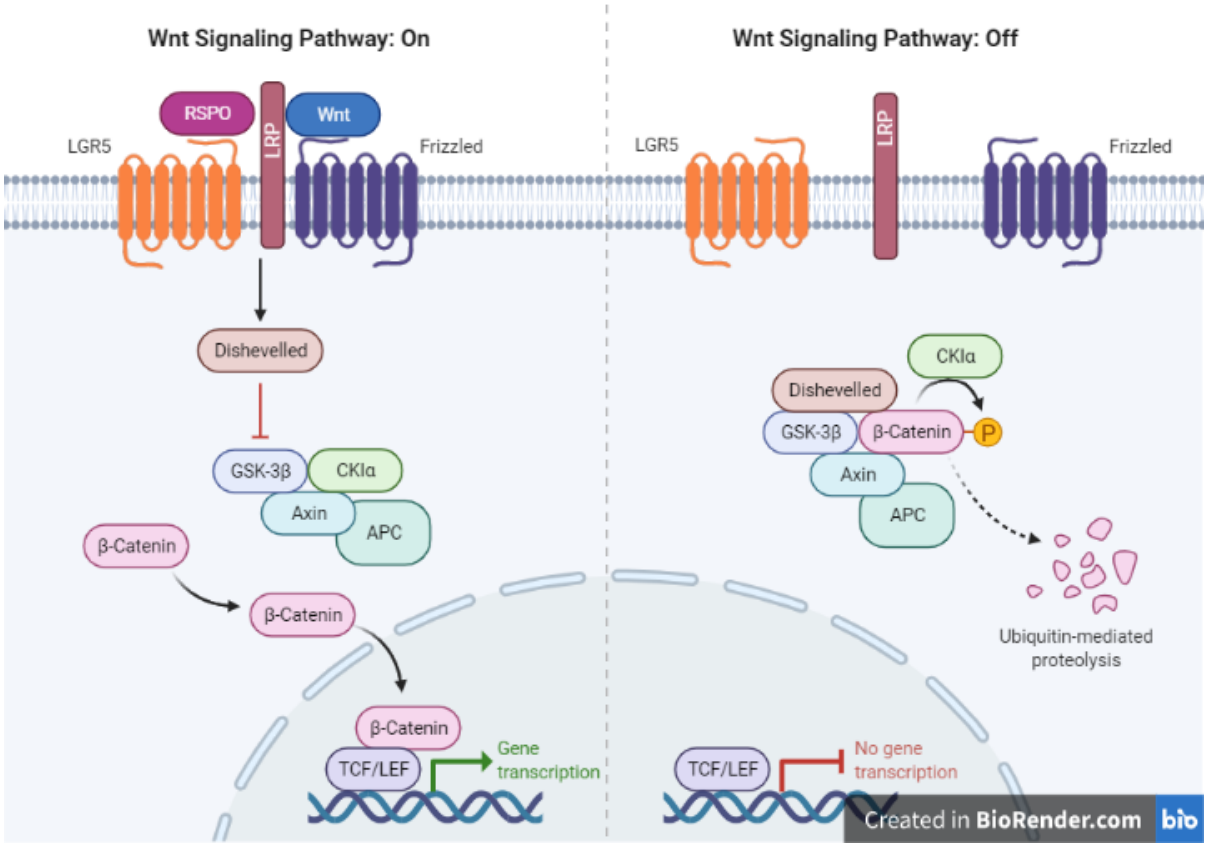


Figure 3: LGR5 modulates the Wnt Pathway. RSPO bound LGR5 interacts with Lrp5/6 and Wnt bound Frizzled, the receptor complex inhibits the destruction complex, stabilizing β-catenin. Activated β-catenin then translocates to the nucleus and engages in transcriptional activity. (Adapted from "Wnt Signaling Pathway Activation and Inhibition", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates)

Some findings suggest that the LGR5/RSPO complex promotes Wnt/β-catenin signaling by sequestering E3 ubiquitin ligases Ring finger protein 43 (Rnf43), and Zinc and Ring Finger 3 (ZNRF3), which negatively regulates Frizzled by inducing receptor degradation[70]. Despite the substantial evidence for the interaction between the related receptor LGR4 and RNF43/ZNFR3, there has been no direct evidence that RSPO-bound LGR5 directly interacts with these E3 ubiquitin ligases[71]. RSPO bound LGR5 has been proven to interact with Wnt receptors LRP6 and Fzd5, forming a signalosome, and is rapidly and constitutively internalized in a Dynamin and Clathrin dependent manner. LGR5 likely stabilizes the co-receptors within the signalosome to potentiate Wnt/β-catenin signaling[68,71,72]. However,

LGR5's role in Wnt activation may also be context dependent, as the silencing of LGR5 was found to be associated with the increased activation of the Wnt pathway and upregulation of several Wnt target genes[73–75]. This suggests that LGR5 may also play a suppressor role in Wnt signaling.

Aside from modulation of canonical Wnt/β-catenin signaling, our lab showed that LGR5 can play a role in altering the actin cytoskeleton structure to increase cell-cell adhesion, independent of RSPO binding[73]. Our lab published findings that depict LGR5's interaction with the intracellular scaffold signaling protein IQ Motif Containing GTPase Activating Protein 1 (IQGAP1). IQGAP1 has been shown to coordinate with various other receptors, such as EGFR and MET, to regulate tumorigenesis, actin cytoskeleton dynamics, adhesion, migration, and other cellular processes[76–79]. When bound to LGR5, IQGAP1 is dephosphorylated leading to increased binding to Ras-related C3 botulinum toxin substrate 1 (Rac1)[73]. Once bound to Rac1, IQGAP1 is likely unable to interact with β-Catenin, leading to increased E-cadherin-β-catenin-α-catenin complex formation and F-actin cross-linking and ultimately stronger cell-cell adhesion[80,81]. This LGR5-IQGAP1 interaction posits an alternative functional role for LGR5 by which it promotes the retention of normal stem cells within their intestinal niche and strengthens the adhesion properties of colorectal CSCs.

1.7 Therapeutic Targeting of LGR5+ CSCs

Since CSCs are able to evade chemotherapy and radiation, the direct targeting of CSC markers has become a next step in improving the treatment of CRC[82]. Antibody-drug conjugates (ADC) are mAbs which have been chemically linked to potent cytotoxic agents[83]. ADCs can be constructed to specifically target a tumor antigen, such as a CSC marker, destroying tumor cells while circumventing damage to normal healthy tissues[84]. LGR5 has been identified as an ideal candidate for ADC targeted therapy, due to its high expression in CRC relative to low expression in healthy tissue and its rapid initialization which can shuttle the ADC into CRC cells[57,68]. Our lab has previously generated two LGR5-targeting ADCs linked with the antimitotic, tubulin-inhibiting agent monomethyl auristatin E (MMAE)[57]. One ADC was composed of a chemical linker that could be cleaved by lysosomal enzymes to release the drug into the cell, whereas the other ADC consisted of a non-cleavable linker which was dependent upon proteolytic degradation for drug release[57]. It was found that in xenograft mouse models of CRC that the ADC with the cleavable linker anti-LGR5-mc-vc-PAB-MMAE, effectively eliminated high LGR5 expressing tumors[57]. In addition to our study, another group demonstrated that ADCs targeting LGR5 suppressed LGR5+ tumor growth in xenografts and genetically engineered mouse models and were also well tolerated[85]. However, our study showed that tumors eventually reemerged when ADC treatment was ceased. This suggests that a subpopulation of LGR5+ CSCs may have interconverted to an LGR5- with a resistant phenotype that was able to eventually re-initiate tumor growth, this process of interconversion is known as stem cell plasticity[57].

1.8 LGR5+ CSC Plasticity in Treatment and Progression

While the traditional CSC model depicts a small subpopulation of self-renewing quiescent cells which are likely responsible for tumor initiation and maintaining tumor growth, this does not account for the reemergence of stem cells after treatment with CSC-targeted therapy. These observations propose that daughter stem cells, including those that have differentiated, may de-differentiate in order to maintain tumor by replenishing the stem cell population[86,87]. This process is referred to as cell plasticity and has been observed in healthy intestinal tissue where committed progenitors of both secretory and enterocyte lineages take on a pluripotent phenotype to reconstitute lost LGR5+ CBCs after damage; this same phenomenon is believed to occur in CRC[88,89].

In fact, CSC plasticity has been demonstrated in xenograft models of human CRC, as selective ablation of LGR5+ CSCs restricted primary tumor growth but failed at tumor regression. The LGR5- CRC cells proliferating and maintaining the tumor, were shown to eventually replenish the LGR5+ CSC pool leading to rapid re-initiation of tumor growth [43,90]. LGR5+ CSCs have also been shown to interconvert to an LGR5- state in response to both chemo- and radio- therapy, and once treatment was ceased, these cells regained LGR5 expression[91,92]. LGR5- CRC cells still maintained a number of other stem cell markers and were shown to have increased drug resistance to 5-FU, oxaliplatin, irinotecan and were also more radio resistant. Additionally, our group showed that shRNA knockdown or CRISPR knockout of LGR5 in CRC cells promoted a more chemo-resistant phenotype[93]. As mentioned previously, we observed cancer cell plasticity by selective targeting of LGR5+ CRC cells with our anti-LGR5 ADC, which did lead to tumor size regression, yet LGR5-low/negative tumors re-emerged in a fraction of the mice once treatment was ceased[57].

Loss of LGR5 along with CRC plasticity may potentially be an important step in metastatic progression of CRC. A recent study by Fumagalli et. al. demonstrated that not only were the majority of disseminated CRC cells in circulation lacking LGR5 expression, but these LGR5- cells were also responsible for seeding and initiation of metastases[94]. Once seeded the LGR5- CRC cells reacquired LGR5 expression which drove proliferation and metastatic outgrowth[90,94]. This suggests that co-targeting LGR5 along with the molecular mechanism(s) that drive CRC plasticity or drug resistance may be essential to successfully treat CRC and prevent progression and relapse.

1.9 STAT3 activation

Signal transducer and activator of transcription 3 (STAT3) is one of seven in the STAT protein family, this family of proteins act as both cytoplasmic signaling proteins and nuclear transcription factors for gene activation[95]. STAT proteins receive and transmit cytoplasmic signaling from tyrosine-kinase receptor activated by either cytokines or growth factors[95,96]. Once STAT3 is activated by phosphorylation on critical tyrosine residues (i.e., Tyr 705), phosphorylated STAT3 dimerizes head-to-tail and translocates to the nucleus (Fig. 4)[97]. Dimerized STAT3 is trafficked to the perinuclear compartment by a signalosome, ensuring efficient nuclear accumulation[98]. Once in the nucleus, STAT3 binds to a promoter region and activates transcription of a number of target genes involved in cellular proliferation (e.g. Cyclin D1), survival (e.g. Bcl-xL), differentiation, and cancer stem cell expansion[99]. STAT3 has been shown to be one of the most important STAT proteins in cancer and its progression, this is due to the overexpression of a number of its upstream modulators such as Interleukin-6 receptor (IL-6R), EGFR, and/or c-MET.

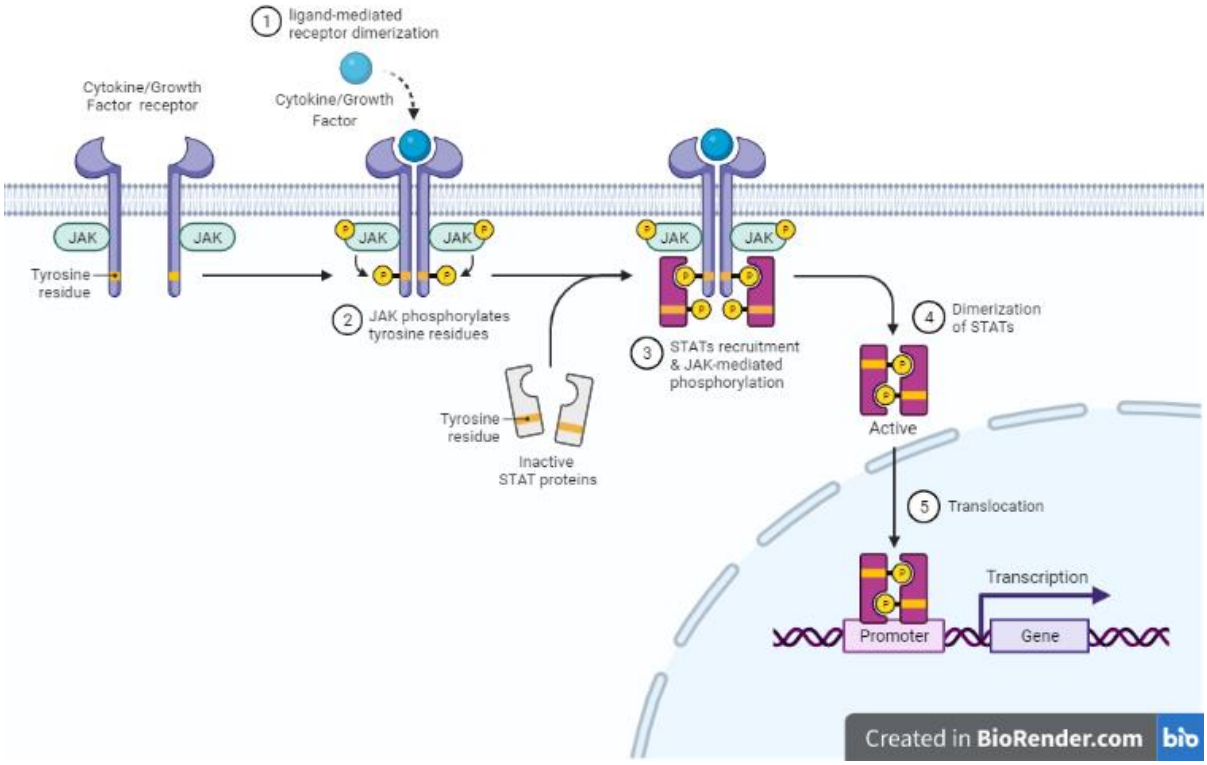


Figure 4: STAT3 Activation by Cytokine or Growth Factor Receptors. A variety of cell membrane receptors, such as interleukin-6 receptor (IL-6R), Epidermal Growth Factor Receptor (EGFR) and HGF/Scatter Factor Receptor (MET) activate Signal Transducer and Activator of Transcription 3 (STAT3) mediated through Janus-Kinase (JAK). Phosphorylated STAT3 dimerizes resulting in translocation to the nucleus where it binds to the promoter region to induce transcription of various genes that mediate cellular processes. (Adapted from "Cytokine Signaling through the JAK-STAT Pathway", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates)

The IL-6/Janus kinase (JAK)/ STAT3 pathway is believed to be a key component in CRC tumorigenesis as IL-6 production has been found elevated in both CRC tissue and patient serum[100]. When IL-6 binds to IL-6R it complexes with Glycoprotein 130 (Gp130), which subsequently phosphorylates JAK leading to STAT3 activation through phosphorylation at its tyrosine 705 position[101]. While Gp130 is ubiquitously expressed in most cells, IL-6R is exclusively expressed in certain cells such as hepatocytes and leukocytes restricting signaling. However, a soluble form of IL-6R exists which can circulate and interact with Gp130 allowing IL-6 to broaden its target cells including epithelial cells to enhance signaling[102].

EGFR is another upstream activator of STAT3 with aberrant expression in CRC cancer and is currently a therapeutic target in late-stage CRC[103,104]. EGFR is a transmembrane receptor tyrosine kinase which is primarily responsible for the activation of mitogen activated protein kinase (MAPK) pathway. MAPK triggers multiple phosphorylation cascades such as the Ras-Raf-Mek-Erk activation and dysregulation of this pathway leads to malignant transformation, tumor progression, proliferation, along with invasion and metastasis[13,105]. In addition to activating MAPK, EGFR has been shown to activate STAT3 through the phosphorylation of JAK or through direct phosphorylation of STAT3[106].

Hepatocyte growth factor/scatter factor (HGF) and its receptor (HGFR) or (mesenchymal epithelial transition receptor) MET also participates in the activation of STAT3. Like EGFR, MET is a transmembrane tyrosine kinase receptor which is overexpressed in CRC and associated with worse survival outcomes[107]. MET expression has been shown to progressively increase with the development and progression of CRC and is especially high in distal metastasis[108–110]. Once bound, HGF dimerizes MET leading to autophosphorylation of its tyrosine residues which act as a docking site for multiple substrates including STAT3[111].

1.10 STAT3 function in CRC and CSCs

STAT3 expression has been shown to be significantly associated with higher mortality in CRC and its increased expression in both the cytoplasm and nucleus have been shown to correlated with depth of tumor invasion and staging[112,113]. Its expression has also been associated with CRC initiation and progression with its over activation enhancing cancer cell proliferation, tumor growth, invasion, migration, and survival[114,115]. Increased STAT3 over activation and expression in CRC has also been correlated with CRC resistance to 5-FU based chemotherapy and radiation therapy. STAT3 expression is also implicated in resistance to anti-EGFR therapy, as its phosphorylation levels in metastatic CRC was increased in patients that had a poor response to EGFR targeted therapy[116–118].

STAT3 signaling was also found to be highly active in CSCs, playing an important role in CSC persistence during and after treatment by stimulating transcription of genes involved in cell cycle progression, DNA synthesis, replication, and repair[119]. In addition to CSC maintenance, STAT3 is also an important player in epithelial-mesenchymal transition (EMT), a process where epithelial cells acquire mesenchymal phenotypes. EMT has been strongly linked to cancer cell plasticity, as many of its markers have been found to be associated with the acquisition of CSC properties[120]. Inhibition of STAT3 through small molecule inhibitors has been shown to sensitize cancer cells to chemotherapy in CRC and is considered a potential drug target for CRC[121].

