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Overview of the Oncogenic Signaling Pathways in Colorectal Cancer: Mechanistic Insights

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Abstract:

Colorectal cancer is a multifaceted disease which is therapeutically challenging. Based on insights gleaned from decades of research, it seems clear that deregulation of spatio-temporally controlled signaling pathways play instrumental role in development and progression of colorectal cancer. High-throughput technologies have helped to develop a sharper and broader understanding of the wide ranging signal transduction cascades which also contribute to development of drug resistance, loss of apoptosis and, ultimately, of metastasis. In this review, we have set the spotlight on role of JAK/STAT, TGF/SMAD, Notch, WNT/β-Catenin, SHH/GLI and p53 pathways in the development and progression of colorectal cancer. We have also highlighted recent reports on TRAIL-mediated pathways and molecularly distinct voltage-gated sodium channels in colorectal cancer.

Introduction:

Colorectal cancer is the 3rd commonest cancer and the 2nd leading cause of cancer-related deaths in the Western world (1). The outlook for disease incidence rates is bleak, reports predict up to a 60% rise in the global burden of CRC in developing countries by 2030 (2). Innovations in disease management are critical.

Conventional treatment remains surgery in combination with a regime of adjuvant chemotherapy. Although targeted molecular inhibitors have found success in a number of cancer types however, such an option is lacking for CRC due to the dearth of validated molecular and phenotypic targets that will direct the development of efficacious treatment strategies. Some success has been found using monoclonal antibodies targeting EGFR and VEGF however, effective use is severely hampered by the lack of robust prognostic indicators for treatment outcome.

Sequencing of cancer genomes has identified a trove of mutations that drive intestinal epithelial transformation and carcinogenesis. By far, the most prevalent genetic events accompanying CRC development are mutations that de-regulate the Wnt signaling cascade. In particular, inactivating mutations in the tumor suppressor adenomatous polyposis coli (APC) are regarded as the earliest genetic lesions sufficient to initiate tumorigenesis. Along the framework of the adenoma-carcinoma sequence hypothesis proposed by Fearon and Vogelstein (3), tumors acquire a sets of driver mutations that de-regulate specific signaling pathways controlling cell growth and differentiation ultimately conferring colorectal tumors with all of the malignant properties of lethal metastatic carcinoma.

The aim of this review is to examine how mutational de-regulation of molecular pathways corrupts cell behavior, conferring malignancy. We will focus on therapeutic tractability of de-regulated molecular pathways and discuss emerging strategies that may be used to target CRC.

Wnt pathway

Oncogenic mutations in the Wnt pathway are the prevalent feature of colorectal cancers (CRCs). Mutations inactivating the Wnt pathway regulatory component adenomatous polyposis coli (APC) are found in >80% of CRCs, arise early in disease development and are believed to initiate malignant transformation of the colorectal epithelia (4). The vast majority of APC mutant colon epithelial tumors are benign and never progress to CRC. However, the acquisition of further genetic changes during the largely asymptomatic 10-15 year period of cancer development drives clonal evolution of lethal metastatic carcinoma (3). A smaller set of CRCs are initiated by alternate genetic lesions and follow unique molecular pathways of cancer development. Nonetheless, oncogenic mutations in the Wnt pathway are found in the majority of cases of these CRCs. Despite the prevalence of mutations driving the oncogenic form of the Wnt pathway in CRC, the effectiveness of therapeutic strategies geared towards inhibiting the pathway remains to be determined. Here we give a brief synopsis of how mutations de-regulating the Wnt pathway are key drivers of CRC. We discuss whether inhibiting the oncogenic form of the Wnt pathway is a viable strategy for targeting CRC as well as factors that have hindered the development of efficacious therapeutic inhibitors of the disease.

Wnt pathway activity spatially restricts the intestinal epithelial stem cell niche

The Wnt pathway has critical roles in defining cell identity and function throughout metazoan development, specified by expression of a context specific transcriptional programme (5, 6). β-catenin is the key effector of the Wnt pathway and its expression levels are regulated by a dedicated protein complex (the β-catenin destruction complex) that earmarks it for proteasome-mediated destruction. The destruction complex acts primarily through phosphorylation of β-catenin at specific serine/threonine residues in its N-terminus and subsequent ubiquitination by β-TRCP for proteasome recruitment. APC is a key limiting regulator of the destruction complex, providing a scaffold for its assembly and ensuring efficient covalent modification of β-catenin (7). Activation of the Wnt pathway by a context-specific cadre of afferent ligands leads to inhibition of the destruction complex, stabilization of β-catenin and its interaction with TCF/LEF family transcription factors at target genes. The subsequent recruitment of additional transcriptional activators drives Wnt pathway target gene expression.

In both the colon and small intestinal epithelia, spatially-restricted molecular cues emanate at the base of invaginations in the intestinal epithelial monolayer called crypts of Lieberkühn and from cells in the underlying mesenchyme. These molecular cues activate the Wnt pathway as well as other molecular pathways that specify stem cell niche homeostasis (8, 9). Detailed studies demonstrate a key role for the Wnt pathway in maintaining the stem cell niche of the murine intestinal epithelia (reviewed in (10)). In the small intestine, Paneth cells provide the source of Wnts and ligands for EGFR (EGF) and NOTCH (DLL4) pathways (11). Signals emanating from the underlying mesenchyme include R-spondins that potentiate ligand-induced Wnt pathway activity (12, 13) and BMP signaling antagonists such as Gremlin that temper differentiation and apoptosis-promoting cues, the consequence of active TGFβ signaling (14).

The molecular cues driving multipotency and indeed their source are less-well defined in the colon intestinal epithelial stem cell niche. A population of a c-Kit and Reg4 expressing cells at the base of murine colon epithelial crypts may act as surrogate colon epithelial Paneth cells, providing a source of EGF and the NOTCH ligands DLL1 and DLL2 (15, 16). Wnt and R-spondin ligands are expressed by cells within the underlying mesenchyme (17).

Oncogenic Wnt pathway is a driver of colorectal cancer

Oncogenic mutations de-regulating the Wnt pathway in CRCs are well-known. Individuals with familial adenomatous polyposis (FAP) patients inherit heterozygous, oncogenic mutations in APC, display hundreds of colon epithelial tumours by adulthood and invariably develop CRC (18). Moreover, in sporadic CRCs, somatic mutations that inactivate APC are prevalent at all stages of colon carcinogenesis consolidating an early if not initiating genetic lesion in disease aetiology (18, 19). Alternatively, up to

30% of CRCs harbor oncogenic mutations in other Wnt pathway regulatory components (see below) reinforcing the notion that Wnt pathway de-regulation is a primary driver of colorectal carcinogenesis. However, a small subset of CRCs (<10%) lack overt somatic changes driving oncogenic Wnt pathway activity (discussed below).

Molecular pathways of CRC development

CRCs can be stratified by their distribution within the colon epithelia and associated genetic alterations that broadly classify molecular pathways of cancer development as chromosomal instability (CIN) and microsatellite instable (MSI):

Chromosomal instability (CIN) is a hallmark of greater than 70% of colon carcinoma tumors (20, 21) that harbour somatic mutations in APC (see APC, below) and predominantly localize to the distal (descending and sigmoidal) colon epithelia (22, 23). CIN CRCs are characterized by karyotype abnormalities and often harbor mutations leading to activation of KRAS, loss of TP53, and loss of heterozygosity for the long arm of chromosome 18 (24). Increased structural defects associated with CIN presumably expand the repertoire of driver mutations fuelling carcinogenesis.

Microsatellite instability (MSI) is a feature of up to 30% of CRCs and is associated with tumors located within the proximal (ascending and transverse) colon. MSI in colon tumors arises through dysfunction of the mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6*, and *PMS2* and leads to numerous mutations, in particular within highly repetitive microsatellite regions (25). Germline mutations in MMR genes are rare in CRC and lead to hereditary nonpolyposis CRC (referred to as Lynch Syndrome) characterized by MSI (26). MSI colorectal epithelial cancers are frequently associated with the *BRAF* V600E mutation.

Dysfunction of MMR in MSI tumours is most often the consequence of gene silencing by promoter methylation. The CpG island methylator phenotype (CIMP) is a feature of most MSI tumours, characterized by a predisposition to DNA hypermethylation and transcriptional silencing of MLH1 and other MMR genes. The root cause of CIMP is unknown. The majority of MSI CRCs have lost expression of MMR genes through epigenetic inactivation of their promoters by DNA methylation. Hence, CIMP CRCs are most often MSI, display a high mutational burden and express oncogenic BRAF (27).