1.11 Project Goal

The goal of this study was to identify a possible targets or mechanisms that are upregulated due to the loss of LGR5 and which are also associated with CRC plasticity and drug resistance. We hypothesize that loss of LGR5 expression in CRC cells promotes drug resistance through activation of STAT3, and thus, inhibition of STAT3 activity may potentially restore drug sensitivity of LGR5-low/negative cells. Our specific aims are: (1) to characterize the effect of LGR5 expression in the regulation of STAT3 signaling, (2) examine the role of LGR5 and STAT3 in drug resistance and CRC cell plasticity in vitro, and (3) determine if STAT3 inhibition enhances drug sensitivity of CRC tumors in vivo. This project may lead to the identification of novel molecular mechanisms that mediate drug resistance and CRC cell plasticity. Co-targeting of LGR5 and these molecular mechanisms may be essential for the improved treatment of CRC.

Chapter 2: Materials and Methods

2.1 Antibodies, Plasmids, and Chemicals

The primary antibodies MET (#8198), STAT3 (#9139), β-Catenin (#8480), EGFR (#4267), JAK (#3230), ERK 1/2 (#4695), MEK 1/2 (#9122), BIM (#2933), Bcl-XL (#2764), Cyclin D1 (#2922), Axin 2 (#2151), SHP-2 (#3397), and β-Actin (#3700), p-MET Y1234/1235 (#3077), p-STAT3 Y705 (#9145), p-STAT3 Y727 (#9134), Non-p-β-Catenin (#8814), p-EGFR Y1068 (#4407), p-EGFR Y992 (#2235), p-JAK (#3776), p-ERK 1/2 T202/Y204 (#9101), p-MEK 1/2 S217/221 (#9121), p-SHP-2 Y580 (#3703), p-SHP-2 Y542 (#3751) were purchased from Cell Signaling Technology. The primary antibodies for LGR5 and p-EGFR Y1173 were purchased from Abcam, and the primary antibody for IQGAP1 was purchased from Bethyl Laboratories. Secondary antibodies anti-rabbit IgG, HRP-linked (#7074) and anti-mouse IgG, HRP-linked (#7076) were also purchased from Cell Signaling. For immunocytochemistry TO-PRO-3 (Thermo Fischer Scientific) was used for nuclear staining, and secondary antibodies goat-anti-mouse-Alexa-488, goat-anti-rabbit-Alexa 488, and goat-anti-human-Alexa 555 were purchased from Invitrogen. The LGR5 (8F2) mAb and cleavable anti-LGR5-MMAE (anti-LGR5-mc-vc-PAB-MMAE) ADC were generated previously as described and contained a drug-to-antibody ratio of 4[57]. The plasmids encoding myc-LGR5 was generated previously[66]. Constitutively active mouse STAT3 (STAT3-CA) plasmid Stat3-C Flag pRc/CMV was a gift from Jim Darnell (Addgene plasmid 8722).

Chemotherapies, irinotecan hydrochloride and irinotecan hydrochloride trihydrate were purchased from Selleck Chemical and 5-fluorouracil (5-FU) was purchased from Acros Organics. The STAT3 inhibitors, stattic and cryptotanshinone (Crypto), and the EGFR

inhibitor Gefitinib were also purchased from Selleck Chemical. The MET inhibitor, crizotinib was purchased from Cell Signaling, GP130 inhibitor SC144 was purchased from Millipore, and the β-catenin inhibitor XAV939 was purchased from Cayman Chemical. Therapeutics used for in vitro experiments were resuspended in Dimethyl Sulfoxide (DMSO, Fisher). For in vivo experiments, stock irinotecan hydrochloride was resuspended in 10% DMSO and 5% sterile dextrose in water (D5W, Fisher Bioreagents), stock stattic was resuspended in 10% DMSO, 35% polyethylene glycol 300 (PEG-6: Fisher Bioreagents), 5% Tween 80 (Spectrum), and D5W.

2.2 Cell Culture, Stable Cell Lines, and Transfection

HEK293T, DLD-1, HT-29, and HCT116 cells were purchased and authenticated by ATCC. LoVo cells were provided by the laboratory of Dr. Shao-Cong Sun (MD Anderson Cancer Center, Houston TX). HEK293T and HCT116 cells were grown in Dulbecco modified Eagle medium (DMEM; Gibco), HCT 116, DLD-1, LoVo and LS180 cells were grown in Roswell Park Memorial Institute medium (RPMI; Gibco). All media was supplemented with 10% fetal bovine serum (FBS) and penicillin /streptomycin (Gibco). Cells were incubated at 37°C with 95% humidity and 5% CO_2. To generate stable cell lines with altered LGR5 expression, LoVo and DLD-1 were infected with lentiviral particles containing shRNA targeting human LGR5 as previously described[73]. Briefly, lentiviral particles were generated through the co-transfection of HEK293T cells with pLKO.1 vector containing the shRNA TRCN0000011586 (shLGR5-1) or TRCN0000011589 (shLGR5-2) (GE Dharmacon) and packing plasmids, psPAX2 and pMD2.G using Fugene 6 (Roche, Basel, Switzerland). Cells were infected and selected in the presence of 1μg/mL puromycin (Life Technologies) or 200

ng/mL G418 (Corning). In LS180, LGR5 was knocked out using a Lenti-CRISPRv2 vector system with the guiding sequence CAG GAG CAC ACC GAG CCG GG, which targeted nucleotides 314-295 in the open reading frame of human LGR5 as described[93]. Transient transfections were performed using jetPRIME (Polypus Transfection).

2.3 Western Blotting

For western blot analysis, cells were harvested with either radio-immunoprecipitation assay (RIPA) buffer; (50 mm Tris-Cl, pH 7.4, 150 mm NaCl, 1 mm DTT, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) or in immunoprecipitation lysis (IP) buffer; (25 mm Tris-HCl, pH 7.4, 150 mm NaCl, 1 mm EDTA, 1% Nonidet P-40, and 5% glycerol) supplemented with protease and phosphatase inhibitors. Lysates were then subjected to sonication and centrifuged at 13,000g for 10 minutes and supernatants were mixed with 1X Laemmli sample buffer (Bio-Rad). Samples were incubated at 37°C for 45 minutes then loaded on to SDS-PAGE 20 gel (Invitrogen) with XCell4 Surelock Midi-Cell (Invitrogen) at 120 V for 120 minutes in 1X Tris-Glycine SDS running buffer (Fisher Scientific). Protein gels were then transferred to nitrocellulose blotting membrane (Amersham by GE Healthcare Life Sciences) at 400 amps for 120 minutes on ice with Criterion Blotter (Bio-Rad) in 1X Tris-Glycine transfer buffer (Fisher Scientific) with 20% methanol. Blots were incubated with indicated primary antibodies in 2.5% non-fat milk (Research Products International) in tris-buffered saline solution (Fisher Scientific) with 1% Tween 20 (TBST, Fisher Bioregents). After primary antibodies were washed from the blots using TBST, HRP-labeled secondary antibodies were added in 2.5% milk TBST solution, then washed again with TBST. Proteins were detected using the standard protocols for Enhanced Chemiluminescence (ECL) purchased

from either Cytiva or KwikQuant. Western blots were exposed by x-ray film or camera imager (KwikQuant).

2.4 Immunocytochemistry and Confocal Microscopy

For immunocytochemistry (ICC) experiments, LoVo, DLD1, or LS180 cells were seeded into an 8-well poly-D-lysine coated chamber slides and allowed to adhere overnight. The following day cells were treated with or without chemotherapies as indicated. Of note, irinotecan hydrochloride trihydrate was used for ICC as it produced less autofluorescence than irinotecan hydrochloride. Cells were washed with PBS, fixed with 4% (vol/vol) paraformaldehyde solution for 15 minutes and permeabilized with 0.3% 1X Triton-X (Life Technologies) or 0.1% saponin (Sigma) for 10 minutes. Cells incubated at room temperature with primary antibody for 1 hour and treated with anti-rabbit-Alexa-488, anti-mouse-Alexa-488, or anti-human-Alexa-555 at room temp for 1 hour. For internalization studies, cells were incubated with anti-LGR5 mAbs at 37°C for 30 minutes prior to fixation. Nuclei were counterstained with TO-PRO-3) at room temperature for 5 minutes. Slides were mounted with ProLong™ Gold Antifade Mountant (ThermoFisher) and images were acquired by confocal microscopy (Leica TCS SP5 microscope) and analyzed with LAS AF Lite software (Leica Microsystems, Inc).

2.5 Wound Healing Assay

For wound healing assays LoVo and DLD1 cells were seeded to near confluence in 12-well plates and then starved in either 1% FBS or serum free-media overnight. The following day cells were scratched with a 200uL pipette tip, cells were then washed with PBS to remove cellular debris. Wells were then refreshed with starve media along with the respective treatment. The wound was then subsequently imaged at 0, 24, and 48 hours and wound closure was evaluated through ImageJ.

2.6 Cell Invasion Assay

For invasion assays, 8-µm pore Transwell migration chambers (BD Bioscience) were coated with a 1:40 mixture of BD BioCoat Matrigel: serum-free RPMI media and allowed to solidify. Cells (5×10^4) were then seeded into the top chamber and allowed to invade at 37°C for 48 hours towards the bottom chamber containing RPMI with 10% FBS. Cells were fixed in 4% paraformaldehyde for 10 minutes, washed with PBS, and then permeated with 100% methanol for 10 minutes before being stained with 0.005% crystal violet (Sigma) solution and rinsed in water. A cotton swab was then used to gently remove any remaining non-invasive cells and rinsed again. Stained cells were examined through light microscopy and 5 individual fields were imaged and quantification (i.e., percent invasion) was determined using ImageJ.

2.7 Clonogenicity

Clonogenicity was analyzed by seeding 5 X 10^3 cells into a 6-well culture plate with and allowed to adhere overnight. Cells were then treated with or without therapeutics as indicated and cultured for 7 days, refreshing media and treatment on day 3. After growth, cells were washed with PBS before being fixed with 4% paraformaldehyde and stained with 0.005% crystal violet and rinsed with sterile water. Stained cell colonies were then imaged and colony count and percent well coverage were analyzed using ImageJ.

2.8 In Vitro Cytotoxicity

For cytotoxicity analysis, cells were seeded at 1.5-2 x 10^3 per well in a white 96 half-well plate (Corning). Plated cells were then treated with serial dilutions of specified therapeutic compounds or ant-LGR5-MMAE ADC then incubated at 37°C for 3-4 days. Cytotoxicity was then quantified using the CellTiter-Glo (Promega) Luminescent Cell Viability Assay and Envision multilabel plate reader (PerkinElmer).

2.9 In Vivo studies

Animal studies were performed in line with the strict recommendations of the Institutional Animal Care and Use Committee of the University of Texas Health and Science Center at Houston (AWC-20-0144)). Female nu/nu mice between 6-8 weeks old (Jackson Laboratory) were subcutaneously inoculated into the lower right flank with 1 x 10^6 LoVo cells in PBS. Once tumors reached an average size of ~100 mm^3, animals were randomized into four treatment groups (vehicle, stattic, irinotecan, or irinotecan + stattic). Irinotecan (20 mg/kg) or vehicle (2% DMSO in D5W) was administered intraperitoneally every five days for three

doses. Stattic (10mg/kg) or vehicle (4% DMSO, 14% PEG300, and 2%Tween in D5W) was administered intraperitoneally every other day for a total of six doses. Tumor volume was measured every 2 days and calculated using the formula: Tumor Volume = (Length x width^2)/2. Mice were euthanized when tumor volume reached approximately 2000 mm^3.

2.10 Statistical Analysis

Statistical analysis was performed and IC50s determined using the Prism 5 (GraphPad Software, Inc.). All in vitro experiments were performed at least three times. The levels of significance between samples were determined through an unpaired two-tailed Student *t* test (mean comparison with one factor) or one-way ANOVA for groups with multiple comparisons. Data are shown as mean ± standard error of the mean (SEM) or standard deviation (SD) as indicated, with P values ≤ 0.05 considered to be statistically significant.

Chapter 3: Results

3.1 Chemotherapy and ADC treatment of CRC cell lines decreases LGR5 expression

To test if LGR5+ CRC cells convert to an LGR5- state during drug treatment, we measured changes in LGR5 expression in response to different chemotherapeutics. CRC cell lines LoVo, DLD1, and LS180 were selected based on for their high expression of LGR5. As we previously published, LoVo cells contain the highest level of LGR5 protein expression as confirmed by western blot and gene expression as indicated by data obtained from the Cancer Cell Line Encyclopedia (CCLE)[57]. CRC cells were treated with 10μM of either 5-FU or irinotecan at indicated time-points; LGR5 expression was then detected by western blot analysis (Figs. 5A-B). LGR5 expression was nearly undetectable after 72 hours of chemotherapy treatment in LoVo and LS180 cells and was found decreased in DLD1 cells (Figs. 5A-B). Loss of LGR5 expression was confirmed through ICC staining, demonstrating a gradual decrease in LGR5 expression at 24 hours and complete loss at 72 hours (Fig. 5C).

To test if CRC cells were entering a similar LGR5- state with targeted therapy, LoVo and LS180 cells were treated with 1μg/mL of anti-LGR5-MMAE ADC at indicated time points. This dose was selected as previous experiments showed it did not result in complete cell death after 72 hours[57]. Like chemotherapy treatment, LGR5 expression was undetectable at 72 hours in LoVo cells and 48 hours in LS180 cells (Fig. 5D). To demonstrate plasticity and determine if LGR5- CRC cells can convert back to an LGR5+ state, LoVo and LS180 were treated with either irinotecan or anti-LGR5-MMAE ADC for 72 hours and cells were either

harvested or washed and allowed to recover for an additional 72 hours. We show that after treatment was ceased, and drug was removed, LGR5 expression re-emerged, providing evidence of CRC cell plasticity (Fig. 5E). Furthermore, this data suggests that the LGR5- state which these cells enter may be more drug resistant with activated survival mechanisms.

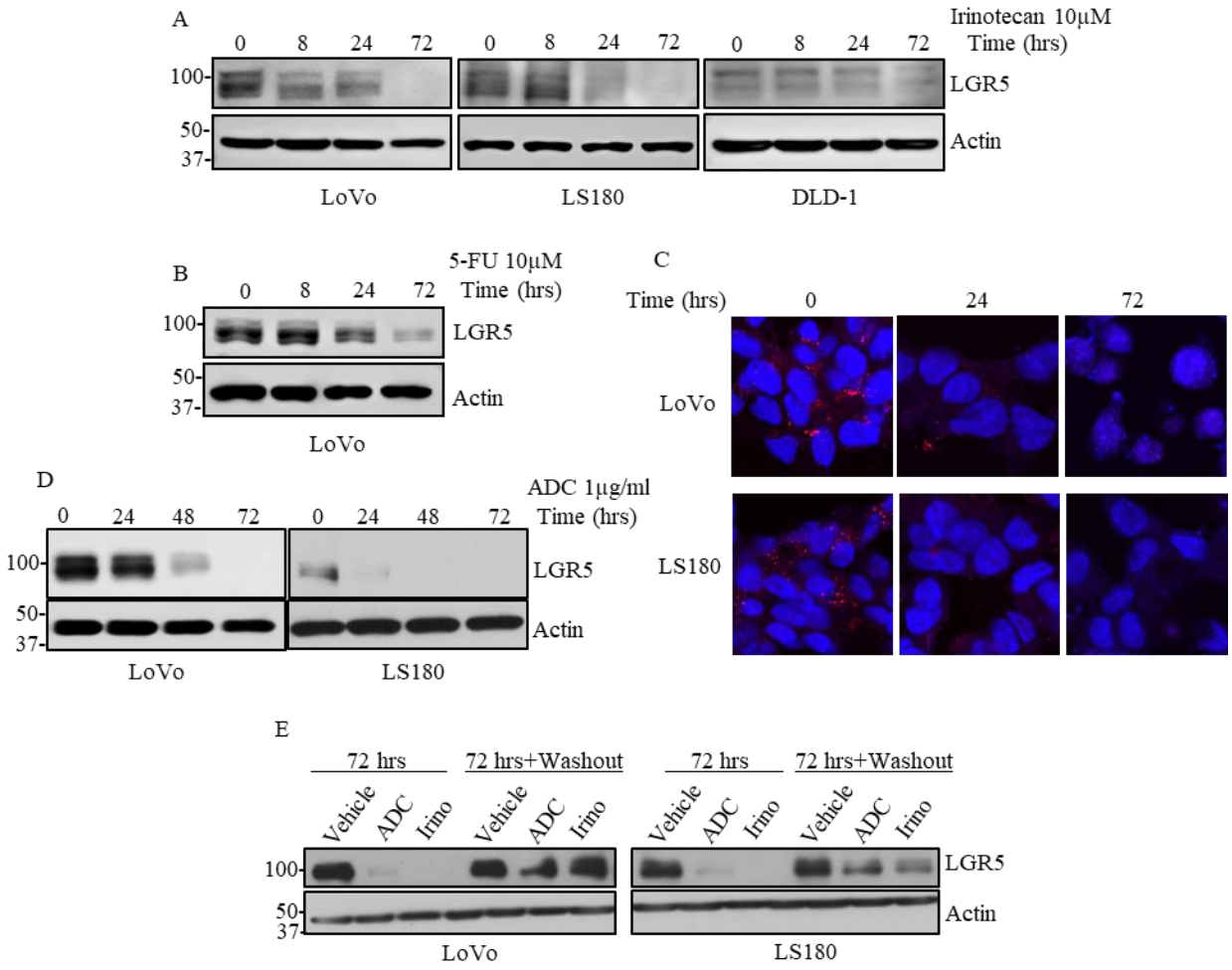


Figure 5. Drug Treatment leads to loss of LGR5 expression. (A-B) LoVo and LS180 CRC cells were treated with 10μM of either (A) irinotecan or (B) 5-FU for 0, 8, 24, and 72 hours. LGR5 expression was detected by western blot. (C) Confocal microscopy images of LGR5 expression in LoVo and LS180 after treatment of 10μM of irinotecan for 0, 24, and 72 hours. (D) LoVo and LS180 cells were treated with 1μg/mL of anti-LGR5-MMAE ADC for 0, 24, 48, and 72 hours and LGR5 expression was detected by western blot. (E) CRC PDX organoids were treated with 10μM irinotecan or 1μg/mL anti-LGR5-MMAE ADC for 3 days and LGR5 expression was detected by western blot. (F) LoVo and LS180 were treated with vehicle, anti-LGR5-MMAE ADC, or irinotecan for 72 hours, washed with PBS, and allowed to recover for 3 days. LGR5 expression was detected by western blot.

3.2 Loss of LGR5 leads to increased survival and invasive phenotype

In a recent publication, our lab generated LGR5 KD LoVo and DLD-1 cell lines using shRNA constructs and LGR5 CRISPR/Cas9 KO LS180 cell lines[93]. We showed that LGR5KD or KO enhanced resistance to both 5-FU and irinotecan supporting the hypothesis that loss of LGR5 conferred a more drug resistant state[93]. Given these findings, we performed survival assays in vitro to test changes in clonogenicity. As shown in Figs. 6A-B, LoVo cells with LGR5KD (shLGR5-1 and shLGR5-2) had around a 3-fold increase in colony formation compared to parental and vector (shCTL) control cells, and an approximate 3-6-fold and 10-fold increase in the presence of 5-FU and irinotecan, respectively (Figs. 6A-B). Next, we evaluated the effect of loss of LGR5 on migration and invasion, which are critical for CRC progression and metastasis. Using wound heal assays, we found that both LoVo LGR5KD cell lines exhibited a 3-4-fold (15-20%) increase in percent wound closure compared to parental and shCTL (5%) (Figs. 6C-D). In the presence of 10μM irinotecan, LGR5KD cells showed an increase in wound closure compared to parental and shCTL cells. Parental and shCTL cells showed a decrease in closure in the presence of irinotecan compared to untreated, likely due to a decrease in cell viability (Figs. 6C-D). Next, we tested effects of loss of LGR5 on cell invasion. We found LGR5KD in LoVo cells increased invasion by 3-4-fold (Fig. 6E). Taken together, loss of LGR5 not only leads to drug resistance but also potentially induces a more metastatic phenotype.

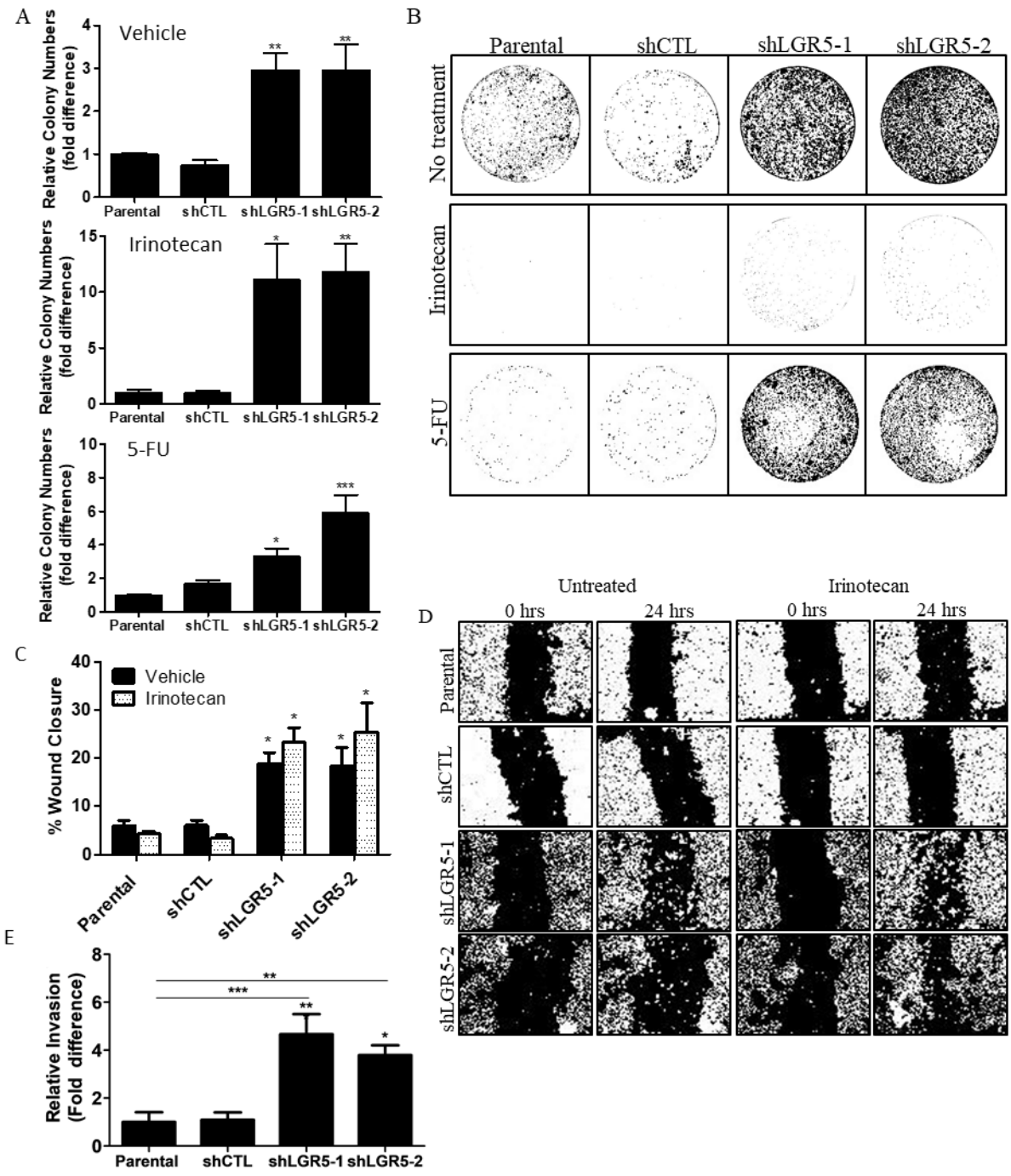


Figure 6. Effect of LGR5 knockdown on CRC cell function. (A-B) Clonogenicity assay of LoVo cells treated with or without 10μM irinotecan or 5-FU and incubated for 7 days, cells were then stained with crystal violet. (A) Quantification of clonogenicity assay and (B) representative images of colonies stained with crystal violet. (C) Quantification of wound healing assay and (D) representative images of wound healing assay. (E) Quantification of invasion assay. Data represent mean +/- S.E.M. of at least three experiments. Statistical analysis was performed using one-way ANOVA, *, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$ compared to controls.

3.3 LGR5 knockdown CRC cells have increased STAT3/MET phosphorylation

To identify signaling mechanisms that may mediate plasticity and drug resistance in LGR5- CRC cells, we performed a western blot analysis to determine changes in protein expression. In LoVo cells, LGR5KD resulted in increased its non-phosphorylation of active β-catenin (as we previously reported)[93], JAK (Y1007/1008), and STAT3 (Y705) (Fig. 7A). Along with increased STAT3 nuclear accumulation we also observed alteration in the expression of STAT3-associated survival proteins that prevent cell cycling arrest and apoptosis. Specifically, loss of LGR5 lead to increased Bcl-xL and Cyclin D and decreased BIM expression (Fig. 7B). These results suggest that loss of LGR5 increases both Wnt and STAT3 activation in CRC cells. Through ICC and confocal analysis, we observed that loss of LGR5 through shRNA KD in LoVo cells leads to increased nuclear accumulation of β-catenin and phosphorylated STAT3 indicating increased Wnt and STAT3 signaling (Fig 7C).

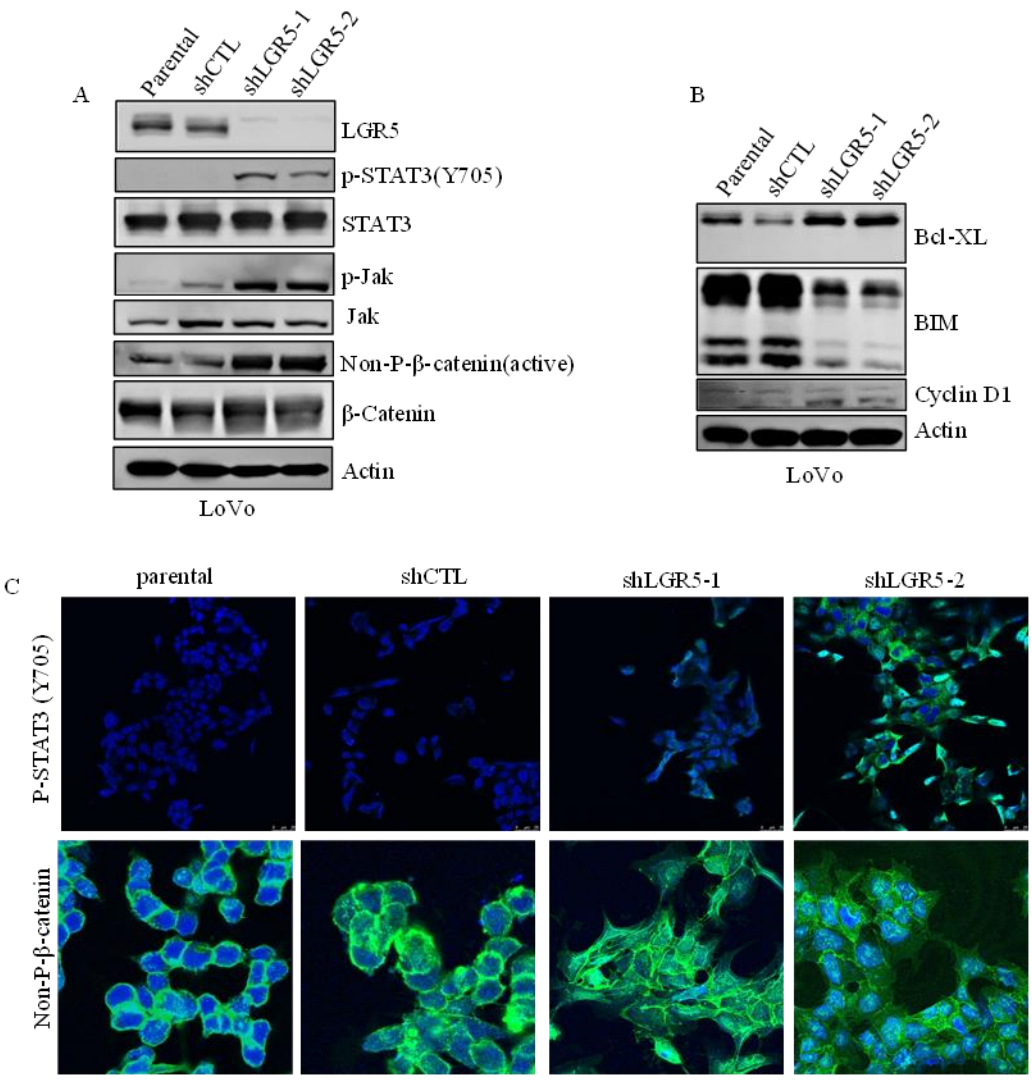


Figure 7. Loss of LGR5 increases phosphorylation of STAT3 and MET. (A) Western blot analysis of changes in protein levels and phosphorylation in LoVo cells with or without LGR5 KD. (B) Western blot of changes in expression of STAT3-associated survival proteins. (C) Confocal microscopy images of STAT3 phosphorylation and activate β-Catenin in LoVo cell lines.

3.4 IL-6R/Gp130 and EGFR pathways do not mediate STAT3 activation in LGR5KD cells.

To identify which upstream mediators may be responsible for increased STAT3 activation, we chemically targeted three cell membrane receptors, IL6/Gp130, EGFR, and MET which are known to specifically phosphorylate STAT3 (Y705). IL6/Gp130 activation has been correlated with CRC disease status and metastasis and is most associated with the activation of STAT3. However, most commercial CRC cell lines do not express high levels of IL6R, which was confirmed for LoVo cells by the CCLE database. We also failed to detect IL6R by western blot in LoVo cells (not shown). To observe if Gp130 activation is playing a role in STAT3 activation, LGR5KD LoVo cells were treated with 10μM of Sc144, a small molecule Gp130 inhibitor, at indicated times. It was found that inhibition Gp130 did not suppress STAT3 activation (Fig. 8A). Therefore, it is likely that IL6R/Gp130 receptors are not responsible for the altered STAT3 signaling.

As mentioned previously, EGFR is often targeted in late-stage CRC and is involved in progression and survival, both of which mediate its activation of several downstream signaling factors like MAPK and STAT3. Through western analysis, no significant increase in EGFR phosphorylation at sites, Y1068, or Y1173 were observed in LGR5KD LoVo cells (Fig. 8B). LGR5 KD LoVo cells treated with 10 μM gefitinib, an EGFR inhibitor at indicated times, showed no significant decrease in STAT3 activation (Fig. 8C). These findings suggest that STAT3 activation is most likely not mediated through EGFR activation.

Since loss of LGR5 also leads to increased levels of active β-catenin, we tested if increased phosphorylation of STAT3 was potentially mediated through Wnt signaling.

LGR5KD LoVo cells were treated with 10 μM XAV939, a tankyrase inhibitor that stimulates β-catenin degradation by stabilizing Axin, at indicated times. XAV939 showed increased stabilization of Axin2, however no significant decrease in STAT3 activation was detected (Fig. 8D). These findings suggest that STAT3 activation is likely not mediated through β-catenin activation.

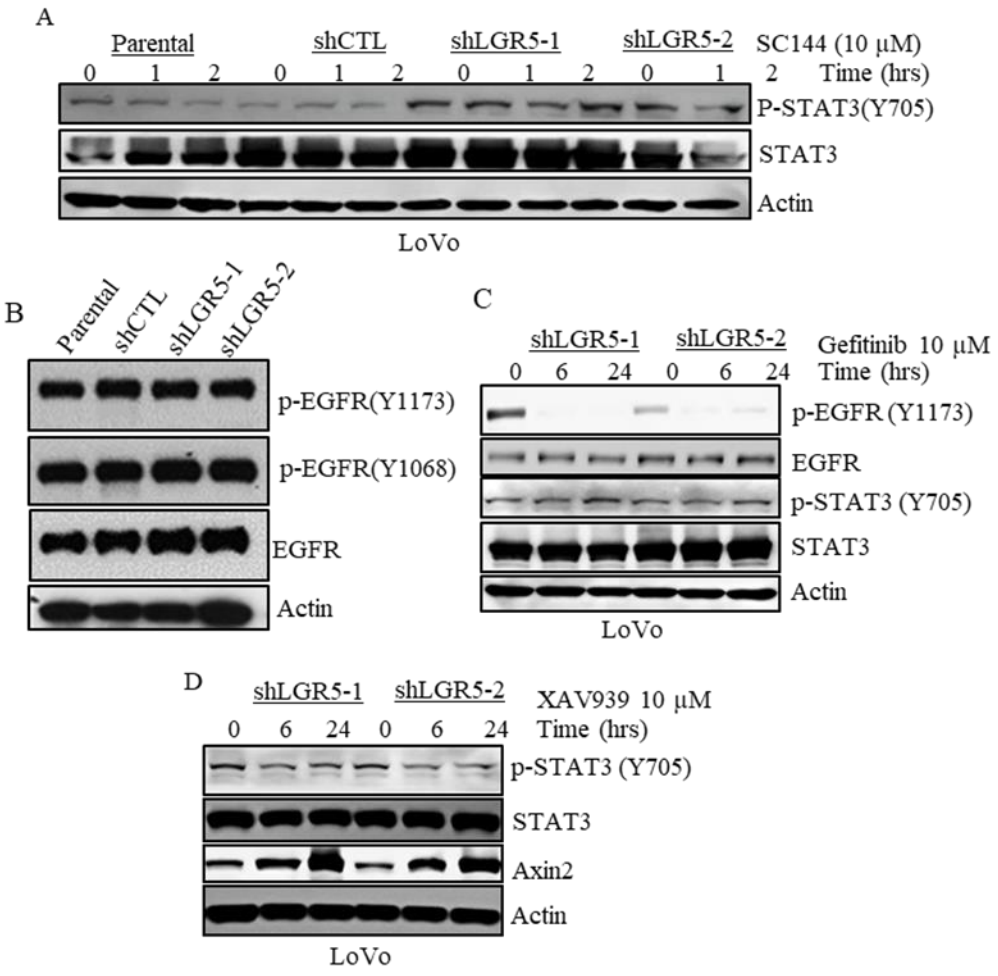


Figure 8. Inhibition of IL6R/Gp130 nor EGFR attenuated STAT3 activation in LGR5 KD cells. (A) Western blot analysis of STAT3 in LoVo cells treated with 10μM Sc144 for 0, 1, and 2 hours. (B) Western blot of EGFR phosphorylation in LoVo cells. (C) Western blot analysis of STAT3 and EGFR and STAT3 phosphorylation in LoVo LGR5 KD cells treated with 10μM gefitinib for 0, 6, and 24 hours. (D) Western blot analysis of STAT3 phosphorylation and Axin2 expression in LoVo LGR5 KD cells treated with 10μM XAV939 for 0, 6, and 24 hours.

3.5 STAT3 activation is mediated through MET Signaling.

Next, to verify if MET plays a role in STAT3 activation we analyzed changes in MET phosphorylation and expression in LoVo cells. We observed an increase in both the mature form of MET and phosphorylation of MET (Y1234/1235). We also detected increased MET and STAT3 phosphorylation in DLD-1 LGR5KD cells and LS180 LGR5KO (1.4 and 1.5) cells (Figs 9A-C). We also found an increase in cytoplasmic and nuclear presence of phosphorylated MET through ICC (Fig. 9D). The effect of MET inhibition on STAT3 was investigated by treating LGR5KD cells with the MET inhibitor crizotinib. We observed by western blot analysis that after 6 hours of treatment there was a complete loss of phosphorylated STAT3 (Fig. 9E). This suggests that STAT3 activation is mediated through activation of MET. In turn, we also observed loss of phosphorylated MET after treating cells with STAT3 inhibitors cryptotanshinone (Crypto) and stattic (Fig. 9E). This suggests that in LGR5KD cells, enhanced MET/STAT3 signaling may rely on a potential positive feedback loop between MET and STAT3 to drive survival and drug resistance.