MSI tumors frequently harbor mutations in Wnt pathway regulatory components that include truncating mutations (the results of insertions and deletions in microsatellite repeat regions) of *APC*, *TCF7L2* and *AXIN2* genes or mutations in *CTNNB1* (the encoding gene for □-catenin) that result in expression of the stabilized form of the protein. The consequence of all of these mutations is oncogenic activation of the Wnt pathway. Interestingly, in MSI CRCs, β-catenin, AXIN2 and APC mutations are mutually exclusive (28) indicating each of these mutations individually may be sufficient to drive oncogenic Wnt pathway activity.

Approximately, one third of CIMP CRCs lack overt oncogenic mutations in *β-catenin*, *AXIN1/2*, *APC*, *RNF43* and *RSPONDIN* genes. This may be due to other, yet unidentified oncogenic mutations, perhaps in other signaling pathways, that counterbalance the anti-neoplastic role of regulated Wnt pathway activity. Alternatively, hypermethylation of *AXIN1/2* and *APC* gene promoters in these CIMP tumors may instead drive oncogenic Wnt pathway activity.

CRC mutations driving the oncogenic Wnt pathway

Oncogenic mutations driving Wnt pathway activity in CRCs confer stem cell niche independence via expression of a stem cell-specifying transcriptional programme. A comprehensive review of Wnt pathway mutations and their frequency in CRCs has recently been published (29); in this review, we instead focus on the most common genetic lesions leading to oncogenic Wnt pathway activity characteristic of the disease.

Mutations in APC

APC is a large multidomain protein with diverse roles including control of Wnt pathway activity and regulation of the actin and microtubule cytoskeletons. All somatic *APC* mutations identified in colon epithelial neoplasms are located within a discrete domain (the mutational cluster region; MCR) and are invariably truncating mutations. Curiously, at least one *APC* allele expressing truncated APC protein is invariably retained in CRCs (3) however, the phenotypic consequence is unclear. The MCR is interspersed amongst protein-protein interaction domains for the Wnt pathway effectors β-catenin and Axin proteins. The C-terminus of APC contains a microtubule-binding domain and an interacting domain for EB1, a protein that binds to and stabilises the dynamic (+) end of microtubules. Therefore, truncating APC mutations found in CRC compromise both APC's regulatory in the Wnt pathway and its function(s) in microtubule dynamics.

Chromosomal instability (CIN)

CIN is a hallmark of APC mutant CRC tumours. Embryonic stem cells homozygous for oncogenic APC mutations rapidly develop aneuploidy and other hallmarks of chromosomes segregation defects characteristic of CIN (30-32). However, the molecular mechanism driving such CIN in CRC remains controversial. Previous studies have shown that expression of mutant APC may compromise the recruitment of microtubules to the kinetochore, preventing proper assembly of the mitotic spindle (30, 31, 33) or trigger the premature onset of anaphase, resulting in defective chromosomal segregation (34). APC mutations have also been shown to also disrupt chromosomal congression or segregation indirectly through increased β-catenin expression and oncogenic activation of the Wnt pathway (32, 35). Nonetheless, all studies agree that inactivating APC mutations can lead to CIN and therefore, may be a defining, functional characteristic, of tumor progression to CRC.

β-catenin mutations

β-catenin mutations are found in at least 10% of CRCs (36). These are most often point mutations within the N-terminal phosphorylation sites rendering it refractory to activity of the β-catenin destruction complex. Mutations in β-catenin driving oncogenic Wnt pathway activity are primarily associated with MSI CRCs and their expression is exclusive of oncogenic mutations in *APC* and *AXIN1/2*.

Mutations in the ubiquitin ligases RNF43 and Znrf3

RNF43 and Znrf3 are E3 ubiquitin ligases that attenuate steady-state levels of the FZ/LRP Wnt pathway co-receptors at the plasma membrane via their ubiquitination, internalization and inactivation. The function of these ubiquitin ligases is inhibited by the stimulation of cells with R-spondins (see below). Inactivating RNF43 mutations are found in approximately 20% of all CRCs and are almost exclusively associated with MSI in the proximal colon (37).

Mutations in R-spondins

Engagement of R-Spondin ligands (RSPOs) with their cognate LGR family receptors leads to the recruitment and inactivation of RNF43 and Znrf3 and increased steady-state levels of Wnt receptors at the plasma membrane (38, 39). Whilst RSPOs do not induce Wnt pathway activity on their own, they act to increase steady-state concentrations of FZ/LRP at the plasma membrane thereby potentiating Wnt pathway activity in response to afferent Wnt ligands. Gene fusion transcripts of RSPO2 and RSPO3 are

found in up to 10% of CRC cases and the expressed fusion protein is sufficient to potentiate Wnt pathway activity in HEK293T cells as well as the CRC cell line HT29 (39, 40).

Mutations in the transcription factor TCF7L2

The *TCF7L2* gene harbors a microsatellite within its terminal exon. Insertions or deletions within the microsatellite results in frameshift mutations and leading to the expression of C-terminal truncated versions of TCF7L2. Whilst the mutant TCF7L2 protein can interact with and activate Wnt target genes it lacks its CRARF and CREB binding protein (CBP)/p300 domains. The CRARF domain is a second DNA binding site required for transcription of a subset of Wnt pathway target genes associated with multipotency in stem cells. CBP and p300 contain binding domains for a number of transcriptional co-activators as well as a histone acetyltransferase domain. Whether these truncating mutations are selected in CRC to temper high levels of Wnt pathway activity or confer neomorphic properties to tumor cells remains to be determined.

Therapeutic tractability of the oncogenic Wnt pathway and consequence in CRC.

The prevalence of mutations driving oncogenic Wnt pathway activity in CRC has sparked a number of initiatives geared towards the development of corresponding molecular inhibitors to target the disease. However, there are few Wnt pathway-specific inhibitors in clinical trials for CRC despite greater than two decades of research since the identification of mutations in APC and the identification and characterization of other key regulators of the Wnt pathway. Moreover, it remains to be formally shown whether targeting the oncogenic form of the Wnt pathway would lead to a beneficial therapeutic outcome. Further initiatives developing oncogenic Wnt pathway inhibitors should first address the following:

A. Determine whether inhibition of oncogenic Wnt pathway activity during colorectal carcinogenesis targets the tumor.

Although clinical trial data demonstrating appropriate patient responses to treatment with Wnt pathway inhibitors is lacking, several lines of experimental evidence from pre-clinical studies argue a beneficial consequence of Wnt pathway blockade. Overexpression of wild type APC in the HT29 CRC cell line (harboring oncogenic APC mutations) promotes apoptosis (41). Additionally expression of a dominant negative version of TCF7L2 (dnTCF) in CRC cell lines reduces cyclinD1 expression leading to arrest of cells in G1 (42, 43). Finally, inducible depletion of β-catenin in APC mutant CRC cell lines xenografts inhibit tumor growth (44).

One *in vivo* study using a mouse model of colon carcinogenesis reported that APC re-expression, on the background of p53 inactivation and expression of oncogenic Kras (genetic lesions commonly found in human CRC), re-establishes stratified colon epithelia from high-grade colon adenocarcinoma (45).

Hence, although published evidence supports ongoing efforts to target oncogenic Wnt pathway activity, it remains to be established whether such therapeutic inhibitors will be effective as interventional agents in human CRC *in situ*. Moreover, faithful indicators of colon cancer tumour attrition upon blockade of oncogenic Wnt pathway activity remain to be identified.

B. Identification of tractable regulatory nodes in the oncogenic form of the Wnt pathway that effectively target CRC.

In the large majority of CRCs the primary genetic drivers in the Wnt pathway are APC and β-catenin, intracellular proteins whose oncogenic activity is little affected by upstream regulatory components. One criteria for Wnt pathway inhibition is therefore the development of cell-permeable agents that can act on

regulatory nodes downstream of APC function either by targeting β-catenin levels or effector proteins directing the Wnt pathway transcriptional programme. However, few of these targets are Wnt pathway specific and amongst these, effective inhibition requires interference with protein-protein interactions.

One reported inhibitor fulfils criteria for inhibition of oncogenic Wnt pathway activity; ICG-001 targets the interaction between β-catenin and the acetyltransferase CBP, inhibiting Wnt pathway-dependent transcription and demonstrating a reasonable therapeutic window in CRC cell lines (46, 47). A second-generation β-catenin-CBP inhibitor, PRI-724, is currently in Phase I/II trials (Clinical trial reference: NCT01302405) for oncological indications including colon cancer and demonstrates acceptable toxicity profile; as yet Phase II data has not been reported.

Alternatively, a number of therapeutic initiatives have developed inhibitors against Wnt pathway regulatory components at the level of ligand – Wnt receptor interaction, that have shown clinical promise in targeting CRC:

A small molecule screen for inhibitors of Wnt pathway activity identified IWP-2, an inhibitor of the S-palmitoyl acyltransferase Porcupine (48). Porcupine inhibition blocks acylation of Wnt, a post-translational modification essential for its secretion and activity (49). LGK974 is a humanized Porcupine acyltransferase inhibitor, currently in Phase I/II clinical trials for metastatic colorectal cancer. Although PORC inhibitors are not expected to target CRCs with oncogenic mutations in intracellular Wnt pathway regulatory components, pre-clinical data demonstrates that CRC cells harboring oncogenic mutations in RNF43 or those expressing RSPO2/3 fusion proteins are particularly sensitive to LGK974 treatment (50).

OMP131R10 is a humanized RSPO3 antibody currently in Phase I/II clinical trials as monotherapy for patients with metastatic colorectal cancer. Pre-clinical data using patient derived tumor xenograft models demonstrates that OMP131R10 treatment reduces tumor growth (51). Another study found that OMP131R10 efficiently targets not only CRCs expressing oncogenic RSPO fusions but also those with oncogenic Wnt pathway mutations in intracellular regulatory components such as APC, □-catenin or RNF43 (52). Whether OMP131R10 can be used as a general therapeutically agent to target CRCs will require further clinical study.

Finally, Foxy-5, a peptide mimetic of Wnt5A is currently in Phase I clinical trials. Pre-clinical data indicates Wnt5A expression is associated with poor prognosis in metastatic colon cancer (53, 54) and treatment of colorectal cancer cell lines impairs cellular migration (54). Although not entirely clear, the suppressive mode of action of Foxy-5 may be through activation of the non-canonical Wnt pathway and/or upregulation of the tumor suppressor 15-hydroxyprostaglandin dehydrogenase (55).

Taken together, although some inhibitors of the oncogenic form of the Wnt pathway are currently under clinical study, late phase clinical trial data demonstrating efficacy in targeting CRC is lacking.

In the upcoming section we will set spotlight on JAK-STAT signaling and summarize how JAK-STAT driven pathway played crucial role in colorectal cancer.

JAK-STAT pathway in Colorectal Cancer:

JAK/STAT pathway has attracted considerable attention because of its ability to regulate cancer progression and metastasis. In this section, we will summarize some of the most ground-breaking discoveries which have identified oncogenic role of JAK-STAT pathway in colorectal cancer. Following section deals with STAT proteins which have been shown to drive colorectal cancer.

Interplay of STAT3 with Long noncoding RNAs:

Long non-coding RNAs (lncRNAs) have been shown to play instrumental role in colorectal cancer [56]. FEZF1-AS1, a highly overexpressed lncRNA was noted to promote STAT3 signaling. RNA pull-down assay provided evidence of binding of FEZF1-AS1 to pyruvate kinase 2 (PKM2). FEZF1-AS1 was noted to regulate subcellular localization of PKM2 [56]. Data clearly suggested that total and nuclear PKM2

levels were significantly decreased in FEZF1-AS1-silenced HCT116 cells and increased in FEZF1-AS1 overexpressing LoVo cells. STAT3 signaling was functionally active in PKM2 or FEZF1-AS1 overexpressing CRC cells and PKM2 inhibition blocked FEZF1-AS1-induced STAT3 activation. Moreover, knockdown of FEZF1-AS1 completely blocked STAT3 activation, which was rescued by overexpression of PKM2 [56].

Another lncRNA, DILC negatively regulated colorectal cancer progression. Overexpression of DILC inhibited the growth and metastasis of CRC cells [57]. Furthermore, lnc-DILC knockdown potentiated proliferation and metastasizing potential of CRC cells. Mechanistically, lnc-DILC suppressed CRC cell progression via inactivation of IL-6/STAT3 signaling [57].

STAT3 Regulation of miRNAs:

miR-572 was significantly upregulated in LS174T cells transfected with a constitutively activated mutant of STAT3 [58]. Whereas, STAT3 inhibition significantly reduced miR-572 expression levels in SW480 cells. Modulator of apoptosis 1 (MOAP-1) was negatively targeted by miR-572 in colorectal cancer cells. Data clearly suggested that STAT3 induced progression of colorectal cancer through miR-572-MOAP-1 pathway [58].

NF-κB and STAT3 transcriptionally upregulated miR-18a in colorectal cancer cells [59]. PIAS3 overexpression or miR-18a knockdown significantly induced regression of tumors in xenografted mice [59].

STAT3 Activation by Different Proteins:

Leukemia inhibitory factor (LIF) promoted STAT3 phosphorylation [60]. Magnolin, a naturally derived molecule dose-dependently decreased mRNA of LIF and inhibited proliferation of colorectal cancer cells [60]. Mechanistically, RAC1-GTP interacted directly with STAT3 and promoted its phosphorylation and consequently promoted EMT of CRC cells (Zhou et al, 2018). So overall these findings highlighted that different pathways may activate STAT3 in colorectal cancer cells.

KDM4B was a positive regulator of STAT3 signaling. Significant downregulation of p-STAT3 was noted in KDM4B knockdown cells [61]. STAT3 overexpression in KDM4B knockdown cells enhanced cell survival in response to IR treatment in colorectal cancer cells. Cyclic-AMP responsive element binding protein (CREB) directly controlled KMD4B by binding to its promoter region. Overexpression of KDM4B in CRC cells correlated with resistance to irradiation [61]. KDM4B-deficient CRC cells exhibited enhanced DNA damage and were found to be more sensitive to irradiation stimuli [61].

Overall these findings clearly suggested that JAK-STAT signaling played a central role in development and progression of colorectal cancer. Recently emerging high-impact research has helps us to uncover wide ranging proteins which strategically regulate STAT proteins. Therefore, effective targeting of various proteins which crosstalk with STATs is also very essential and need of the hour for result-oriented targeting of JAK-STAT pathway.

Next we discuss how TGF/SMAD signaling plays role in development and progression of colorectal cancer.

TGF/SMAD pathway in Colorectal Cancer:

TGF-β superfamily members acted through a heteromerically organized receptor complex that comprised of type I and type II receptors present at the surface of cancer cells that transduced signals intracellularly through SMAD complex. TGFRII trans-phosphorylated TGFRI to ignite SMAD-dependent signaling. In SMAD-dependent pathway, phosphorylated R-SMADs (SMAD2 or 3) formed functionally active complexes with SMAD4 and moved into the nucleus to transcriptionally regulate myriad of target genes. However, this pathway is negatively regulated by SMAD7. SMAD7 prevented phosphorylation of R-SMADs and targeted R-SMADs for degradation by SMURF. SMURF roleplay in colorectal cancer development and progression will be discussed in a separate part. In this section we have summarized how TGF-SMAD signaling pathway modulated colorectal cancer.

In a latest study, multiplexed fluorescent immunohistochemistry was used to quantitatively analyze levels of TGFβ, TGFRI, TGFRII, SMAD4, p-SMAD2/3 and SMAD1/5/9 in cancerous tissues [62]. TGFβ, TGFRI, TGFRII, SMAD4, p-SMAD2/3 and SMAD1/5/9 were considerably enhanced in cancerous tissues [62].

SMAD1 promoted migration of colorectal cancer cells by simultaneous induction of oncogenic proteins Snail and Ajuba [63]. E-cadherin, a Snail/Ajuba target gene was downregulated by SMAD1 expression. Furthermore, Ajuba depletion in HCT116 cells significantly abrogated the cell migratory ability of SMAD1 overexpressing cancer cells [63]. Data clearly suggested that SMAD1 inhibited E-cadherin mainly through Snail and Ajuba.

TGFβ –treated colon adenocarcinoma cells were injected into the spleen of mice and to evaluate growth of tumors [64]. TGFβ treatment drastically enhanced primary tumor growths and metastases of liver. However, treatment of mice with TGFβ1-targeting peptides P17 and P144 induced regression of the tumors. TGFβ treatment induced activation of SMAD1/5/8, SMAD3, SMAD2 and promoted invasion and trans-endothelial migration [64].

It is also very exciting to note that cancer cells secrete microvesicles which are taken up by recipient cells and the molecules delivered through microvesicles trigger different signaling pathways. Colorectal cancer cells have been reported to actively secrete miR-1246 and TGFβ via microvesicles [65]. Incubation of Human umbilical vein endothelial cells (HUVECs) with colorectal cancer cells derived microvesicles promoted the proliferative, migratory and tube forming abilities of HUVECs. SMAD1/5/8-driven signaling was observed in HUVECs co-incubated with cancer cells derived microvesicles [65].

Moreover, increasingly it is being realized that secretion of microvesicles play central role in switching the TGF/SMAD signaling. It is relevant to mention that very few studies are available related to secretion of extracellular vesicles from colorectal cancer cells. Therefore, future studies must converge on detailed analysis of vesicles derived from colorectal cancer cells and how these vesicles trigger activation of SMAD signaling in recipient cells.