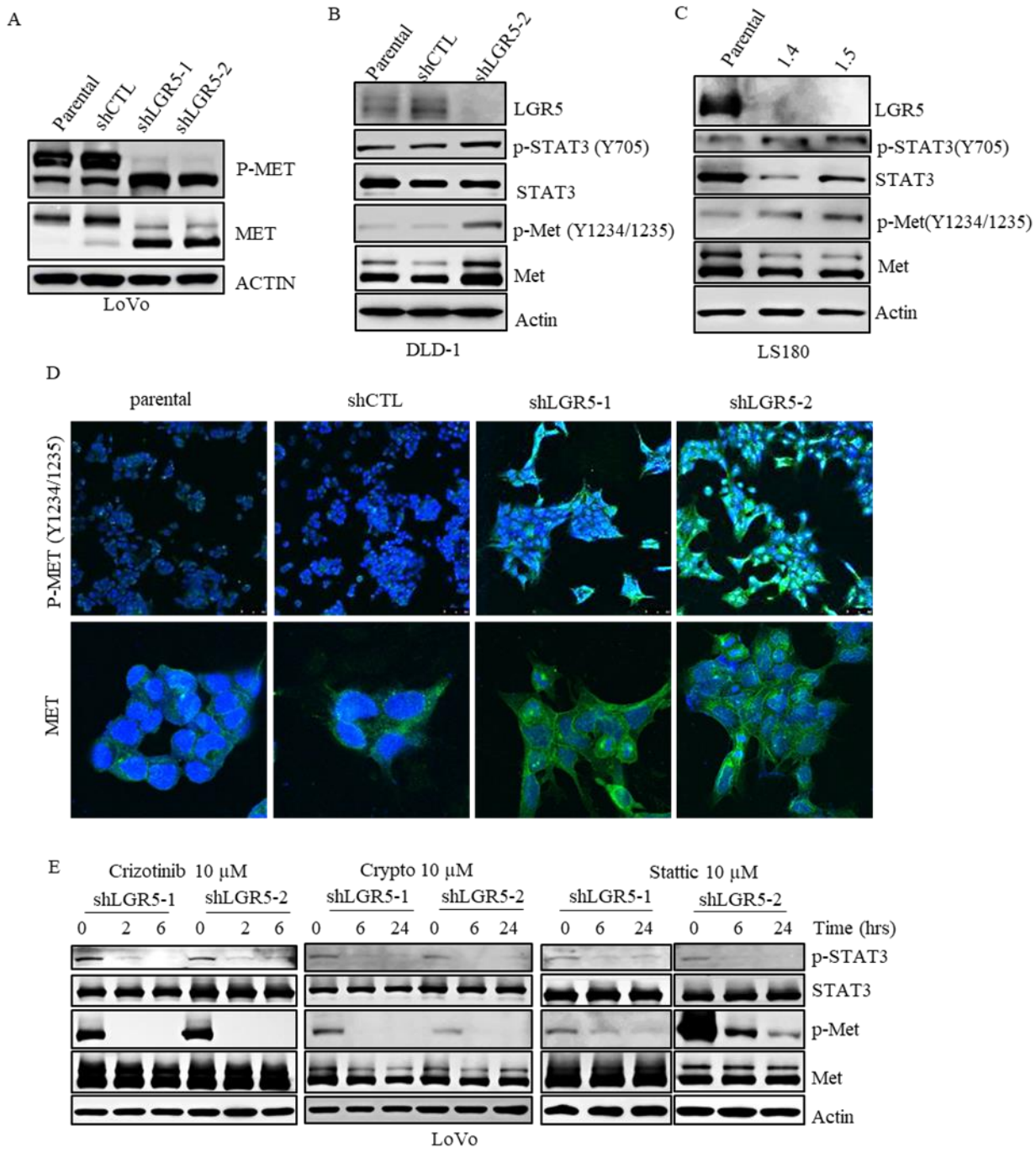


Figure 9. MET mediates STAT3 activation through a potential positive feedback loop. (A) Western blot analysis of changes in MET phosphorylation in LoVo cells with or without LGR5 KD. (B-C) Western blot of changes in phosphorylation of STAT3 and MET in (B) DLD1 with or without LGR5 KD and (C) LS180 parental or LGR5 KO clonal cells lines 1.4 and 1.5. (D) Confocal microscopy images of phosphorylated and total MET in LoVo cell lines. (E) Western blot analysis of changes in MET and STAT3 phosphorylation in LoVo LGR5 KD cells treated with 10 μM crizotinib, cryptotanshinone, or stattic at indicated time-points.

3.6 Chemotherapy and ADC treatment of CRC cell lines increases MET/STAT3 activation

Since treatment with irinotecan or anti-LGR5-MMAE ADC was shown to convert LGR5+ CRC cells to an LGR5- state (Fig. 1), we tested if levels of MET and STAT3 were also changed. As shown in Fig. 10A-B, increased MET and with STAT3 activation was detected in LoVo and LS180 cells treated with 10 μM irinotecan. CRC cells treated with 1 μg/mL anti-LGR5-MMAE ADC also led to increased phosphorylation of MET and STAT3 (Fig. 10C-D). These findings show that drug-induced loss of LGR5 in CRC cells leads to activation of MET/STAT3 in a manner similar to LGR5KD or KO.

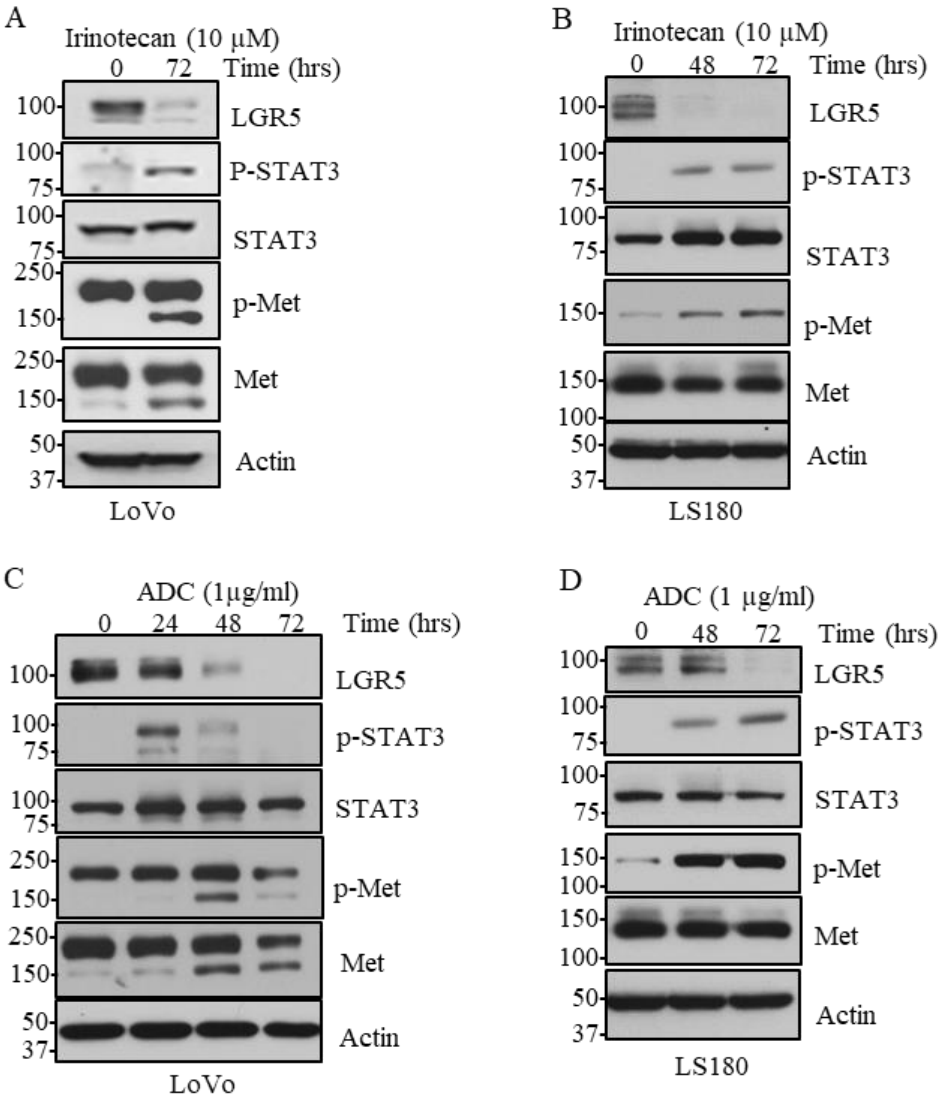
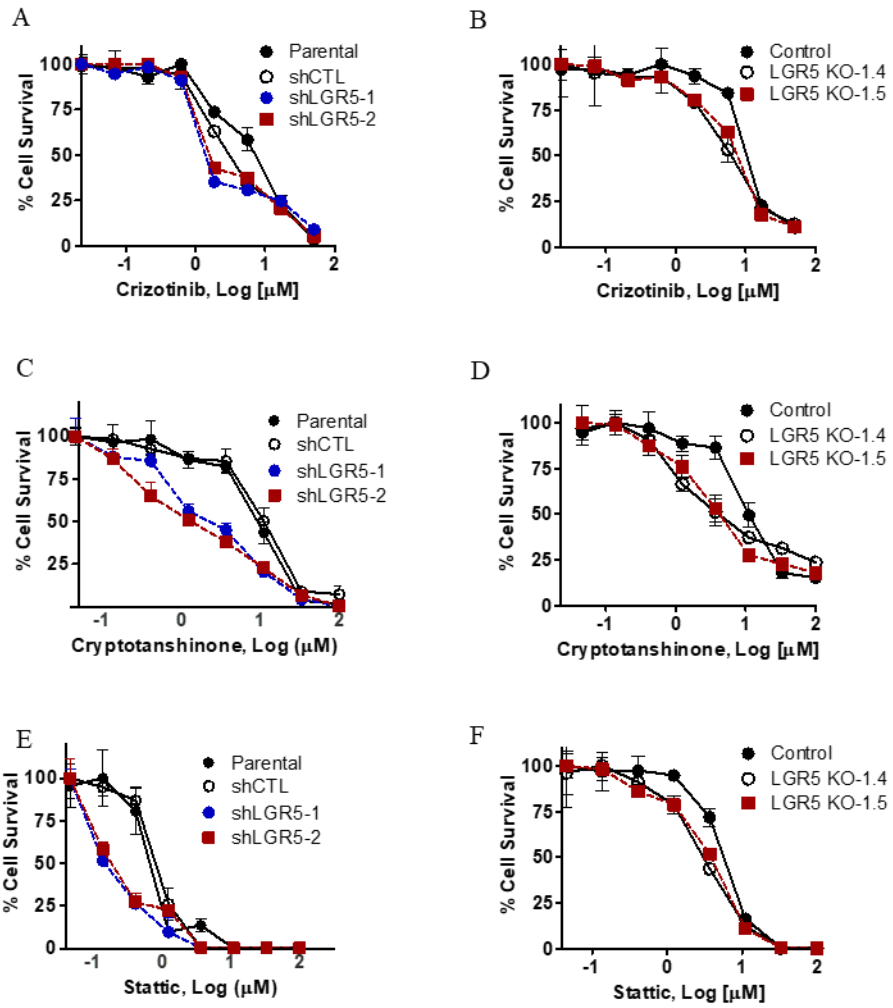


Figure 10. Drug treatment leads to increased Met/STAT3 signaling with loss of LGR5. (A-B) Western blot analysis of MET and STAT3 phosphorylation in (A) LoVo and (B) LS180 cells treated with irinotecan at different time-points. (C-D) Western blot analysis of MET and STAT3 phosphorylation in (C) LoVo and (DB) LS180 cells treated with anti-LGR5-MMAE ADC at different time-points.

3.7 MET/STAT3 activation mediates drug resistance in LGR5 KD cells

To determine if LGRKD cells dependent on MET/STAT3 activation, we tested the impact of MET and STAT3 inhibitors on cell viability. We found that LGR5KD LoVo and LGR5KO LS180 cells were more sensitive to the MET inhibitor and STAT3 inhibitors stattic, and cryptotanshinone (Fig. 11A-F). The IC50s were approximately 4 to 8-fold and 1.5 to 6-fold lower for LoVo LGR5KD and LS180 LGR5KOs compared to control cell lines, respectively (Fig. 11G). This suggests that MET/STAT3 activation may be integral for the survival of LGR5KD cells.



G

CRC cell line	IC_{50} (μM) ± SD		
LoVo	**Crizotinib**	**Crytptotanshinone**	**Stattic**
parental	6.7 ± 1.8	6.5 ± 2.9	5.3 ± 0.4
shCTL	6.9 ± 2.2	4.2 ± 1.4	6.9 ± 0.07
shLGR5-1	1.8 ± 0.09	1.4 ± 0.7	0.6 ± 0.02
shLGR5-2	1.5 ± 0.01	1.1 ± 0.1	1.0 ± 0.03
LS180			
control	12 ± 5.3	11.3 ± 1.8	7 ± 1.0
LGR5 KO 1.4	7.7 ± 1.5	1.9 ± 0.2	4.3 ± 0.9
LGR5 KO 1.5	5.9 ± 1.7	2.4 ± 0.4	4.6 ± 1.17

Figure 11. LGR5 knockdown and knockout cells are more sensitive to inhibitors of MET and STAT3. (A-B) Cytotoxicity assays of (A) LoVo and (B) LS180 cells treated with MET inhibitor, Crizotinib. (C-D) Cytotoxicity assays of (C) LoVo and (D) LS180 cells treated with STAT3 inhibitor, Cryptotanshinone. (E-F) Cytotoxicity assays of (E) LoVo and (F) LS180 cells treated with STAT3 inhibitor, Stattic. Cytotoxicity assays were performed after 4 days using CellTiter-Glo. (G) Table of average IC_{50}s for 2-3 different experiments performed in triplicate.

To further elucidate the relationship of LGR5 expression and MET/STAT3 activation on drug resistance, constitutively activate STAT3 (STAT3-CA) was overexpressed in both LoVo and LS180 cells by transient transfection. Through western blot analysis, it was found that STAT3-CA led to loss of LGR5 (Fig. 12A). Cytotoxicity assays also showed STAT3-CA cells had increased resistance to irinotecan, supporting its role in drug resistance for LGR5- CRC cells (Fig. 12B-C). We next tested if overexpression of LGR5 leads to decreased MET/STAT3 activation. We selected to use HCT116 cells, a CRC cell line that does not endogenously express LGR5 but has higher levels of activated MET/STAT3. LGR5 was overexpressed by transient transfection using increasing amounts of DNA. We show gradual loss of MET and STAT3 activation with increasing amounts of transfected LGR5 plasmids (Fig. 12D). Using CellTiter-Glo assay, LGR5 overexpression was also found to sensitize HCT116 cells to irinotecan treatment, decreasing IC50 by 5-fold (Fig. 12E). These findings further support a role for MET/STAT3 signaling for driving drug resistance in LGR5- CRC cells.

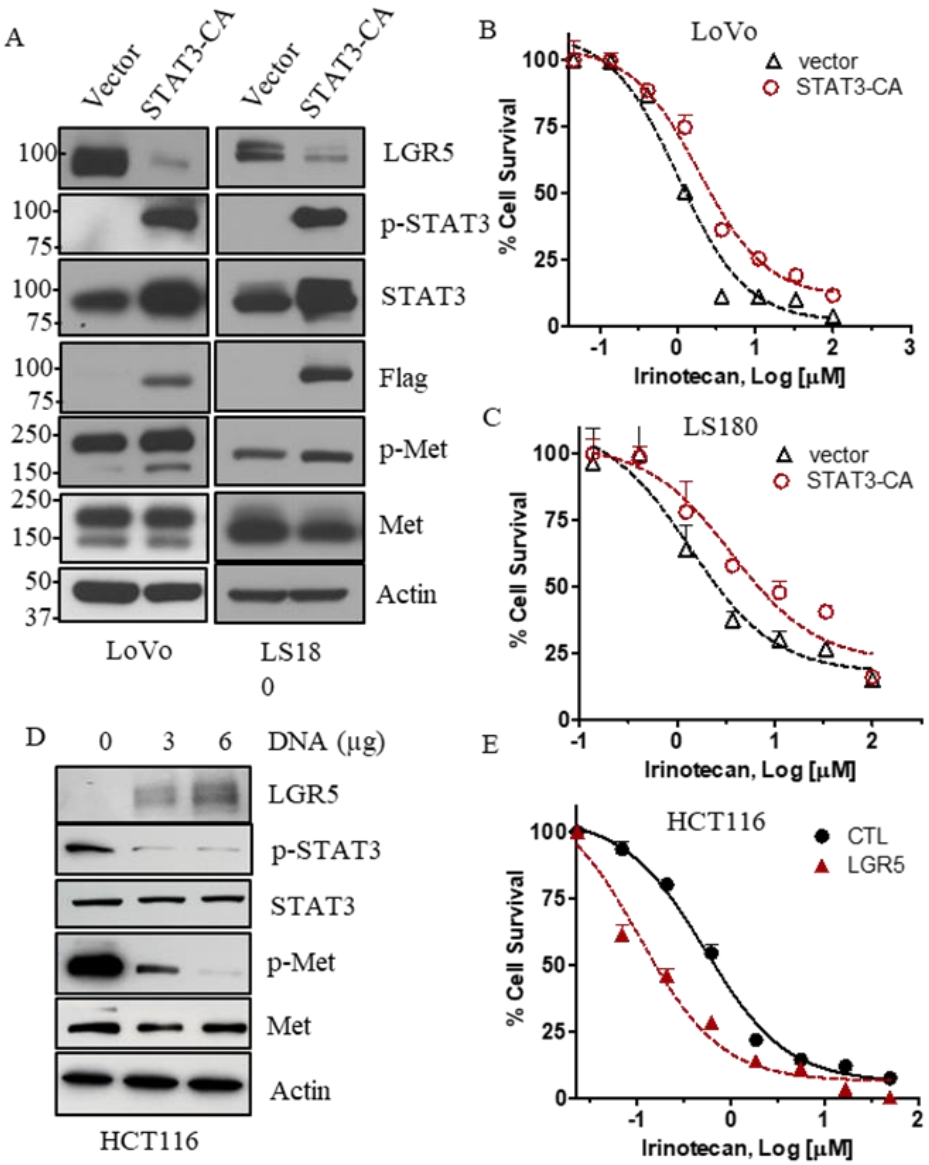


Figure 12. Effects constitutively active STAT3 and LGR5 overexpression on irinotecan resistance. (A) Western blot of LoVo and LS180 cells transiently transfected with Flag-tagged constitutively active STAT3 (STAT3-CA) and effects on MET and STAT3 phosphorylation. (B-C) Cytotoxicity assay of (B) LoVo and (C) LS180 cells transfected with vector or STAT3-CA and treated with irinotecan for 4 days. (D) Western blot of HCT116 cells transiently transfected with increasing amounts of LGR5 and effects on MET and STAT3 phosphorylation. (E) Cytotoxicity assay of HCT116 cells transfected with vector or LGR5 and treated with irinotecan for 4 days. HCT116 experiments were performed with Ashlyn Parkhurst.