Tissue associated macrophages (TAMs) promoted metastasizing potential of colorectal cancer cells via TGFβ secretion [66]. TGFβ induced SMAD/Snail/E-cadherin signaling axis in colorectal cancer cells. SMAD induced Snail further downregulated E-cadherin [66]. Therefore, TGF-SMAD signaling functionalized different proteins to inhibit metastasis suppressor proteins.

Follistatin-like protein 1 (FSTL1), a target gene of SMAD3 promoted colorectal cancer metastases via activation of focal adhesion signaling pathway [67]. Chromatin immunoprecipitation assay provided evidence of binding of SMAD3 to promoter region of FSTL1 [67].

These findings clearly suggested that TGF/SMAD signaling tactfully induced EMT mainly through inhibition of metastasis suppressor proteins.

Colorectal cancer arises from precursor lesions known as polyps. However, molecular features have not yet been fully characterized which can categorize benign and malignant polyps [68]. For molecular characterization of polyps, Cancer Free Polyp (CFP) and Cancer Adjacent Polyp (CAP) patients were used. SMAD2 was found to be exclusively mutated in CAPs [68].

SMAD proteins as Tumor Suppressors:

Nitrilase 1 (NIT1), belonged to carbon-nitrogen hydrolase super-family and played contributory role in suppression of tumor [69]. SMAD anchor for receptor activation (SARA) interacted directly with SMAD2 and SMAD3 and recruited SMAD2 and SMAD3 to the TGFR. Co-immunoprecipitation analyses revealed that NIT1, SMAD2/3 and SARA interacted with each other in colorectal cancer cells. SMAD3 was noted to transcriptionally upregulate NIT1 [69]. Data proposed that NIT1 suppressed proliferation of colorectal cancer cells through a positive feedback loop between NIT1 and TGFβ-SMAD signaling pathway. These findings are seemingly opposite to the generally known role of TGF/SMAD signaling in colorectal cancer. Another report also highlighted tumor suppressive effects of TGF signaling. TGFβ inhibited ezrin phosphorylation at 567^{th} threonine residue which resulted in protein kinase-A (PKA) activation and apoptosis. TGFβ1 treatment time-dependently decreased ezrin phosphorylation [70]. Cyclic-AMP-dependent protein kinase A-anchoring proteins (AKAPs) structurally interacted with PKA and targeted these supra-molecular complexes to particular subcellularly localized regions where they phosphorylated different proteins. Ezrin inhibition mediated activation of PKA was found to be dependent on AKAP149 in colorectal cancer cells [70].

It seems exciting to note that TGF/SMAD demonstrated diametrically opposed roles in CRC. However, it will be essential to identify context dependent roles of different SMADs in CRC.

SMURF role in Colorectal Cancer:

SMURF proteins have largely been known as negative regulators of TGF/SMAD signaling. However, in this section we will discuss a different portfolio of SMURF proteins in CRC which is independent of TGF/SMAD signaling.

SMURF1 played positive role in colorectal cancer development and progression. In this section, we will focus on the "triggers" which modulate activity of SMURF1.

Detailed mechanistic insights revealed that Interleukin-1 receptor-associated kinase-2 (IRAK2) phosphorylated threonine residues of SMURF1 and promoted its self-degradation by ubiquitylation to induce apoptosis [71]. Structural studies revealed that protein kinase domain of IRAK2 interacted with HECT domain of SMURF1. IRAK2 overexpression triggered downregulation of SMURF1. IRAK2 was found to significantly promote protein turnover of wild-type SMURF1 whereas C699A mutant (ligase-inactive) was unable to produce similar results. On the contrary, knockdown of endogenous IRAK2 induced accumulation of SMURF1 protein. Treatment of cells with Thapsigargin resulted in downregulation of endogenous SMURF1. More importantly, Thapsigargin treatment time- and dose-dependently induced SMURF1 degradation and IRAK2 accumulation [71].

SMURF1 also played central role in abrogating sensitivity of colorectal cancer cells to chemotherapeutic drugs particularly 5-fluorouracil [72]. miR-497 was noted to effectively target SMURF1 in colorectal cancer cells. Enforced expression of miRNA-497 induced an increase in the 5-fluorouracil sensitivity in SW480 cells. Whereas, use of inhibitors-against miR-497 enhanced growth and reduced drug sensitivity of LoVo cells [72].

Cysteine residues played active role in auto-neddylation therefore Cysteine residues in the SMURF1 HECT domain were mutationally converted into alanine and neddylation assay was performed for evaluation. Mutational conversion of 426th cysteine into alanine (C426A) resulted in a reduction in the levels of auto-neddylated SMURF1 [73]. Neddylation of SMURF1 occurred at multiple lysine residues in the C2, WW HECT linker region and HECT domains. Reintroduction of SMURF1-C530A or wild type-SMURF1 significantly enhanced tumor volume and weight in nude mice and promoted proliferation and invasive potential of colorectal cancer cells. However, reintroduction of SMURF1 C699A or C426A into colorectal cancer cells slightly increased tumor growth and invasion. Data clearly suggested that auto-neddylation mediated SMURF1 activation was essential for its tumor-promoting effects [73].

SMURF1 overexpression markedly enhanced growth of HCT116 and SW480 cancer cells [74]. Treatment of cells with Rapamycin suppressed cell cycle-regulated induction of SMURF1. CKIP-1 repressed activity of AKT in colon cancer SW480 and HCT116 cells that consequently resulted in SMURF1 reduction. Henceforth, during cell cycle, regulation of SMURF1 by CKIP-1 may probabilistically be due to AKT inactivation. Intriguingly, inhibition of PI3K or mTOR markedly impaired EGF-modulated induction of SMURF1. Likewise, overexpression of CKIP-1 severely abrogated EGF-driven SMURF1 induction [74].

SMURF has been shown to be a negative regulator of TGF/SMAD signaling. However, surprisingly, SMURF role has not been studied in context of TGF/SMAD signaling in CRC. Overall these findings clearly suggested that SMURF promoted CRC and targeting of SMURF is important to kill colorectal cancer cells. However, these are missing pieces of an incomplete jig-saw puzzle. We still have to deeply investigate how SMURF controlled SMAD proteins in colorectal cancer cells. Truthfully there are more questions than answers!

NOTCH Pathway in Colorectal Cancer

Ligand-receptor interaction induced proteolytic processing of the receptor. Proteolytically processed NICD translocated and interacted with the nuclear binding partner Recombination signal-binding protein-1 for J-Kappa (RBPJ) and converted repressor complex to functionally active complex (Fig. 1). NOTCH pathway has been extensively investigated and we now know that NOTCH pathway enhanced stem-like properties and chemoresistance in colorectal cancer cells. In this section we have gathered some exciting

pieces of evidence which highlight oncogenic role of NOTCH pathway. We will also set spotlight on the microRNAs which negatively regulate NOTCH pathway to kill colorectal cancer cells. Moreover, there are different oncogenic miRNAs which promote NOTCH pathway-mediated cancer promoting effects.

Negative Regulation of NOTCH Pathway by microRNAs:

miR-34a negatively regulated NOTCH1 and restored sensitivity of colorectal cancer cells to 5-fluorouracil (5-FU) [75]. 3'UTR of NOTCH2 and RBPJ had binding sites for miR-195-5p. Enforced expression of miR-195-5p in SW620 CRC cells markedly reduced stemness and drug resistance [76]. Transient transfection of miR-206 mimics into the SW480 and SW620 cells markedly reduced the levels of NOTCH3, inhibited uncontrolled proliferation of cells, migration, and induced apoptosis [77]. JAG1 overexpression induced epithelial-to-mesenchymal transition (EMT) and promoted metastasis of CRC cells [78]. However, overexpression of miR-598 significantly inhibited JAG1 and substantially suppressed metastasis [78].

Different proteins have to been shown to enhance NOTCH pathway via inhibition of tumor suppressor miRNAs. Doublecortin- and Calmodulin Kinase-Like-1 (DCAMKL1) enhanced NOTCH pathway via inhibition of miR-144 [79]. Nanotechnologically delivered siDCAMKL-1 increased miR-144 and inhibited colorectal cancer mainly through NOTCH1 dependent mechanism [79].

Long Non-coding RNAs Join Hands with NOTCH Pathway:

Long non-coding RNA, FAM83H antisense RNA-1 (FAM83H-AS1) promoted colorectal cancer cell proliferation [80]. NOTCH pathway was noted to positively regulate its expression. DAPT (GSI-IX) is a novel γ-secretase inhibitor markedly reduced NOTCH1 that consequently resulted in downregulation of FAM83H-AS1 [80]. These findings provided clue that NOTCH pathway inhibition may inhibit different long non-coding RNAs.

FOXD2-AS1 (FOXD2-AS1), another lncRNA worked harmoniously with NOTCH pathway and promoted colorectal cancer progression [81] (Fig. 2). FOXD2-AS1 knockdown increased E-cadherin and decreased levels of N-cadherin and Snail. FOXD2-AS1 knockdown also drastically reduced HES-1 and NICD [81]. Underlying mechanisms of regulation of NOTCH pathway by FOXD2-AS1 have not been studied in detail. Therefore, it will be exciting to see if FOXD2-AS1 regulated NOTCH pathway by interacting with NOTCH pathway-targeting miRNAs or it worked with transcriptional machinery to stimulate expression of NOTCH pathway target genes.

Competing endogenous RNA (ceRNA)-crosstalk network has emerged as an important aspect in cell signaling [82]. CeRNAs are RNA transcripts having unique microRNA-binding sites called miRNA response elements (MREs). Many lncRNAs have MREs which enable them to compete for shared miRNAs [82]. Recently, FOXD2-AS1 has been shown to associate with miR-185-5p in colorectal cancer cells. miR-185-5p negatively regulated Cell division cycle-42 (CDC42) and inhibited proliferation of colorectal cancer cells [83] (Fig. 2). However, FOXD2-AS1 competitively interacted with miR-185-5p and interfered with its repressive effects on CDC42 [83].

Interplay of NOTCH Pathway with Different Proteins:

Runt-related transcription factor 3 (RUNX3), a transcriptional factor has been reported to be involved in regulation of NOTCH pathway in different cancers. Expression levels of Jagged 1, Notch1, HEY1, HES1 and NICD were found to be upregulated in RUNX3 silenced SW620 cells [84]. RUNX3 inhibited NOTCH pathway to impair proliferation and metastasizing capacity of SW620 cells [84]. It was exciting to note that RUNX3 interacted with NICD and RBP-J and transcriptionally downregulated HES1 by loading of this multi-component repressor machinery to RBP-J recognition motif of HES-1 promoter region [85] (Fig. 1). This mechanistic interaction of RUNX3, NICD and RBP-J has been unveiled in hepatocellular carcinoma cells however, it will be exciting to see if similar molecular network also operates in colorectal cancer cells!

Family with sequence similarity 83, member D (FAM83D) inhibition promoted upregulation of F-box and WD repeat domain-containing 7 protein (FBXW7) in colorectal cancer cells [86]. FBXW7, an E3 ubiquitin ligase tagged proteins including NOTCH for degradation [86]. Increased NOTCH activity was

also observed in cancer patients heterozygously mutated for FBW7 [87]. Moreover, loss of FBXW7 altered homeostasis of the intestinal epithelium in wild-type mice. Activation of NOTCH pathway was observed to be associated with loss of FBXW7 that consequently resulted in development of adenomas at 9-10 months of age [88].

Recent studies on Polycomb repressive complex-2 (PRC2) have expanded our perspectives on its function and regulation and it has been shown that mechanistically, Serine/Threonine kinase receptor-associated protein (STRAP) induced dissociation of PRC2 assembly by interfering with structural interactions of PRC2 subunits SUZ12 and EZH2 [89]. More importantly, JAG2, NICD and HES1 were lower in STRAP silenced colorectal cancer cells. Luciferase assays provided clues of significantly reduced promoter activity of HES1 and HES5 in STRAP silenced cancer cells. STRAP interfered with proteins of PRC2 assembly and transcriptionally upregulated NOTCH target genes (HES1 and HES5) in colorectal cancer cells [89] (Fig. 1). There was markedly reduced tumor growth and better sensitivity of the tumors to chemotherapeutic drugs in mice xenografted with STRAP silenced HCT116 cancer cells [89].

Disabled-1 (DAB1) was found to be under direct control of NOTCH pathway. DAB1 inhibition effectively suppressed cancer invasion and metastasis in the NOTCH signaling-activated mice [90]. DAB1 was phosphorylated by ABL tyrosine kinase, which activated ABL reciprocally. Additionally, RAC/RHOGEF protein TRIO was reported be another protein substrate of ABL and phosphorylation at 2681st tyrosine residue (pY2681) induced RHO activation in colorectal cancer cells [90]. Its unphosphorylatable mutant form TRIO Y2681F reduced activity of RHOGEF and inhibited invasive potential of colorectal cancer cells [90]. Transgenic mice generated by conditional activation of the NOTCH1 receptor and a p53 deletion in the digestive epithelium (NICD/p53($^{-/-}$)) developed metastatic tumors and had high penetrance [91].

HES1 overexpressing colon cancer cells were found to be resistant to 5-Fu treatment [92]. E-cadherin was downregulated and N-cadherin was noted to be upregulated in HES1 overexpressing cancer cells. Moreover, HES1 overexpression also induced an upregulation of ABCC1, ABCC2 and P-gp1. Data clearly suggested that HES1 potentiated chemoresistance to 5-Fu by induction of EMT and upregulation of several ABC transporter genes [92].

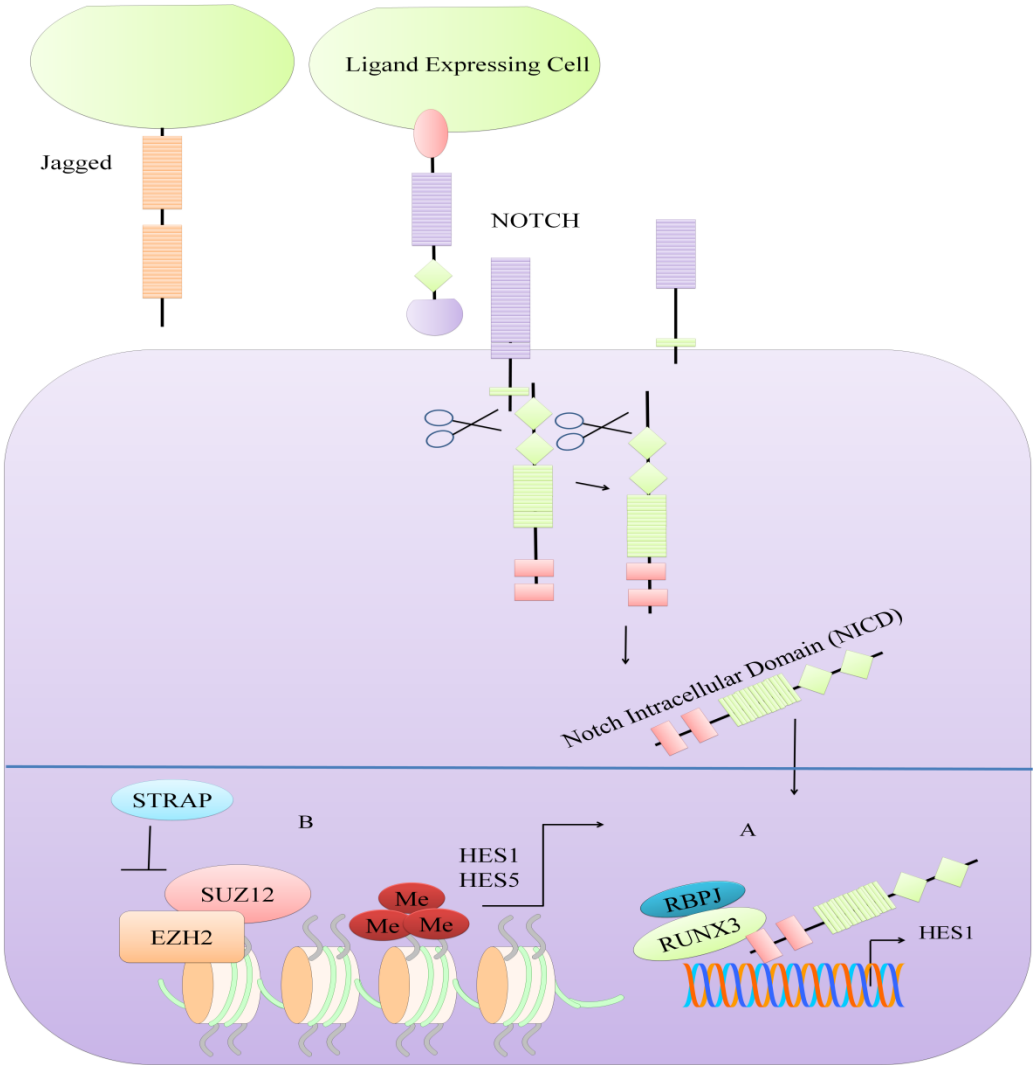

Figure 1 shows (A) NOTCH signaling. Ligand-receptor interaction induces proteolytic processing of the receptor. Proteolytically processed NICD translocates into the nucleus and transcriptionally upregulates HES1. RUNX3 is also an important member of transcriptional machinery that stimulates HES1. (B) STRAP interferes with PRC-2 complex to trigger transcription of NOTCH target genes.