3.8 MET/STAT3 inhibitors enhance efficacy of chemotherapy and ADCs in vitro

To verify if MET/STAT3 activation is at least partially responsible for increased drug resistance, we first compared combination treatment of STAT3 or MET inhibitors with irinotecan. In LoVo cells we found that both the MET inhibitor, crizotinib, and the STAT3 inhibitor, stattic, synergized with irinotecan to increase efficacy (Figs.13A-B). The effect was much greater in LGR5 KD cells, as they have increased levels of active MET and STAT3. Combination treatment of MET and STAT3 inhibitors also synergized with irinotecan in LS180 cells (Fig. 13C). We then tested if MET and STAT3 inhibitors would enhance therapeutic efficacy of anti-LGR5-MMAE ADC treatment. As shown in Figs. 9D-E, both inhibitors enhanced efficacy of ADC treatment in LoVo and LS180 cells, however the effect was less in LoVo cells since they are already sensitive to anti-LGR5-MMAE ADCs. Together with previous findings, we show combination therapy enhances efficacy of chemotherapy and ADCs in CRC cells, potentially due to increased activation of MET/STAT3 signaling.

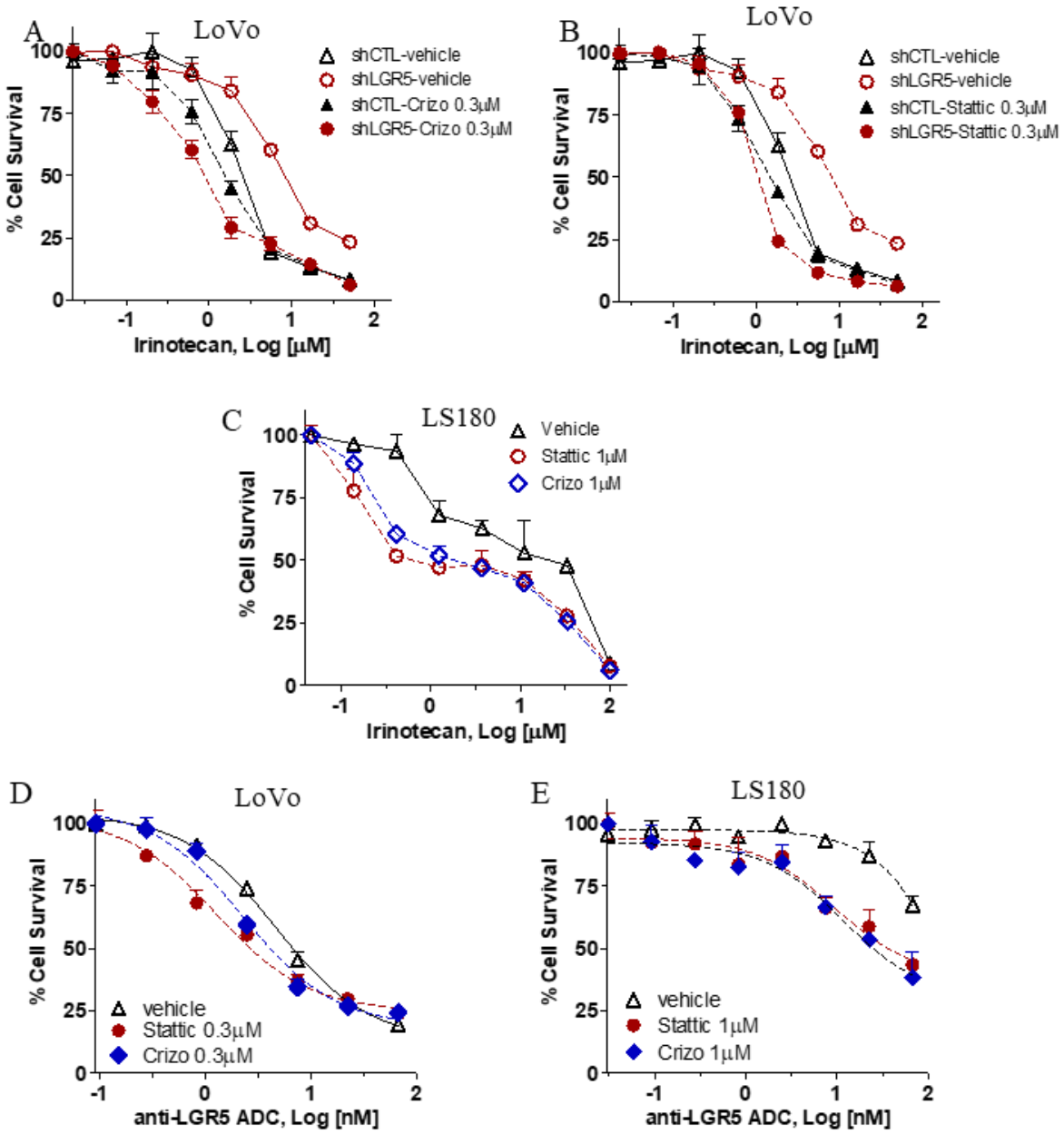


Figure 13. MET and STAT3 inhibitors synergize with irinotecan and anti-LGR5 ADCs. (A-B) Cytotoxicity assays of LoVo shCTL and shLGR5 cells treated with increasing concentrations of irinotecan in combination with (A) 0.3 μM Crizotinib and (B) 0.3 μM Stattic. (C) LS180 cells treated with irinotecan alone or in combination with 1 μM Crizotinib or Stattic. (D-E) Cytotoxicity assays of (D) LoVo and (E) LS180 cells treated with increasing doses of anti-LGR5-MMAE ADC alone or in combination with Crizotinib or Stattic as indicated. Experiments were performed 2-3 times in triplicate.

3.9 Combination treatment of Irinotecan with STAT3 inhibitor enhances anti-tumor efficacy and survival

To test the efficacy of combination treatment of irinotecan with a STAT3 inhibitor in vivo, nu/nu mice were injected with 1.0-2.0 $X10^6$ LoVo or LS180 CRC cells. Once tumors reached an average of approximately $100mm^3$ they were randomized into four groups. Mice were treated with vehicle, 20 mg/kg irinotecan every 5 days, 10 mg/kg stattic every 2 days, or combination treatment of irinotecan and stattic (Fig. 14). Treatment was terminated after the first vehicle control tumor reached $2000mm^3$. In LoVo xenografts, each treatment resulted in a significant decrease in tumor growth, with combination treatment having the most pronounced effect (Fig. 14A). Similar effects were observed in LS180 xenografts (Fig. 14B). However, stattic treatment alone did not result in a significant effect, likely due to the rapid growth of LS180 cells compared to LoVo cells in vivo. Combination treatment in both CRC models significantly enhanced efficacy in both models, with 82% and 64% inhibition in tumor growth for LoVo and LS180 xenografts, respectively (Figs. 14A-B). Irinotecan treatment alone only resulted in ~50-55% inhibition of tumor growth. We next monitored the effects on survival after treatment was terminated and found that combination treatment increased survival in both LoVo and LS180 xenograft models (Fig. 14C-D). No significant effect in body weight nor overt toxicity was observed during treatment (Figs. 14E-F). These data provide evidence of improved therapeutic efficacy in targeting STAT3 along with standard chemotherapy.

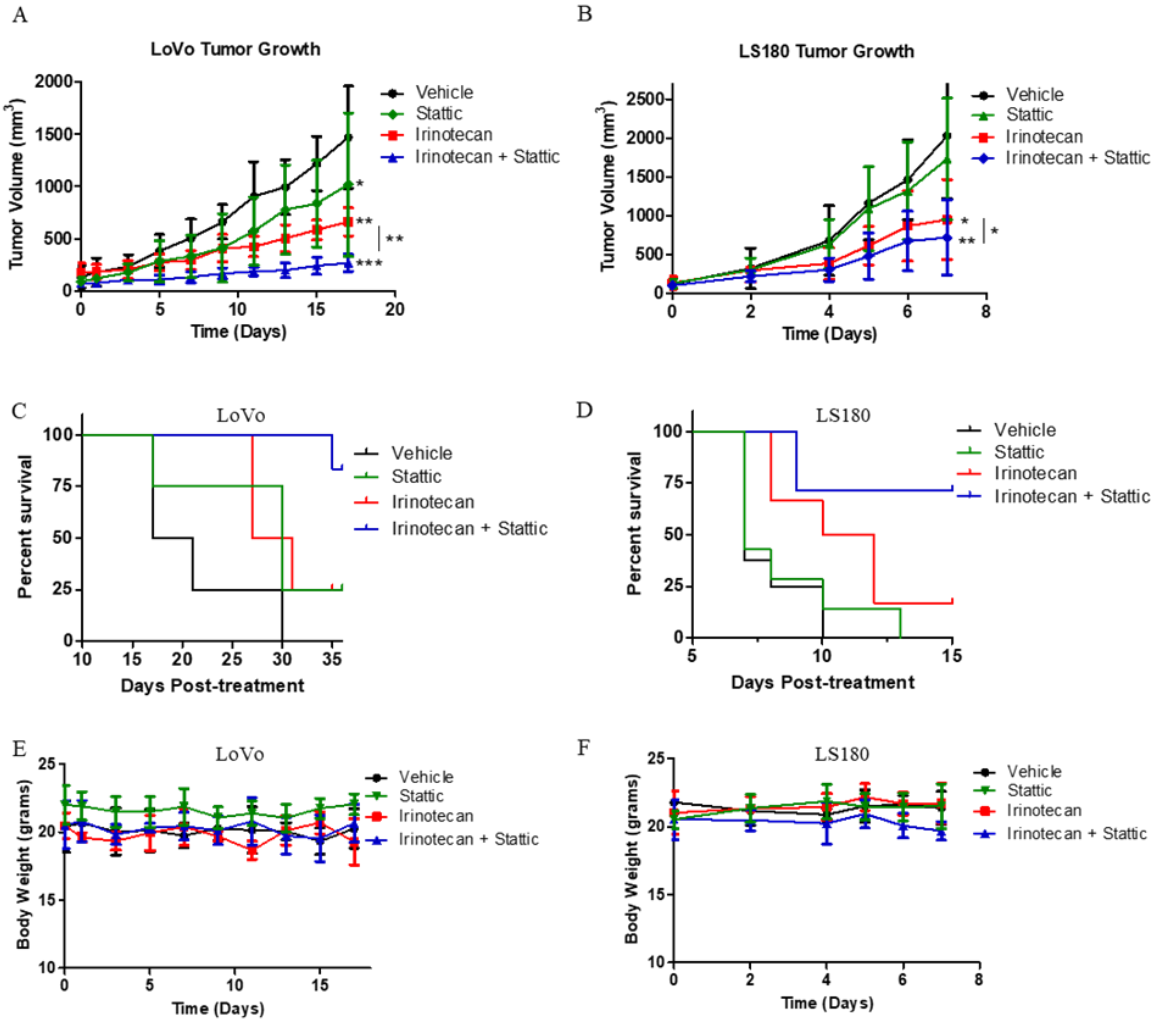


Figure 14. Combination treatment of Irinotecan with STAT3 inhibitor in vivo. (A) Tumor growth curve of LoVo xenograft mice treated with vehicle (n=5), 10 mg/kg stattic (n=4), 20mg/kg irinotecan (n=4), or combination (n=7). (B) Tumor growth curve of LS180 xenograft mice treated with vehicle (n=8), 10 mg/kg stattic (n=6), 20mg/kg irinotecan (n=7), or combination (n=6). Statistical analysis was performed using one-way ANOVA and Dunnett's multiple comparison test. *, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.001$ compared to vehicle unless otherwise indicated. (C-D) Kaplan−Meier survival plot for (C) LoVo and (D) LS180 xenografts days post-treatment initiation. (E) Average body weight mice over the course of the study. Error bars are SD.

Chapter 4: Discussion

While LGR5+ cells have been consistently confirmed as a tumor initiating CSCs, able to regenerate all cancer cell type needed for proliferative growth, the actual role of LGR5 in plasticity, drug resistance, and metastasis has been of recent interest. Through LGR5 gene knockdown and overexpression studies, LGR5 expression has been linked to both tumor suppression and pro-oncogenic effects in CRC[122]. In this study, we showed that therapeutic treatment with standard chemotherapies, irinotecan and 5-FU, in CRC cells induces loss of LGR5 expression (Fig. 5A-C). Loss of LGR5 is due to gradual decrease in expression and not selection against LGR5+ vs LGR5- cells as the CRC cell lines are rather homogenous for LGR5 expression (Fig. 5C). Previously, we published that LGR5KD and LGR5KO cells have increased drug resistance to both 5-FU and irinotecan compared to their respective LGR5+ control cell lines[93]. These findings are consistent with prior reports showing both chemotherapy and radiotherapy initiate a switch from an LGR5+ state to a more drug resistant LGR5- state[91,92].

As previously mentioned, our lab generated an anti-LGR5-MMAE ADC by conjugating the antimitotic, tubulin-inhibiting agent monomethyl auristatin E (MMAE) by a cleavable chemical linker[57]. While targeting LGR5 resulted in cancer cell death in vitro and inhibited tumor growth and recurrence in vivo, once treatment was ceased, tumors re-emerged in some of the mice[57]. This suggested that tumor re-emergence was potentially due to the presence of LGR5- cancer cells which may have converted back to LGR5+ CSCs for tumor proliferation, in a manner similar to other findings[43,88,94]. Here we show that chemotherapy or ADC treatment in CRC cells induce loss of LGR5 expression (Fig. 5B-D); we further

confirmed that CRC cells indeed interconvert between LGR5+ and LGR5- states as removal of treatment leads to the re-emergence of LGR5 expression (Fig. 5E).

In addition to increased drug resistance and proliferation, which we and others have previously reported, we showed that loss of LGR5 in LoVo cells leads to increased clonogenicity, migration, and invasion (Fig. 6). Similar findings have been reported by other groups using different CRC cell lines[74,123] and relates to previous findings that showed the invasive front of CRCs consist of LGR5- cells and the majority of circulating cancer cells and seeding cells which are primarily LGR5- [94,124]. These findings support the notion that the loss of LGR5 expression may drive disease resistance and relapse after treatment and may also play an important role in the progression of CRC to metastasis. However, while these cells are capable of sustaining the tumor, the re-emergence of LGR5 expression is still necessary for rapid tumor growth, possibly to generate a more favorable microenvironment for growth[125].

Because of the increased tumorigenicity and drug resistance of LGR5KD cells, we wanted to identify signaling mechanisms which are mediating these phenotypic changes. Based on recent findings, mechanistically, LGR5 binds to an RSPO ligand to potentiate Wnt/β-catenin signaling[68]. However, in both normal and cancer cells loss of LGR5 expression led to increased activation and nuclear accumulation of β-catenin (Figs. 7A-B), and an increase in levels of markers related to activated Wnt/β-catenin signaling. This observation suggests that LGR5 may play a role in the negative regulation of Wnt signaling and as LGR5 is a transcriptional target gene of Wnt, it may act in a self-regulation manner [49,73,75]. Along with β-catenin activation, STAT3 and its upstream mediator JAK were found to have increased activation in LGR5KD LoVo cells (Fig. 7A). We also observed increased nuclear localization of STAT3, indicating increased transcriptional activation (Fig. 7B). This was further confirmed

through an observed an increased expression of Bcl-xL and Cyclin D1, which are transcriptional targets of STAT3 which promote cell survival, and drug resistance. Consistently, BIM, which directly antagonizes Bcl-xL was decreased in LGR5KD cells (Fig. 7C)[121,126,127]. We also showed that loss of LGR5 expression in DLD-1 and LS180 cells also resulted in increased STAT3 phosphorylation (Figs. 9B-C). These findings indicate that LGR5KD/KO cells may rely on STAT3 signaling for driving drug resistance and the metastatic phenotype.

Crosstalk and interactions between STAT3 and the Wnt/β-catenin signaling pathways has been previously reported[128,129,130,131]. However, LGR5KD CRC cells treated with XAV939, a small molecule inhibitor that enhances β-catenin degradation through stabilization of Axin2 a component of the destruction complex, did not dampen activation of STAT3 (Fig. 8D)[132,133]. These findings suggest that enhanced Wnt activation is likely not responsible for STAT3 activation; at least in LoVo cells. In fact, in findings by Ibraham et.al., show that β-catenin may be a transcriptional target for STAT3, leading to its upregulation[134]. Furthermore, suppression of STAT3 through targeted inhibition has been shown to prevent Wnt signaling activation[131]. However, since we observed nuclear accumulation of both STAT3 and β-catenin in LGR5KD cells (Fig. 7C), it is possible that they may interact to promote transcriptional activity.

As STAT3 signaling has been shown to be mediated through IL6R/Gp130, EGFR and MET[97,135], we investigated which possible upstream mediator may activate STAT3 in response to loss of LGR5. Since our CRC cell lines did not express IL6R and the Gp130 inhibitor SC144 failed to inhibit STAT3 activation in our LGR5KD cells (Fig. 8A), these findings suggest that increased STAT3 activation was not mediated by the IL-6R/Gp130 pathway. EGFR is an

important therapeutic target in CRC which has also been linked to activation of the JAK-STAT3 signaling pathway in CSCs and is believed to be responsible for maintaining CSC survival[104,136]. However, no significant increase in EGFR activation was observed in LGR5KD cells and treatment with EGFR inhibitor gefitinib failed to ablate STAT3 activation (Figure 8B-C). Together, these findings reveal that neither EGFR nor IL-6R/Gp130 were responsible for mediating STAT3 activation in LGR5KD CRC cells.