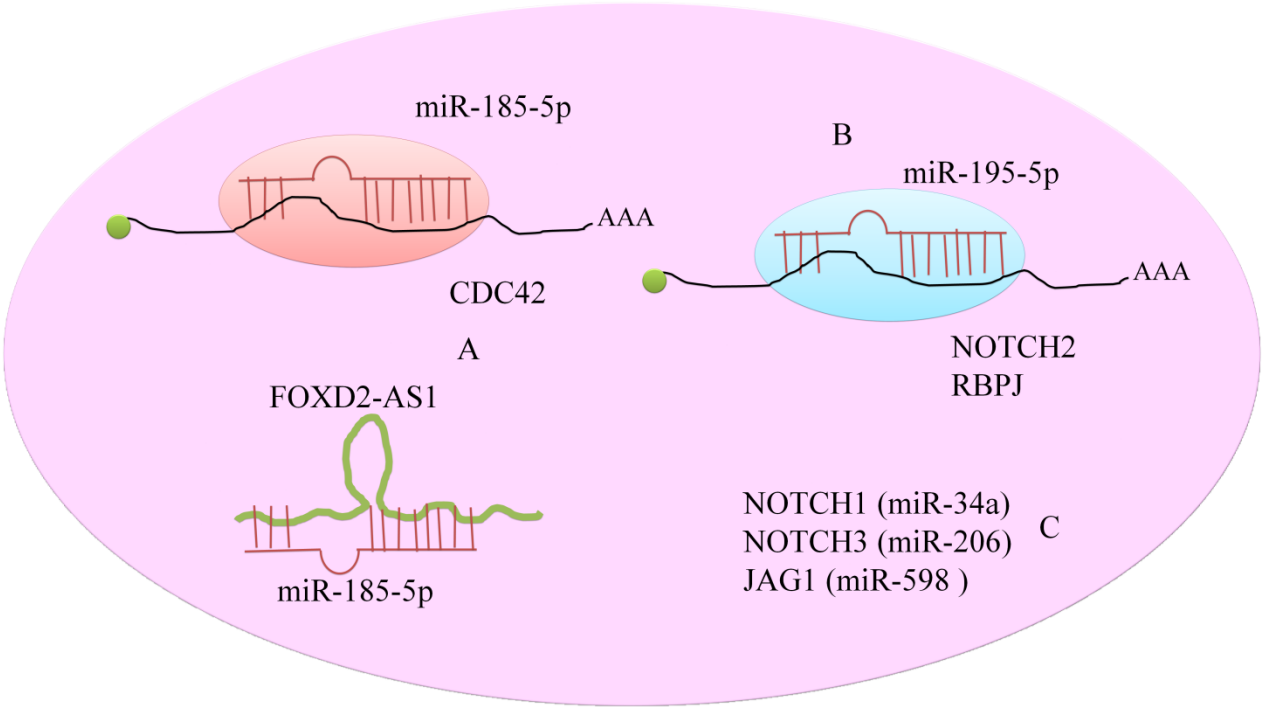

Figure 2 shows regulation of NOTCH pathway by different microRNAs and Long noncoding RNAs. (A) FOXD2-AS1, a long noncoding RNA facilitates NOTCH pathway. It inhibits NOTCH pathway targeting miRNAs. FOXD2-AS1 interacts with miR-185-5p. (B) Regulation of NOTCH1, NOTCH2, NOTCH3, JAG1 and RBPJ by different miRNAs.

SHH/GLI Signaling in Colorectal Cancer

Cancer Promoting Role of SHH Signaling:

Aberrantly active Hh signaling promoted proliferation of CRC cells by directly binding to the promoter of FoxM1 and increasing its transactivational activity [93]. FoxM1 inhibition dramatically reduced colony formation and growth rate of HCT116 cells [93].

Three-dimensional organoid culture models were prepared from tissues of colorectal cancer patients. Culture model contained numerous CSCs and showed resistance to Irinotecan and 5-fluorouracil (5-FU) [94]. Treatment with Hedgehog signaling pathway inhibitors (GANT61, AY9944) decreased the cellular viability of organoids as compared to NOTCH and WNT signaling inhibitors. Treatment with GANT61 or AY9944 inhibited expression levels of stem cell markers c-Myc, Nanog and CD44, mainly through inhibition of GLI-1. Combinatorial treatment strategies consisting of AY9944 or GANT61 with 5-FU or Irinotecan effectively reduced colony forming capacity of HCT116 and SW480 cells [94].

Retinoid X Receptor-Alpha (RXRα) transcriptionally regulated speckle-type POZ protein (SPOP). Hypermethylation of promoter region of SPOP interfered with binding of transcriptional factor RXRα to promoter region of SPOP [95]. Moreover, SPOP structurally interacted with GLI2 and directed ubiquitination and degradation of GLI2 in colorectal cancer cells. SPOP overexpression in HCT116 cells notably enhanced apoptosis. Additionally, tumor growth was markedly reduced in BALB/c nude mice subcutaneously transplanted with SPOP overexpressing CRC cells [95].

Tumor Suppressive Effects of HH Signaling:

It has previously been reported that stroma-specific Hh activation in mice significantly reduced the tumor load and blocked progression of advanced neoplasms, partially through regulation of BMP signaling and restriction of the colonic stem cell signature [96]. However, inactivation of Hh signaling accelerated colonic tumorigenesis [96].

Targeting of SHH Signaling:

Resveratrol, a naturally occurring bioactive molecule inhibited viability and migration of HCT116 cells, promoted apoptosis through inhibition of PTCH, SMO, and GLI-1[97]. GLI1 transcriptionally induced expression of Bcl-2 and IGFBP6 genes but cyclopamine treatment exerted repressive effects on GLI1 mediated upregulation of target genes [98].
Extract of Hedyotis diffusa Willd (HDW), traditionally used Chinese herb was found to downregulate target genes (VEGF-A and VEGFR2) of SHH signaling [99]. Ethanolic extracts of HDW reduced mRNA levels of SHH, PTCH-1, SMO and GLI1 in mice xenografted with colorectal cancer cells [99]. Similarly, another Chinese herbal medicine, Scutellaria barbata D. Don also effectively inhibited SHH pathway [100].

TRAIL mediated signaling in Colorectal Cancer:
Selective targeting of cancer cells has been pursued intensively and doubtlessly remained an overarching goal of biomedical researchers. TNF-related apoptosis-inducing ligand (TRAIL) had emerged as a premium cancer killing molecule and because of initial high-quality results entered into clinical trials but tone of some contemporary research-reports reflected disappointment with the perceived outcome. Scientists started to work on demystification of the underlying mechanisms associated with development of resistance to TRAIL in different cancers. Confluence of information suggested that inactivation of pro-apoptotic molecules, overexpression of anti-apoptotic proteins and downregulation of TRAIL-specific receptors (DR, DR5) played contributory role in development of resistance to TRAIL-based therapeutics. Access to the complete sequences of genomes has helped us in developing a better knowledge about re-balancing of pro- and anti-apoptotic proteins to restore apoptosis in TRAIL resistant cancers. TRAIL signaled through death receptors and induced apoptosis via formation of multi-molecular assembly termed as death inducing signaling complex (DISC). DISC was formed by assembly of FAS-associated protein with death domain (FADD) and pro-caspase-8 at death receptor (Fig.3). Pro-caspase-8 was converted into its functionally active form caspase-8 and its activated its downstream effector caspase-3 to trigger apoptosis extrinsically. Apoptosis has branching trajectories and mitochondrially controlled apoptosis is also a very essential mechanism to induce apoptosis. Caspase-8 has the ability to proteolytically process Bid protein. Truncated Bid (tBid) moved into the mitochondrion and facilitated the release of cytochrome c and second mitochondria-derived activator of caspase (SMAC) to the cytoplasm (Fig.3). Cytochrome c formed a complex (apoptosome) with pro-caspase-9 and adaptor protein apoptotic protease activating factor-1 (APAF1) (Fig.3). Functionally active caspase-9 also activated caspase-3 to induce apoptosis.
Downregulation of death receptors severely impaired TRAIL-induced apoptosis in cancer cells. MARCH-8, a family-member of membrane-associated RING-CH (MARCH) ubiquitin ligases targeted DR4 for degradation [101] (Fig.4). N-myc downstream-regulated gene-1 (NDRG1) prevented MARCH-8 mediated DR4 degradation. NDRG1 overexpression markedly enhanced DR4 protein in the membrane fractions. DR4 was poly-ubiquitinated in NDRG1 depleted colorectal cancer cells. Co-immunoprecipitation assay provided evidence of interaction between NDRG1 and MARCH-8 in RKO and HCT116 cells [101].
It seems clear that different strategies which can either stimulate death receptors on surface of cancer cells or effectively inhibit anti-apoptotic proteins can prove to be helpful in improving the treatment outcome of colorectal cancer.
ONC201/TIC10, a first-in-class anti-cancer molecule was noted to efficiently deplete $CD44^+$ cancer stem cells in SW480, HCT116 and DLD1 cells [102]. Moreover, ONC201/TIC10 dose- and time-dependently depleted $CD133^+$ cancer stem cells in DLD1 cells. ONC201/TIC10 dose-dependently increased $TRAIL^+$ cells within the $CD44^+$ population in SW480 cells. ONC201/TIC10 considerably enhanced cell surface expression of DR5 in $CD44^+$ and $CD44^-$ cells. ONC201/TIC10 reduced levels of p-ERK, AKT, p-AKT, FOXO3A and p-FOXO3A in both CSCs and non-CSCs [102].
Zerumbone and celecoxib effectively promoted transcriptional upregulation of DR5 in p53 deficient colorectal cancer cells [103]. Mechanistically it was revealed that Zerumbone and celecoxib promoted

upregulation of DR5 by increasing the levels of activating transcription factor 3 (ATF3). Binding sites for ATF/CREB, CHOP and p53 were identified in promoter region of DR5 gene [103].

Goniothalamin, naturally occurring styryl-lactone compound obtained from Goniothalamus spp considerably upregulated DR5 and simultaneously reduced c-FLIP in colorectal cancer cells [104]. (Fig.3) Parthenolide efficiently upregulated DR5 in TRAIL-resistant (HT-29) and TRAIL-sensitive (HCT116) cells [105].

Azithromycin, a macrolide antibiotic increased the levels of DR4 and DR5 in HCT116 and SW480 cells [106]. Diaminothiazoles (DAT) are anti-mitotic compounds having unique ability to stimulate expression of death receptors in p53-deficient HCT116 cancer cells (Fig. 4). DAT also activated ERK and promoted its nuclear accumulation to stimulate expression of DR5 [107].

Myeloid cell leukemia sequence-1 (MCL-1) and BCL2 antagonist killer (BAK) are anchored to the outer mitochondrial membrane (OMM) by specific trans-membrane domains. Clitocine, a natural product sequestered MCL-1 away from BAK and potentiated TRAIL-driven apoptosis [108]. Ginsenoside compound K exerted inhibitory effects on MCL-1, BCL-2, survivin, XIAP and c-FLIP. Ginsenoside compound K upregulated BAX, tBid and cytochrome c and induced DR5 [109]. Bee venom also effectively enhanced the levels of DR4, DR5, BAX, caspase-8, -9 and caspase-3 [110]. Gefitinib enhanced cell surface expression of DR4 and DR5 in HCT116 cells via activation of c-Jun N-terminal kinase (JNK) [111] (Fig. 4). Tunicamycin triggered JNK/CHOP/DR5 pathway to induce apoptosis in TRAIL-resistant colon cancer cells [112].

Myristoylated alanine-rich C-kinase substrate (MARCKS) positively regulated TRAIL-induced apoptosis. MARCKS overexpression considerably amplified TRAIL-mediated apoptotic cell death in SW707 and SW480 cells [113]. BAY61-3606, a tyrosine kinase inhibitor promoted p53-dependent upregulation of DR4 in colon cancer cells [114].

Hyperthermia directed depletion of c-FLIP from cytosolic fractions, without apparent degradation, has been shown to prevent recruitment of c-FLIP into the DISC assembly [115]. Hyperthermia-triggered c-FLIP depletion was independent of c-FLIP ubiquitination and degradation [115].

Trimeric variants of recombinant TRAIL are currently being tested for efficacy. DISC formation and caspase-8 activation was notable in TRAIL-resistant colorectal HT-29 cells [116]. Moreover, TRAIL-mediated apoptosis was enhanced upon treatment with ABT-737 (Bcl-2 family inhibitor) [116]. TRAIL mutant membrane penetrating peptide alike (TMPPA) TRAIL-Mu3 was found to be effective against colorectal cancer cells [117]. Lipid nanoparticles decorated with sTRAIL (LUV-TRAIL) promoted clustering of DR5 on surface of cancer cells. Moreover, TRAIL molecules were arranged into highly ordered oligomers only in LUV-TRAIL [118].

NK cells were transduced with a lentiviral vector expressing secretion signal domain, extracellular domain and a trimerization domain of the TRAIL gene [119]. Excitingly, engineered NK cells secreted a glycosylated TRAIL fusion protein that triggered apoptosis. Intraperitoneal injection of NK cells effectively delayed tumor growth. There was significant accumulation of TRAIL expressing NK cells at tumor sites, superior infiltration within tumor tissues and higher apoptotic rate [119].

It is encouraging that researchers are focusing on different strategies to improve the efficacy of TRAIL molecules. However, carefully designed clinical trials will further inform us about the potential of these agents to gain entry into the mainstream medicine.

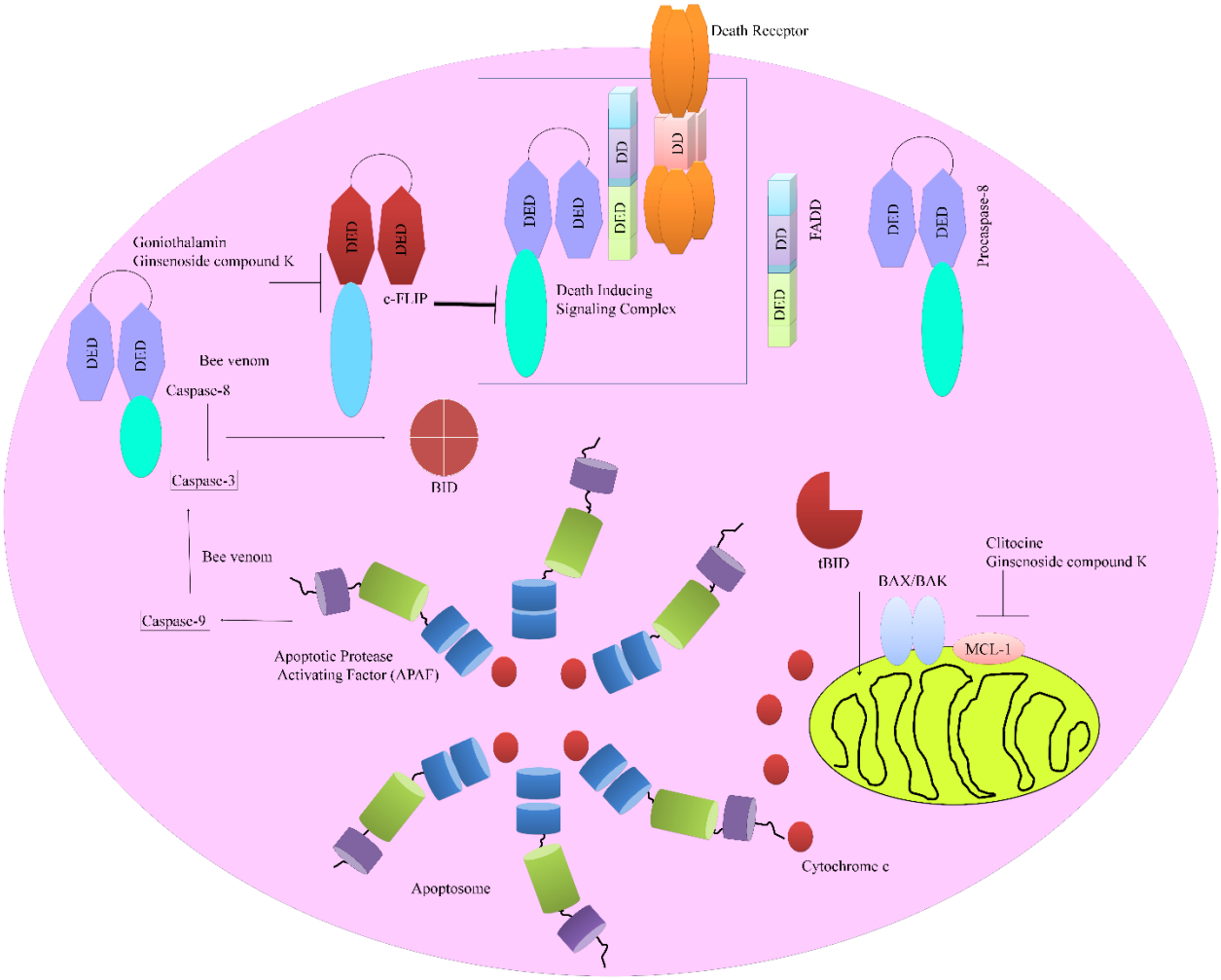

Figure 3 shows TRAIL-induced signaling. Interaction of TRAIL with death receptors (DR4, DR5) induced assembly of FADD and pro-caspase-8. Death receptor, FADD and pro-caspase-8 together formed DISC. C-FLIP interfered with formation of DISC and played central role in inhibition of TRAIL-triggered apoptosis. Activation of caspase-8 was necessary for downstream activation of caspase-3. There is another pathway termed as intrinsic pathway. Intrinsically controlled pathway was triggered via shuttling of truncated Bid (tBid) into mitochondrion. Cytochrome c moved into the cytoplasm and formed a complex with apoptotic protease activating factor (APAF) to induce activation of caspase-9. Different drugs and natural products have been shown to re-balance stoichiometric ratios of pro-apoptotic and anti-apoptotic proteins to induce apoptosis. Bee venom induced activation of caspase-8,-9 and caspase-3. Ginsenoside compound K and Clitocine inhibited MCL-1. Goniothalamin and Ginsenoside compound K inhibited c-FLIP.