Overexpression of MET has been shown to play a critical role in progression and invasion in CRC, correlating with tumor staging and lymph/liver metastasis, making it a promising target in late-stage CRC treatment[107–109]. MET mutations are rarely found in patients with CRC (2-5%), however overexpression of HGF/MET was observed in 50-70% of CRCs[108,110]. This has been attributed to amplification of HGF-MET through either paracrine or autocrine loops as MET activation has been shown to enhance HGF transcriptional activation[137]. MET is also known to play a vital role in cell survival and acquired drug resistance through JAK-STAT3 signaling activation in CRC[98]. Interestingly, increased MET phosphorylation was observed in our LGR5KD/KO cells along with increased levels in the cytoplasm and nucleus (Figs. 9A-D). Increased MET/STAT3 activation was also detected in CRC cells with corresponding loss of LGR5 expression in response to either chemotherapeutic treatment or targeted LGR5 ADC treatment (Figure 10A-D), suggesting that MET/STAT3 activation plays a role in therapy induced CRC plasticity. Consistently, LGR5 overexpression in the LGR5- HCT116 CRC cell line resulted in decreased MET/STAT3 activation and increased irinotecan sensitivity, whereas constitutively active STAT3 led to downregulation of LGR5 expression along with enhanced drug resistance (Fig. 12). When treating our LGR5KD cells with the MET inhibitor, crizotinib, we observed loss of phosphorylated STAT3 (Fig. 9E).

These observations suggest that STAT3 activation with LGR5KD/KO is mediated by MET activation. This is significant as MET mediated activation of STAT3 has been implicated in EGFR and IL-6 targeted therapeutic resistance in CRC[138–140]. Another recent report showed enhanced MET/STAT3 signaling mediated resistance to MEK inhibitors in KRAS mutant CRC Cancer[58,141,142]. In our study, to test the potential effects of STAT3 inhibition on MET activation, we used stattic, a non-peptidic small molecule which inhibits STAT3 dimerization, and cryptotanshinone, a cell-permeable diterpenoid anthraquinone originally derived from *Salvia miltiorrhiza* which inhibits signaling of the JAK-STAT3 pathway[143–145]. Inhibition of STAT3 with either stattic or cryptotanshinone in LGR5KD cells led to loss of MET phosphorylation (Figs. 9E), indicating the existence of a possible positive feedback loop where MET phosphorylates STAT3, and in turn, STAT3 potentiates MET activation. It is currently unclear how STAT3 activation leads to activation of MET, but one study has posited that MET activated STAT3 enhances HGF transcriptional activity[146].

We demonstrated that MET or STAT3 inhibition synergized with irinotecan and anti-LGR5 ADCs in vitro (Fig. 13). LGR5KD/KO cells were more sensitive to STAT3 and MET inhibitors compared to their LGR5+ counterparts (Figs. 11A-G and Figs. 13A-B), indicating that LGR5- cells are more dependent on MET/STAT3 signaling for survival and integral for their enhanced drug resistance. Combination treatment of STAT3 inhibitor, stattic, with irinotecan enhanced therapeutic efficacy and survival in CRC xenograft models. Though STAT3 inhibitors have shown poor clinical efficacy and/or toxicity[147], several more STAT3 inhibitors are currently in development which appear more promising[148–150]. These findings indicate that STAT3 or MET-targeted agents may be highly effective when used in conjunction

with irinotecan or LGR5-targeted ADCs to treat CRC. Further in vivo studies of these combination therapies need to be further tested.

As mentioned previously, LGR5 plays a role in the dephosphorylation of IQGAP1, thus loss of LGR5 leads to accumulation of phosphorylated IQGAP1[73]. Importantly, IQGAP1 is phosphorylated at tyrosine sites by MET and shown to play an integral role in HGF induced MET activation[78,143.] This was demonstrated in a study which showed that inhibition of MET by crizotinib resulted in decreased phosphorylation of IQGAP1 and loss of IQGAP1 ablated HGF expression leading to MET deactivation[77]. Thus, we postulate that accumulation of phosphorylated IQGAP1 may be what is initiating the MET-STAT3 signaling activation and the positive feedback loop (Figure 12A-C). For instance, in LGR5- CRC, phosphorylated IQGAP1 may preferentially bind to the tyrosine kinase terminal on MET and increase co-localized kinase activation, or block activation and interaction of a phosphatase, such as SHP-2. SHP-2 is associated with the dephosphorylation of STAT3, inactivating its function[151,152]. As IQGAP1 also has been shown to shown to shuttle signaling transducer to the nucleus to potentiate activation, it may also play this role in localizing STAT3 to the perinuclear region promoting its translocation to the nucleus after it is activated by MET[79]. We propose a model which depicts LGR5 playing an antagonistic role in MET-STAT3 activation through its dephosphorylation of IQGAP1. This model sheds some light as to why loss of LGR5 leads to a more metastatic phenotype and why the majority of circulating tumor cells which seed metastases are LGR5-, as increased phosphorylation of IQGAP1 leads to decreased cell-to-cell adhesion promoting cell release from the primary tumor. This work concomitantly with activation of MET-STAT3 which leads to increased invasion and drug resistance. However, this proposed mechanism requires further investigation[73,109,124].

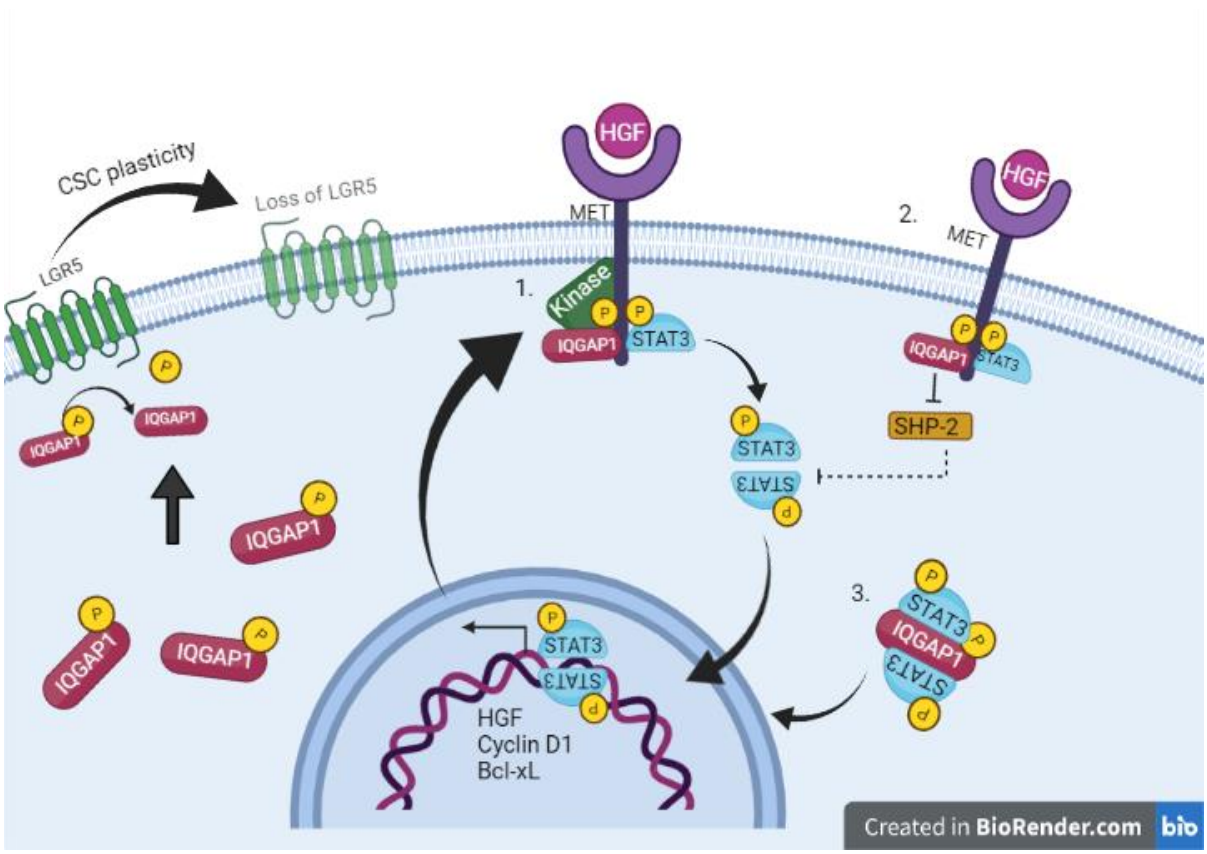


Figure 15: Proposed MET-IQGAP1-STAT3 Model. With loss of LGR5, phosphorylated IQGAP1 accumulates and may promote MET-STAT3 signaling by 1.) increasing kinase interaction with the intracellular MET-domain[77], 2.) IQGAP1 inhibiting SHP-2, a phosphatase which inhibits STAT3[151,152], 3) IQGAP1 acts as a shuttle to promote nuclear localization of STAT3[79], to promote transcription of target genes (i.e. HGF). (Created with BioRender.com)

CHAPTER 5: FUTURE DIRECTIONS

Going forward, our lab will be further exploring the relationship between LGR5-IQGAP1 and MET-STAT3 using other CRC cell lines and tumor organoids. We will perform co-immunoprecipitation and ICC and confocal analysis to detect changes in protein-protein interactions and localization in control vs. LGR5KD/KO cells. We will also identify new protein modulators that may be playing a role in this mechanism to drive plasticity and drug resistance. We will also continue in vivo experiments by testing in other STAT3 and MET inhibitors in combination with irinotecan or our anti-LGR5-targeted ADCs. Altogether, these proposed combination therapies may be an improved approach to overcome drug resistance and plasticity to eradicate CRC.

Bibliography

1. Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*. 2021;71(3):209-249.
2. Siegel, R.L., Miller, K.D., Goding Sauer, A., Fedewa, S., Butterly, L., Anderson, J., Cercek, A., Smith, R., Jemal, A. Colorectal cancer statistics, 2020. *CA: A Cancer Journal for Clinicians*. 2020;70(3):145-164.
3. Arnold, M., Sierra, M.S., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F. Global patterns and trends in colorectal cancer incidence and mortality. *Gut*. 2017;66(4):683-691.
4. Campos, F.G. Colorectal cancer in young adults: A difficult challenge. *World Journal of Gastroenterology*. 2017;23(28):5041-5044.
5. Weinberg, B.A., Marshall, J.L., Salem, M.E. The Growing Challenge of Young Adults with Colorectal Cancer. *Oncology (Williston Park)*. 2017;31(5):381-389.
6. US Preventive Services Task Force. Screening for Colorectal Cancer: US Preventive Services Task Force Recommendation Statement. *JAMA*. 2021;325(19):1965-1977.
7. Balzora, S., Issaka, R.B., Anyane-Yeboa, A., Gray, D.M., May, F.P. Impact of COVID-19 on colorectal cancer disparities and the way forward. *Gastrointestinal Endoscopy*. 2020;92(4):946-950.
8. Del Vecchio Blanco, G., Calabrese, E., Biancone, L., Monteleone, G., Paoluzi, O.A. The impact of COVID-19 pandemic in the colorectal cancer prevention. *International Journal of Colorectal Disease*. Published online June 4, 2020:1-4.
9. Lin, P.C., Yeh, Y.M., Wu, P.Y., Hsu, K.F., Chang, J.Y., Shen, M.R. Germline susceptibility variants impact clinical outcome and therapeutic strategies for stage III colorectal cancer. *Scientific Reports*. 2019;9(1):3931.
10. Kuipers, E.J., Grady, W.M., Lieberman, D., Seufferlein, T., Sung, J.J., Boelens, P.G., van de Velde, C.J.H., Colorectal cancer. *Nature Reviews Disease Primers*. 2015;(1):15065.
11. Fodde, R. The APC gene in colorectal cancer. *European Journal of Cancer*. 2002;38(7):867-871.
12. Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K., Kanamaru, R., Kanegae, Y., Saito, I., Nakamura, Y., Shiba, K., Noda, T. Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science*. 1997;278(5335):120-123.

13. Malki, A., El Ruz, R.A., Gupta, I., Allouch, A., Vranic, S., Al Moustafa, A.E. Molecular Mechanisms of Colon Cancer Progression and Metastasis: Recent Insights and Advancements. *International Journal of Molecular Science*. 2020;22(1): 130

14. Huang, D., Sun, W., Zhou, Y., Li, P., Chen, F., Chen, H., Xia, D., Xu, E., Lai, M., Wu, Y., Zhang, H. Mutations of key driver genes in colorectal cancer progression and metastasis. *Cancer Metastasis Review*. 2018;37(1):173-187

15. Müller, M.F., Ibrahim, A.E.K., Arends, M.J. Molecular pathological classification of colorectal cancer. *Virchows Archive: an International Journal of Pathology*. 2016;469(2):125-134.

16. De Rosa, M., Pace, U., Rega, D., Costabile, V., Duraturo, F., Izzo, P., Delrio, P. Genetics, diagnosis and management of colorectal cancer (Review). *Oncology Reports*. 2015;34(3):1087-1096.

17. American Joint Committee on Cancer. Chapter 20 - Colon and Rectum. In: AJCC Cancer Staging Manual. 8th ed. New York, NY: *Springer*; 2017.

18. Binefa, G., Rodríguez-Moranta, F., Teule, A., Medina-Hayas, M. Colorectal cancer: from prevention to personalized medicine. *World Journal of Gastroenterology*. 2014;20(22):6786-6808.

19. Costas-Chavarri, A., Nandakumar, G., Temin, S., Lopes, G., Cervantes, A., Cruz Correa, M., Engineer, R., Hamashima, C., Fuang Ho, G., Huitzil, F., Moghani, M., Sharara, A., Stern, M.C., The, C., Vazquez Manjarrez, S.E., Verjee, A., Yantiss, R., Shah, M.A. Treatment of Patients With Early-Stage Colorectal Cancer: ASCO Resource-Stratified Guideline. *Journal of Global Oncology*. 2019;5: 1-19.

20. Meyerhardt, J.A., Mayer, R.J. Systemic therapy for colorectal cancer. *New England Journal of Medicine*. 2005;(352):476–487.

21. Sauer, R., Becker, H., Hohenberger, W., Rodel, C., Wittekind, C., Fietkau, R., Martus, P., Tschmelitsch, J., Hager, E., Hess, C.F., Karstens, J.H., Liersch, T., Schmidberger, H., Raab, R. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *New England Journal of Medicine*. 2004;351(17):1731-1740.

22. Bosset, J.F., Collette, L., Calais, G., Mineur, L., Maingon, P., Radosevic-Jelic, L., Daban, A., Bardet, E., Beny, A., Ollier, J. Chemotherapy with preoperative radiotherapy in rectal cancer. *New England Journal of Medicine*. 2006;355(11):1114-1123.

23. Efficacy of adjuvant fluorouracil and folinic acid in colon cancer. International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators. *Lancet*. 1995;345(8955):939-944.

24. André, T., Boni, C., Mounedji-Boudiaf, L., Navarro, M., Tabernero, J., Hickish, T., Topham, C., Zaninelli, M., Clingan, P., Bridgewater, I., Tabah-Fisch, I., de Gramont, A. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *New England Journal of Medicine*. 2004;350(23):2343-2351.

25. Vodenkova, S., Buchler, T., Cervena, K., Veskrnova, V., Vodicka, P., Vymetalkova, V. 5-fluorouracil and other fluoropyrimidines in colorectal cancer: Past, present and future. *Pharmacology & Therapeutics*. 2020; 206:107447.

26. Akhtar, R., Chandel, S., Sarotra, P., Medhi, B. Current status of pharmacological treatment of colorectal cancer. *World Journal of Gastrointestinal Oncology*. 2014;6(6):177-183.

27. Wellstein, A., Giaccone, G., Atkins, M.B., Sausville, E.A. Cytotoxic Drugs. In: Brunton LL, Hilal-Dandan R, Knollmann BC, eds. *Goodman & Gilman's: The Pharmacological Basis of Therapeutics*. 13th ed. McGraw-Hill Education; 2017.

28. Haggar, F.A., Boushey, R.P. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clinic in Colon Rectal Surgery*. 2009;22(4):191-197.

29. Davies, J.M., Goldberg, R.M. Treatment of Metastatic Colorectal Cancer. *Seminars in Oncology*. 2011;38(4):552-560.

30. Piawah, S., Venook, A.P. Targeted therapy for colorectal cancer metastases: A review of current methods of molecularly targeted therapy and the use of tumor biomarkers in the treatment of metastatic colorectal cancer. *Cancer*. 2019;125(23):4139-4147.

31. Duineveld, L.A.M., van Asselt, K.M., Bemelman, W.A., Smits, A. B., Tanis, P.J., van Weert, H.C.P.M., Wind, J. Symptomatic and Asymptomatic Colon Cancer Recurrence: A Multicenter Cohort Study. *Annals of Family Medicine*. 2016;14(3):215-220.

32. Farhat, W., Azzaza, M., Mizouni, A., Ammar, H., Ben Ltaifa, M., Lagha, S., Kahloul, M., Gupta, R., Ben Mabrouk, M., Ben Ali, A. Factors predicting recurrence after curative resection for rectal cancer: a 16-year study. *World Journal of Surgical Oncology*. 2019;17(1):173.

33. Douillard, J.Y., Cunningham, D., Roth, A.D., Navarro, M., James, R.D., Karasek, P., Jandik, P., Iveson, T., Carmichael, J., Alakl, M., Gruia, G., Awad, L., Rougier, P. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet*. 2000;355(9209):1041-1047.

34. Van der Jeught, K., Xu, H.C., Li, Y.J., Lu, X.B., Ji, G. Drug resistance and new therapies in colorectal cancer. *World Journal of Gastroenterology*. 2018;24(34):3834-3848.

35. Van Cutsem, E., Köhne, C.H., Láng, I., Folprecht, G., Nowacki, M.P., Cascinu, S., Shchepotin, I., Maurel, J., Cunningham, D., Tejpar, S., Schlichting, M., Zubel, A., Celik, I., Rougier, P., Ciardiello, F. Cetuximab Plus Irinotecan, Fluorouracil, and Leucovorin As First-Line Treatment for Metastatic Colorectal Cancer: Updated Analysis of Overall Survival According to Tumor *KRAS* and *BRAF* Mutation Status. *Journal of Clinical Oncology*. 2011;29(15):2011-2019.

36. Schwartzberg, L.S., Rivera, F., Karthaus, M., Fasola, G., Canon, J., Hecht, J., Yu, H., Oliner, K.S., Go, W.Y. PEAK: a randomized, multicenter phase II study of panitumumab plus modified fluorouracil, leucovorin, and oxaliplatin (mFOLFOX6) or bevacizumab plus mFOLFOX6 in patients with previously untreated, unresectable, wild-type KRAS exon 2 metastatic colorectal cancer. *Journal of Clinical Oncology*. 2014;32(21):2240-2247.

37. Sforza, V., Martinelli, E., Ciardiello, F., Gambardella, V., Napolitano, S., Martini, G., della Corte, C., Cardone, C., Ferrara, M.L., Reginelli, A., Liguori, G., Belli, G., Troiani, R. Mechanisms of resistance to anti-epidermal growth factor receptor inhibitors in metastatic colorectal cancer. *World Journal of Gastroenterology*. 2016;22(28):6345-6361.

38. de Gramont, A., Van Cutsem, E., Schmoll, H.J., Tabernero, J., Clarke, S., Moore, M.J., Cunningham, D., Cartwright, T.H., Hecht, J.R., Rivera, F., Im, S., Bodoky, G., Salazar, R., Maindault-Goebel, F., Shacham-Shmueli, E., Bajetta, E., Makrutzki, M., Shang, A., Andre, T., Hoff, P.M. Bevacizumab plus oxaliplatin-based chemotherapy as adjuvant treatment for colon cancer (AVANT): a phase 3 randomised controlled trial. *Lancet Oncology*. 2012;13(12):1225-1233.

39. Xie, Y.H., Chen, Y.X., Fang, J.Y. Comprehensive review of targeted therapy for colorectal cancer. *Signal Transduction Targeted Therapy*. 2020;5(1):1-30.

40. Ganesh, K., Stadler, Z.K., Cercek, A., Mendelsohn, R.B., Shia, J., Segal, N.H., Diaz, L.A. Immunotherapy in colorectal cancer: rationale, challenges and potential. *Nature Reviews, Gastroenterology & Hepatology*. 2019;16(6):361-375.

41. Punt, C.J.A., Koopman, M., Vermeulen, L. From tumour heterogeneity to advances in precision treatment of colorectal cancer. *Nature Reviews, Clinical Oncology*. 2017;14(4):235-246.

42. Kreso, A., Dick, J.E. Evolution of the cancer stem cell model. *Cell Stem Cell*. 2014;14(3):275-291.

43. Shimokawa, M., Ohta, Y., Nishikori, S., Matano, M., Takano, A., Fujii, M., Date, S., Sugimoto, S., Kanai, T., Sato, T. Visualization and targeting of LGR5+ human colon cancer stem cells. *Nature*. 2017;545(7653):187-192.

44. Greenow, K., Clarke, A.R. Controlling the stem cell compartment and regeneration in vivo: the role of pluripotency pathways. *Physiological Reviews*. 2012;92(1):75-99.

45. Humphries, A., Wright, N.A. Colonic crypt organization and tumorigenesis. *Nature Reviews Cancer*. 2008;(8):415-424.

46. Ricci-Vitiani, L., Pagliuca, A., Palio, E., Zeuner, A., De Maria, R. Colon cancer stem cells. *Gut.* 2008;57(4):538-548.

47. Zheng, S., Xin, L., Liang, A., Fu, Y. Cancer stem cell hypothesis: a brief summary and two proposals. *Cytotechnology*. 2013;65(4):505-512.

48. Schepers, A.G., Snippert, H.J., Stange, D.E., van den Born, M., van Es, J.H., van de Wetering, M., Clevers, H. Lineage tracing reveals Lgr5+ stem cell activity in mouse intestinal adenomas. *Science*. 2012;337(6095):730-735.

49. Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., Clevers, H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*. 2007;449(7165):1003-1007.

50. Leblond, C.P., Stevens, C.E. The constant renewal of the intestinal epithelium in the albino rat. *The Anatomical Record*. 1948;100(3):357-377.

51. Sun, X., Terakawa, J., Clevers, H., Barker, N., Daikoku, T., Dey, S.K. Ovarian LGR5 is critical for successful pregnancy. *FASEB*. 2014;28(5):2380-2389.

52. Huch, M., Dorrell, C., Boj, S.F., van Es, J.H., Li, V.S.W., van de Wetering, M., Sato, T., Hamer, K., Sasaki, N., Finegold, M.J., Haft, A., Vries, R.G., Grompe, M., Clevers, H. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature*. 2013;494(7436):247-250.

53. Barker, N., Bartfeld, S., Clevers, H. Tissue-resident adult stem cell populations of rapidly self-renewing organs. *Cell Stem Cell*. 2010;7(6):656-670.

54. Barker, N., Huch, M., Kujala, P., van de Wetering, M., Snippert, H.J., van Es, J.H., Sato, T., Stange, D.E., Begthel, H., van den Born, M., Danenberg, E., van den Brink, S., Korving, J., Abo, A., Peters, P.J., Wright, N., Poulsom, R., Clevers, H. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell*. 2010;6(1):25-36.

55. Kemper, K., Prasetyanti, P.R., De Lau, W., Rodermond, H., Clevers, H., Medema, J.P. Monoclonal antibodies against Lgr5 identify human colorectal cancer stem cells. *Stem Cells*. 2012;30(11):2378-2386.

56. Leng, Z., Xia, Q., Chen, J., Li, Y., Xu, J., Zhao, E., Zheng, H., Ai, W., Dong, J. Lgr5+CD44+EpCAM+ Strictly Defines Cancer Stem Cells in Human Colorectal Cancer. *Cellular Physiology and Biochemistry*. 2018;46(2):860-872.

57. Gong, X., Azhdarinia, A., Ghosh, S.C., Xiong, W., An, Z., Liu, Q., Carmon, K.S. LGR5-Targeted Antibody-Drug Conjugate Eradicates Gastrointestinal Tumors and Prevents Recurrence. *Molecular Cancer Therapy.* 2016;15(7):1580-1590.

58. Junttila, M.R., Devasthali, V., Cheng, J.H., Castillo, J., Metcalfe, C., Clermont, A. C., Den Otter, D., Chan, E., Bou-Reslan, H., Cao, T., Forrest, W., Nannini, M.A., French, D., Carano, R., Merchant, M., Hoeflich, K.P., Singh, M. Modeling Targeted Inhibition of MEK and PI3 Kinase in Human Pancreatic Cancer. *Molecular Cancer Therapy*. 2015;14(1):40-47.

59. Fan, X.S., Wu, H.Y., Yu, H.P., Zhou, Q., Zhang, Y.F., Huang, Q. Expression of Lgr5 in human colorectal carcinogenesis and its potential correlation with beta-catenin. *International Journal of Colorectal Disease.* 2010;25(5):583-590.

60. Uchida, H., Yamazaki, K., Fukuma, M., Yamada, T., Hayashida, T., Hasegawa, H., Kitajima, M., Kitagawa, Y., Sakamoto, M. Overexpression of leucine-rich repeat-containing G protein-coupled receptor 5 in colorectal cancer. *Cancer Science*. 2010;101(7):1731-1737.

61. Gao, F.J., Chen, J.Y., Wu, H.Y., Shi, J., Chen, M., Fan, X., Huang, Q. Lgr5 over-expression is positively related to the tumor progression and HER2 expression in stage pTNM IV colorectal cancer. *International Journal of Clinical & Experimental Pathology*. 2014;7(4):1572-1579.

62. Ziskin, J.L., Dunlap, D., Yaylaoglu, M., Fodor, I.K., Forrest, W.F., Patel, R., Ge, N., Hutchins, G.G., Pine, J.K., Quirke, P., Koeppen, H., Jubb, A.M. In situ validation of an intestinal stem cell signature in colorectal cancer. *Gut*. 2013;62(7):1012-1023.

63. Chen, Q., Zhang, X., Li, W.M., Ji, Y.Q., Cao, H.Z., Zheng, P. Prognostic value of LGR5 in colorectal cancer: a meta-analysis. *PloS One.* 2014;9(9):107013.

64. Hsu, S.Y., Liang, S.G., Hsueh, A.J. Characterization of two LGR genes homologous to gonadotropin and thyrotropin receptors with extracellular leucine-rich repeats and a G protein-coupled, seven-transmembrane region. *Molecular Endocrinology*. 1998;12(12):1830-1845.

65. Schuijers, J., Clevers, H. Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. *EMBO J*. 2012;31(12):2685-2696.

66. Carmon, K.S., Gong, X., Lin, Q., Thomas, A., Liu, Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/β-catenin signaling. *PNAS.* 2011;108(28):11452-11457.

67. De Lau, W., Barker, N., Low, T.Y., Koo, B., Li, V.S.W., Teunissen, H., Kujala, P., Haegebarth, A., Peters, P.J., van de Wetering, M., Stange, D.E., van Es, J., Guardavaccaro, D., Schasfoort, R.B.M., Mohri, Y., Nishimori, K., Mohammed, S., Heck, A.J.R., Clevers, H. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature*. 2011; (476):293-297.

68. Carmon, K.S., Lin, Q., Gong, X., Thomas, A., Liu, Q. LGR5 Interacts and Cointernalizes with Wnt Receptors to Modulate Wnt/β-Catenin Signaling. *Molecular Cellular Biology*. 2012;32(11):2054-2064.

69. Glinka, A., Dolde, C., Kirsch, N., Huang, Y., Kazanskaya, O., Ingelfinger, D., Boutros, M., Cruciat, C., Niehrs, C. LGR4 and LGR5 are R-spondin receptors mediating Wnt/β-catenin and Wnt/PCP signaling. *EMBO Reports*. 2011;12(10):1055-1061.

70. de Lau, W., Peng, W.C., Gros, P., Clevers, H. The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. *Genes & Development*. 2014;28(4):305-316.

71. Park, S., Wu, L., Tu, J., Yu, W., Toh, Y., Carmon, K.S., Liu, Q. Unlike LGR4, LGR5 potentiates Wnt–β-catenin signaling without sequestering E3 ligases. *Science Signaling*. 2020; 660(13)

72. Snyder, J.C., Rochelle, L.K., Lyerly, H.K., Caron, M.G., Barak, L.S. Constitutive internalization of the leucine-rich G protein-coupled receptor-5 (LGR5) to the trans-Golgi network. *Journal of Biological Chemistry*. 2013;288(15):10286-10297.

73. Carmon, K.S., Gong, X., Yi, J., Wu, L., Thomas, A., Moore, C.M., Masuho, I., Timson, D.J., Martemyanov, K.A., Liu, Q.J. LGR5 receptor promotes cell-cell adhesion in stem cells and colon cancer cells via the IQGAP1-Rac1 pathway. *Journal of Biological Chemistry*. 2017;292(36):14989-15001.

74. Walker, F., Zhang, H.H., Odorizzi, A., Burgess, A.W. LGR5 is a negative regulator of tumourigenicity, antagonizes Wnt signalling and regulates cell adhesion in colorectal cancer cell lines. *PloS One*. 2011;6(7):22733.

75. Garcia, M.I., Ghiani, M., Lefort, A., Libert, F., Strollo, S., Vassart, G. LGR5 deficiency deregulates Wnt signaling and leads to precocious Paneth cell differentiation in the fetal intestine. *Developmental Biology*. 2009;331(1):58-67.

76. McNulty, D.E., Li, Z., White, C.D., Sacks, D.B., Annan, R.S. MAPK scaffold IQGAP1 binds the EGF receptor and modulates its activation. *Journal of Biological Chemistry*. 2011;286(17):15010-15021.

77. Hedman, A.C., McNulty, D.E., Li, Z., Gorisse, L., Annan, R.S., Sacks, D.B. Tyrosine phosphorylation of the scaffold protein IQGAP1 in the MET pathway alters function. *Journal of Biological Chemistry*. 2020;295(52):18105-18121.

78. Johnson, M., Sharma, M., Henderson, B.R. IQGAP1 regulation and roles in cancer. *Cell Signaling*. 2009;21(10):1471-1478.

79. Smith, J.M., Hedman, A.C., Sacks, D.B. IQGAPs choreograph cellular signaling from the membrane to the nucleus. *Trends in Cellular Biology*. 2015;25(3):171-184.

80. Noritake, J., Watanabe, T., Sato, K., Wang, S., Kaibuchi, K. IQGAP1: a key regulator of adhesion and migration. *Journal of Cellular Science*. 2005;118(Pt 10):2085-2092.

81. Noritake, J., Fukata, M., Sato, K., Nakagawa, M., Watanabe, T., Izumi, N., Wang, S., Fukata, Y., Kaibuchi, K. Positive Role of IQGAP1, an Effector of Rac1, in Actin-Meshwork Formation at Sites of Cell-Cell Contact. *Molecular Biology of the Cell*. 2004;15(3):1065-1076.

82. Ma, Y.S., Li, W., Liu, Y., Shi, Y., Lin, Q.L., Fu, D. Targeting Colorectal Cancer Stem Cells as an Effective Treatment for Colorectal Cancer. Technology in Cancer Research & Treatment. 2020;19:1-9

83. Leal, M., Sapra, P., Hurvitz, S.A., Senter, P., Wahl, A., Schutten, M., Shah, D.K., Haddish-Berhane, N., Kabbarah, O. Antibody-drug conjugates: an emerging modality for the treatment of cancer. *Annals of the New York Academy of Sciences*. 2014;1321:41-54.

84. Hafeez, U., Parakh, S., Gan, H.K., Scott, A.M. Antibody-Drug Conjugates for Cancer Therapy. *Molecules*. 2020;25(20):4764.

85. Junttila, M.R., Mao, W., Wang, X., Wang, B., Pham, T., Flygare, J., Yu, S., Yee, S., Goldenberg, D., Fields, C., Eastham-Anderson, J., Singh, M., Vij, R., Hongo, J., Firestein, R., Schutten, M., Flagella, K., Polakis, P., Polson, A.G. Targeting LGR5+ cells with an antibody-drug conjugate for the treatment of colon cancer. *Science Translational Medicine*. 2015;7(314):314ra186.

86. Batlle, E., Clevers, H., Cancer stem cells revisited. *Nature Medicine*. 2017; (23):1124-1134.

87. Vlashi, E., Pajonk, F. Cancer stem cells, cancer cell plasticity and radiation therapy. *Seminars in Cancer Biology*. 2015; 31:28-35.

88. Tetteh, P.W., Basak, O., Farin, H.F., Wiebrands, K., Kretzschmar, K., Begthel, H., van den Born, M., Kroving, J., de Sauvage, F., van Es, J.H., van Oudenaarden, A., Clevers, H. Replacement of Lost Lgr5-Positive Stem Cells through Plasticity of Their Enterocyte-Lineage Daughters. *Cell Stem Cell*. 2016;18(2):203-213.

89. Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Schroyer, N.F., van de Wetering, M., Clevers, H. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature*. 2011;469(7330):415-418.

90. de Sousa e Melo, F., Kurtova, A.V., Harnoss, J.M., Kljavin, N., Hoeck, J.D., Hung, J., Anderson, J.E., Storm, E.E., Modrusan, Z., Koeppen, H., Dijkgraaf, G.J.P., Piskol, R., de Sauvage, F.J. A distinct role for Lgr5+ stem cells in primary and metastatic colon cancer. *Nature*. 2017;543(7647):676-680.

91. Asfaha, S., Hayakawa, Y., Muley, A., Stokes, S., Graham, T.A., Ericksen, R., Westphalen, C.B., von Burstin, J., Mastracci, T.L., Worthley, D.L., Guha, C., Quante, M., Rustgi, A.K., Wang, T.C. Krt19(+)/Lgr5(−) cells are radioresistant cancer initiating stem cells in the colon and intestine. *Cell Stem Cell*. 2015;16(6):627-638.

92. Kobayashi, S., Yamada-Okabe, H., Suzuki, M., Natori, O., Kato, A., Matsubara, K., Chen, Y.J., Yamazaki, M., Funahashi, S., Yoshida, K., Hashimoto, E., Watanabe, Y., Mutoh, H., Ashihara, M., Kato, C., Watanabe, T., Yoshikubo., T., Tamaoki, N., Ochiya, T., Kuroda, M., Levine, A.J., Yamazaki, T. LGR5-positive colon cancer stem cells interconvert with drug-resistant LGR5-negative cells and are capable of tumor reconstitution. *Stem Cells*. 2012;30(12):2631-2644.

93. Zhang, S., Chatterjee, T., Godoy, C., Wu, L., Liu, Q.J., Carmon, K.S. GPR56 drives colorectal tumor growth and promotes drug resistance through upregulation of MDR1 expression via a RhoA-mediated mechanism. *Molecular Cancer Research*. 2019;17(11):2196-2207.

94. Fumagalli, A., Oost, K.C., Kester, L., Morgner, J., Bornes, L., Bruens, L., Spaargaren, L., Azkanaz, M., Schelfhorst, T., Beerling, E., Heinz, M.C., Postrach, D. Seinstra, D., Sieuwers, A.M., Martens, J.W.M., van der Elst, S., van Baalen, M., Bhowmick, D., Vrisekoop, N., Ellenbroek, S.I.J., Suijkerbuijk, S.J.E., Snippert, H.J., van Rheenan, J. Plasticity of Lgr5-Negative Cancer Cells Drives Metastasis in Colorectal Cancer. *Cell Stem Cell*. 2020;26(4):569-578.

95. Darnell, J.E., Kerr, I.M., Stark, G.R. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*. 1994;264(5164):1415-1421.

96. Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D. How cells respond to interferons. *Annual Review of Biochemistry*. 1998; 67:227-264.

97. Mohan, C.D., Rangappa, S., Preetham, H.D., Nayaka, S.C., Gupta, V.K., Basappa, S., Sethi, G., Rangappa, K. Targeting STAT3 signaling pathway in cancer by agents derived from Mother Nature. *Seminars in Cancer Biology*. Published online April 20, 2020: S1044-579X(20)30082-1.

98. Barrow-McGee, R., Kermorgant, S. Met endosomal signalling: in the right place, at the right time. *International Journal of Biochemistry & Cell Biology*. 2014; 49:69-74.

99. Bournazou, E., Bromberg, J. Targeting the tumor microenvironment: JAK-STAT3 signaling. *JAK-STAT*. 2013;2(2):e23828.

100. Chung, Y.C., Chang, Y.F. Serum interleukin-6 levels reflect the disease status of colorectal cancer. *Journal of Surgical Oncology*. 2003;83(4):222-226.

101. Wang, S.W., Sun, Y.M. The IL-6/JAK/STAT3 pathway: potential therapeutic strategies in treating colorectal cancer (Review). *International Journal of Oncology*. 2014;44(4):1032-1040.

102. Rose-John, S., Scheller, J., Elson, G., Jones, S.A. Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. *Journal of Leukocyte Biology*. 2006;80(2):227-236.

103. Hutchinson, R.A., Adams, R.A., McArt, D.G., Salto-Tellez, M., Jasani, B., Hamilton, P.W. Epidermal growth factor receptor immunohistochemistry: new opportunities in metastatic colorectal cancer. *Journal of Translational Medicine*. 2015;13:217.

104. Krasinskas, A.M. EGFR Signaling in Colorectal Carcinoma. *Pathology Research International*. 2011; 2011:932932.

105. Ruzzo, A., Graziano, F., Canestrari, E., Magnani, M. Molecular predictors of efficacy to anti-EGFR agents in colorectal cancer patients. *Current Cancer Drug Targets*. 2010;10(1):68-79.

106. Park, O.K., Schaefer, T.S., Nathans, D. In vitro activation of Stat3 by epidermal growth factor receptor kinase. *PNAS*. 1996;93(24):13704-13708.

107. Di Renzo, M.F., Olivero, M., Giacomini, A., Porte, H., Chastre, E., Mirossay, L., Nordlinger, B., Bretti, S. Bottardi, S., Giordano, S. Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. *Clinical Cancer Research*. 1995;1(2):147-154.

108. Gayyed, M.F., Abd El-Maqsoud, N.M.R., El-Hameed El-Heeny, A.A., Mohammed, M.F. c-MET expression in colorectal adenomas and primary carcinomas with its corresponding metastases. *Journal of Gastrointestinal Oncology*. 2015;6(6):618-627.

109. Yao, J.F., Li, X.J., Yan, L.K., He, S., Zheng, J.B., Wang, X.R., Zhou, P.H., Zhang, L., Wei, G.B., Sun, X.J. Role of HGF/c-Met in the treatment of colorectal cancer with liver metastasis. *Journal of Biochemical and Molecular Toxicology*. 2019;33(6):22316.

110. Kondo, S., Ojima, H., Tsuda, H., Hashimoto, J., Morizane, C., Ikeda, M., Ueno, H., Tamura, K., Shimada, K., Kanai, Y., Okusaka, T. Clinical impact of c-Met expression and its gene amplification in hepatocellular carcinoma. *International Journal of Clinical Oncology*. 2013;18(2):207-213.

111. Joosten, S.P.J., Zeilstra, J., van Andel, H., Mijnals, R. C., Zaunbrecher, J., Duivenvoorden, A.A.M., van de Wetering, M., Clevers, H., Spaargaren, M., Pals, S.T. MET Signaling Mediates Intestinal Crypt-Villus Development, Regeneration, and Adenoma Formation and Is Promoted by Stem Cell CD44 Isoforms. *Gastroenterology*. 2017;153(4):1040-1053.

112. Morikawa, T., Baba, Y., Yamauchi, M., Kuchiba, A., Nosho, K., Shima, K., Tanaka, N., Huttenhower, C., Frank, D.A., Fuchs, C.S., Ogino, S. STAT3 expression, molecular features, inflammation patterns, and prognosis in a database of 724 colorectal cancers. *Clinical Cancer Research.* 2011;17(6):1452-1462.

113. Kusaba, T., Nakayama, T., Yamazumi, K., Yakata, Y., Yoshizaki, A., Inoue, K., Nagayasu, T., Sekine, I. Activation of STAT3 is a marker of poor prognosis in human colorectal cancer. *Oncology Reports*. 2006;15(6):1445-1451.

114. Corvinus, F.M., Orth, C., Moriggl, R., Tsareva, S.A., Wagner, S., Pfitzner, E.B., Baus, D., Kaufmann, R., Huber, L. A., Zatloukal, K., Beug, H., Ohschlager, P., Schutz, A., Halbhuber, K.J., Friedrich, K. Persistent STAT3 Activation in Colon Cancer Is Associated with Enhanced Cell Proliferation and Tumor Growth. *Neoplasia.* 2005;7(6):545-555.

115. Xiong, H., Zhang, Z.G., Tian, X.Q., Sun, D.F., Liang, Q.C., Zhang, Y.J., Lu, R., Chen, Y.X., Fang, J.Y. Inhibition of JAK1, 2/STAT3 Signaling Induces Apoptosis, Cell Cycle Arrest, and Reduces Tumor Cell Invasion in Colorectal Cancer Cells. *Neoplasia*. 2008;10(3):287-297.

116. Spitzner, M., Emons, G., Kramer, F., Gaedcke, J., Rave-Frank, M., Scharf, J.G., Burfeind, P., Becker, H., Beissbarth, T., Ghadimi, B.M., Ried, T., Grade, M. A gene expression signature for chemoradiosensitivity of colorectal cancer cells. *International Journal of Radiatation, Oncology, Biology, Physics.* 2010;78(4):1184-1192.

117. Barré, B., Vigneron, A., Perkins, N., Roninson, I.B., Gamelin, E., Coqueret, O. The STAT3 oncogene as a predictive marker of drug resistance. *Trends in Molecular Medicine.* 2007;13(1):4-11.

118. Dobi, E., Monnien, F., Kim, S., Ivanaj, A., N'Guyen, T., Demarchi, M., Adotevi, O., Thierry-Vuillemin, A., Jary, M., Kantelip, B., Pivot, X., Godet, Y., Degano, S.V., Borg, C. Impact of STAT3 phosphorylation on the clinical effectiveness of anti-EGFR-based therapy in patients with metastatic colorectal cancer. *Clinical Colorectal Cancer*. 2013;12(1):28-36.

119. Park, S.Y., Lee, C.J., Choi, J.H., Kim, J.H., Kim, J.W., Kim, J.Y., Nam, J.S. The JAK2/STAT3/CCND2 Axis promotes colorectal Cancer stem cell persistence and radioresistance. *Journal of Experimental & Clinical Cancer Research.* 2019;38(1):399.

120. Shih, P.C., Mei, K.C. Role of STAT3 signaling transduction pathways in cancer stem cell-associated chemoresistance. *Drug Discovery Today.* 2021;26(6):1450-1458.

121. Qin, A., Yu, Q., Gao, Y., Tan, J., Huang, H., Qiao, Z., Qian, W. Inhibition of STAT3/cyclinD1 pathway promotes chemotherapeutic sensitivity of colorectal cancer. *Biochemical Biophysical Research Communications.* 2015;457(4):681-687.

122. Morgan, R.G., Mortensson, E., Williams, A.C. Targeting LGR5 in Colorectal Cancer: therapeutic gold or too plastic? *British Journal of Cancer*. 2018;118(11):1410-1418.

123. Zhou, X., Geng, L., Wang, D., Yi, H., Talmon, G., Wang, J. R-Spondin1/LGR5 Activates TGFβ Signaling and Suppresses Colon Cancer Metastasis. *Cancer Research.* 2017;77(23):6589-6602.

124. Ganesh, K. Plasticity in Motion: Shape-Shifting Lgr5− Cells Initiate Colorectal Cancer Metastasis. *Cell Stem Cell.* 2020;26(4):469-471.

125. Chen, X., Wei, B., Han, X., Zheng, Z., Huang, J., Liu, J., Huang, Y., Wei, H. LGR5 is required for the maintenance of spheroid-derived colon cancer stem cells. *International Journal of Molecular Medicine.* 2014;34(1):35-42.

126. Leslie, K., Lang, C., Devgan, G., Azare, J., Berishaj, M., Gerald, W., Kim, Y.B., Paz, K., Darnell, J.E., Albanese, C., Sakamaki, T., Pestell, R., Bromberg, J. Cyclin D1 is transcriptionally regulated by and required for transformation by activated signal transducer and activator of transcription 3. *Cancer Research.* 2006;66(5):2544-2552.

127. Lassmann, S., Schuster, I., Walch, A., Gobel, H., Jutting, U., Makowiec, F., Hopt, U., Werner, M. STAT3 mRNA and protein expression in colorectal cancer: effects on STAT3-inducible targets linked to cell survival and proliferation. Journal of Clinical Pathology. 2007;60(2):173-179.

128. Sinnberg, T., Makino, E., Krueger, M.A., Velic, A., Macek, B., Rothbauer, U., Groll, N., Potz, O., Czemmel, S., Niessner, H., Meier, F., Ikenberg, K., Garbe, C., Schittek, B. A Nexus Consisting of Beta-Catenin and Stat3 Attenuates BRAF Inhibitor Efficacy and Mediates Acquired Resistance to Vemurafenib. *EBioMedicine.* 2016; 8:132-149.

129. Ahmad, R., Kumar, B., Chen, Z., Chen, X., Muller, D., Lele, S.M., Washington, M.K., Batra, S.K., Dhawan, P., Singh, A.B. Loss of claudin-3 expression induces IL6/gp130/Stat3 signaling to promote colon cancer malignancy by hyperactivating Wnt/β-catenin signaling. *Oncogene.* 2017;36(47):6592-6604.

130. Chen, M.W., Yang, S.T., Chien, M.H., Hua, K.T., Wu, C.J., Hsiao, S.M., Lin, H., Hsiao, M., Su, J.L., Wei, L.H. The STAT3-miRNA-92-Wnt Signaling Pathway Regulates Spheroid Formation and Malignant Progression in Ovarian Cancer. *Cancer Research*. 2017;77(8):1955-1967.

131. Phesse, T.J., Buchert, M., Stuart, E., Flanagan, D.J., Faux, M., Afshar-Sterle, S., Walker, F., Zhang, H.H., Nowell, C.J., Jorissen, R., Tan, C.W., Hirokawa, Y., Eissmann, M.F., Poh, A.R., Malaterre, J., Pearson, H.B., Kirch, D.G., Provero, P., Poli, V., Ramsay, R.G., Sieber, O., Burgess, A.W., Huszar, D., Vincan, E., Ernst, M. Partial inhibition of gp130-Jak-Stat3 signaling prevents Wnt–β-catenin–mediated intestinal tumor growth and regeneration. *Science Signaling*. 2014;7(345):ra92-ra92.

132. Jung, Y.S., Park, J.I. Wnt signaling in cancer: therapeutic targeting of Wnt signaling beyond β-catenin and the destruction complex. *Experimental Molecular Medicine*. 2020;52(2):183-191.

133. Wu, X., Luo, F., Li, J., Zhong, X., Liu, K. Tankyrase 1 inhibitior XAV939 increases chemosensitivity in colon cancer cell lines via inhibition of the Wnt signaling pathway. *International Journal of Oncology*. 2016;48(4):1333-1340.

134. Ibrahem, S., Al-Ghamdi, S., Baloch, K., Muhammed, B., Fadhil, W., Jackson, D., Nateri, A.S., Ilyas, M. STAT3 paradoxically stimulates β-catenin expression but inhibits β-catenin function. *International Journal of Experimental Pathology*. 2014;95(6):392-400.

135. Jin, W. Role of JAK/STAT3 Signaling in the Regulation of Metastasis, the Transition of Cancer Stem Cells, and Chemoresistance of Cancer by Epithelial–Mesenchymal Transition. *Cells*. 2020;9(1):217.

136. Cheng, C.C., Liao, P.N., Ho, A.S., Lim, K.H., Chang, J., Su, Y.W., Chen, C.G.S., Chiang, Y.W., Yang, B.L., Lin, H.C., Chang, Y.C., Chang, C.C., Chang, Y.F. STAT3 exacerbates survival of cancer stem-like tumorspheres in EGFR-positive colorectal cancers: RNAseq analysis and therapeutic screening. *Journal of Biomedical Science*. 2018;25(1):60.

137. Kentsis, A., Reed, C., Rice, K.L., Sanda, T., Rodig, S.J., Tholouli, E., Christie, A., Valk, P.J.M., Delwel, R., Ngo,V., Kutok, J.L., Dahlberg, S.E., Moreau, L.A., Bryers, R.J., Christensen, J.G., Woude, G.V., Licht, J.D., Kung, A.L., Staudt, L.M., Look, A.T. Autocrine activation of the MET receptor tyrosine kinase in acute myeloid leukemia. *Nature Medicine*. 2012;18(7):1118-1122.

138. Wu, K., Chang, Q., Lu, Y., Qiu, P., Chen, B., Thakur, C., Sun, J., Li, L., Kowluru, A., Chen, F. Gefitinib resistance resulted from STAT3-mediated Akt activation in lung cancer cells. *Oncotarget*. 2013;4(12):2430-2438.

139. Huang, X., Li, E., Shen, H., Wang, X., Tang, T., Zhang, X., Xu, J., Tang, Z., Guo, C., Bai, X., Liang, T. Targeting the HGF/MET Axis in Cancer Therapy: Challenges in Resistance and Opportunities for Improvement. *Frontiers in Cell and Developmental Biology*. 2020; 8:152.

140. Giannoni, P., de Totero, D. The HGF/c-MET axis as a potential target to overcome survival signals and improve therapeutic efficacy in multiple myeloma. *Cancer Drug Resistance*. 2021;4(4):923-933.

141. Van Schaeybroeck, S., Kalimutho, M., Dunne, P.D., Carson, R., Allen, W., Jithesh, P.V., Redmond, K.L., Sasazuki, T., Shirasawa, S., Blayney, J., Michieli, P., Fenning, C., Lenz, H., Lawler, M., Longley, D.B., Johnston, P.G. ADAM17-Dependent c-MET-STAT3 Signaling Mediates Resistance to MEK Inhibitors in KRAS Mutant Colorectal Cancer. *Cell Reports*. 2014;7(6):1940-1955.

142. Jin, J., Guo, Q., Xie, J., Jin, D., Zhu, Y. Combination of MEK Inhibitor and the JAK2-STAT3 Pathway Inhibition for the Therapy of Colon Cancer. *Pathology & Oncology Research*. 2019; (25):769-775.

143. Schust, J., Sperl, B., Hollis, A., Mayer, T.U., Berg, T. Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. *Chemistry & Biology*. 2006;13(11):1235-1242.

144. Chen, W., Lu, Y., Chen, G., Huang, S. Molecular evidence of cryptotanshinone for treatment and prevention of human cancer. Anticancer *Agents in Medicinal Chemistry*. 2013;13(7):979-987.

145. Wu, Y.H., Wu, Y.R., Li, B., Yan, Z.Y. Cryptotanshinone: A review of its pharmacology activities and molecular mechanisms. *Fitoterapia.* 2020; 145:104633.

146. Sam, M.R., Elliott, B.E., Mueller, C.R. A novel activating role of SRC and STAT3 on HGF transcription in human breast cancer cells. *Molecular Cancer*. 2007;6(1):69.

147. Zou, S., Tong, Q., Liu, B., Huang, W., Tian, Y., Fu, X. Targeting STAT3 in Cancer Immunotherapy. *Molecular Cancer*. 2020;19(1):145.

148. Qin, J., Shen, X., Zhang, J., Jia, D. Allosteric inhibitors of the STAT3 signaling pathway. *European Journal Medicinal Chemistry*. 2020; 190:112122.

149. Cerulli, R.A., Shehaj, L., Tosic, I., Jiang, K., Wang, J., Frank, D.A., Kritzer, J.A. Cytosolic delivery of peptidic STAT3 SH2 domain inhibitors. *Bioorganic & Medicinal Chemistr*y. 2020;28(12):115542.

150. Dong, J., Cheng, X.D., Zhang, W.D., Qin, J.J. Recent Update on Development of Small-Molecule STAT3 Inhibitors for Cancer Therapy: From Phosphorylation Inhibition to Protein Degradation. *Journal of Medicinal Chemistry*. 2021;64(13):8884-8915

151. Gagné-Sansfaçon, J., Coulombe, G., Langlois, M.J., Langlois, A., Paquet, M., Carrier, J., Feng, G., Qu, C., Rivard, N. SHP-2 phosphatase contributes to KRAS-driven intestinal oncogenesis but prevents colitis-associated cancer development. *Oncotarget*. 2016;7(40):65676-65695.

152. Yamaoka-Tojo, M., Tojo, T., Kim, H.W., Hilenski, L., Patrushev, N.A., Zhang, L., Fukai, T., Ushio-Fukai, M. IQGAP1 Mediates VE-Cadherin–Based Cell–Cell Contacts and VEGF Signaling at Adherence Junctions Linked to Angiogenesis. *Arteriosclerosis Thrombosis and Vascular Biology*. 2006;26(9):1991-1997.

Vita

Tressie Alexandra Capri Posey graduated from Caney Creek High School, Conroe Texas in 2014, and then was admitted to Texas State University in San Marcos Texas. She received her degree in Bachelors of Science in Biochemistry with a minor in Biology in May, 2018, while also working with Dr. L. Kevin Lewis as an undergraduate researcher studying Non-Homologous End-Joining (NHEJ) in double stranded break repair. After graduation, she worked at Biobridge Global as a Lab Technician until August 2019 where she began pursuing her Master's in biomedical sciences at the University of Texas MD Anderson Cancer Center UTHealth Graduate School of biomedical Sciences with a focus on Therapeutics. During her master's she worked as a research assistant at the Institution of Molecular Medicine with Dr. Kendra Carmon studying cancer stem cell plasticity and drug resistance.

Permanent Address:

7777 Greenbriar Dr. Apt. 3100

Houston, Tx, 77030