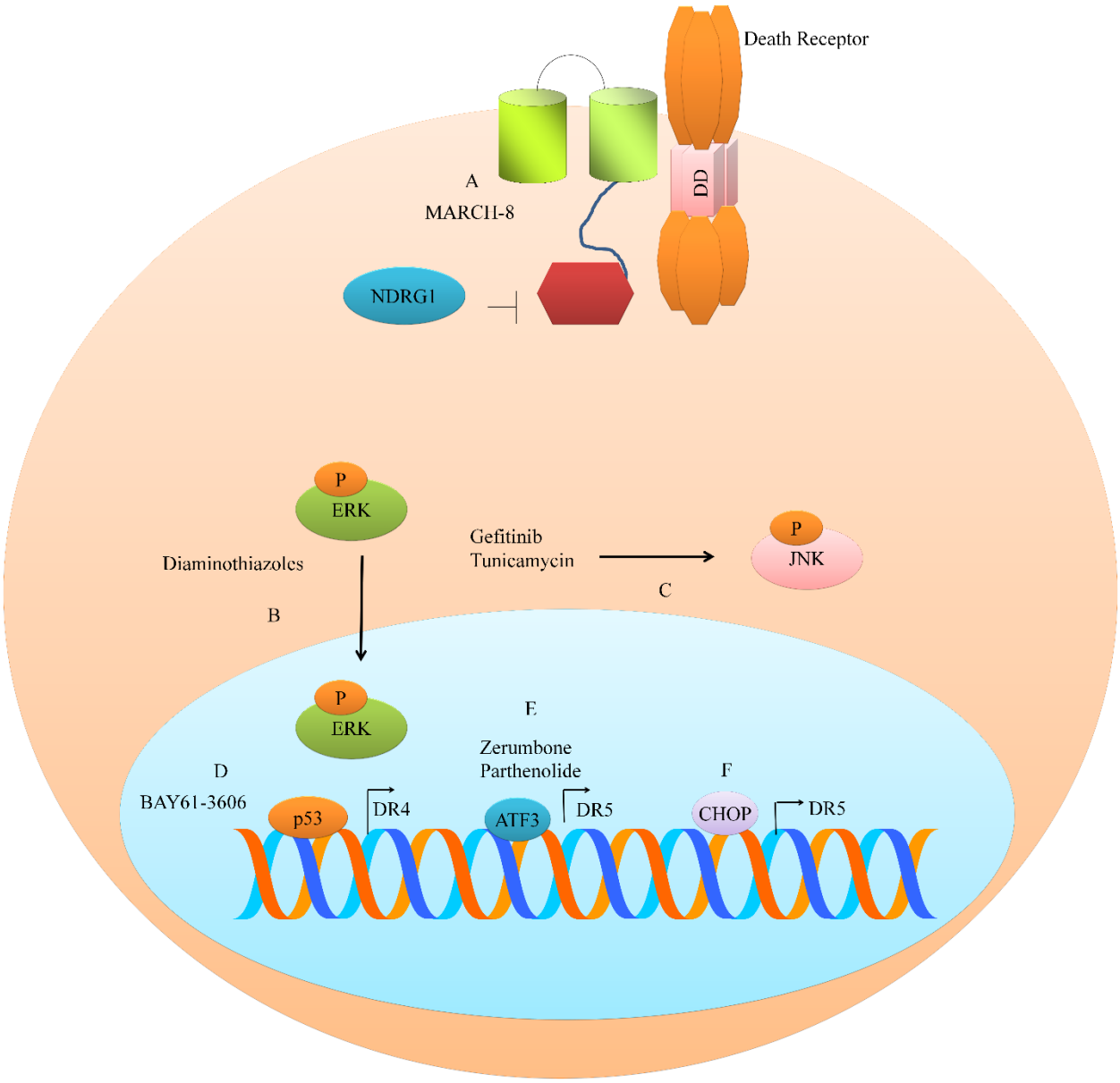

Figure 4 shows regulation of TRAIL pathway at different levels. (A) Death receptors were targeted for degradation by MARCH-8. However, NDRG1 protected death receptors from MARCH-8 mediated degradation. (B) Diaminothiazoles promoted nuclear accumulation of functionally active ERK for transcriptional upregulation of death receptors. Gefitinib and tunicamycin regulated activation of JNK. (D) BAY61-3606 transcriptionally upregulated DR4 via p53. (E) Zerumbone and parthenolide activated DR5 via ATF3. (F) JNK/CHOP was noted to stimulate expression of DR5.

P53 Pathway in Colorectal Cancer:

The p53 protein is a critical transcription factor that has a major function in suppressing tumor formation and growth through multiple pathways [120, 121]. It is often referred to as the 'Guardian of the Genome' because of its important role in maintaining integrity of the DNA, but p53signaling is often deregulated in colorectal cancer. Normally, under homeostatic conditions, the activity of p53 in cells is kept in check by its binding to the negative regulators MDM2 and MDM4, which together induce ubiquitination and proteosomal degradation of p53 and keep cellular concentrations of the tumor suppressor at ineffective low levels (see Figure 5). However, the initial onset of cellular stress following DNA damage propagates signals from specialized stress-sensing proteins to transducer and effector kinases, which then post-translationally modify p53. The resulting conformational change then inhibits MDM2/MDM4 binding to p53 and allows p53 to stabilize and become active [122, 123]. Once activated, the p53 protein can bind to DNA in a sequence-specific manner to transactivate a variety of down-stream gene targets. A major target is the cyclin-dependent kinase (CDK) inhibitor p21, which inhibits the cell cycle to allow cells to repair the DNA damage and survive. Indeed, the p53 protein can further facilitate the process by upregulating components of the DNA repair machinery [121]. Where damage is excessive, the p53 protein will transactivate apoptotic genes, such as Bax, Puma and Noxa, and transrepress tumorigenic and anti-apoptotic genes, such as survivin and Pdk2. This shifts the balance in favor of apoptotic cell death so that the potentially cancer-causing damaged DNA is not passed on to daughter cells [122, 124, 125].

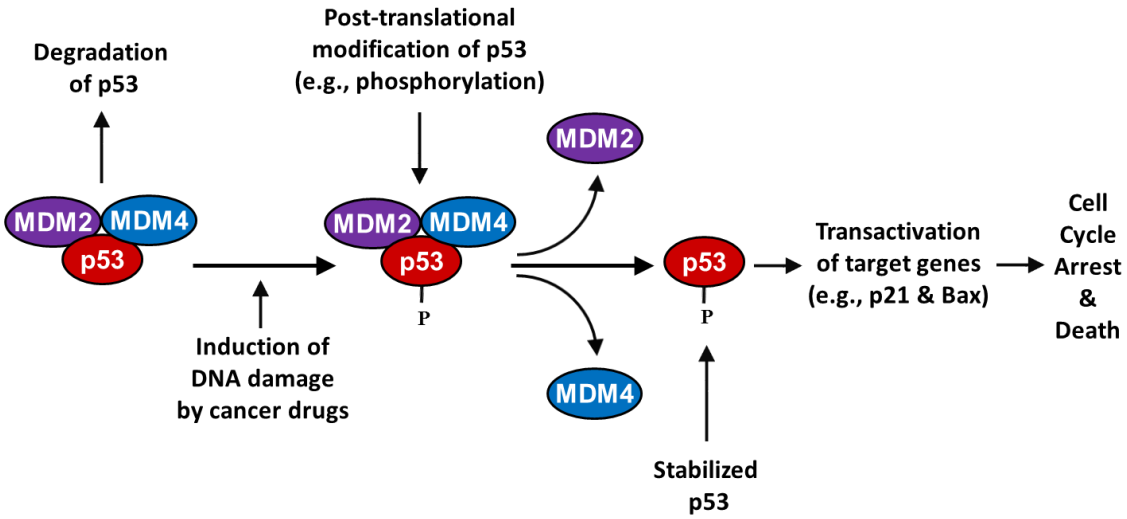

Figure 5. Regulation of p53 by MDM2 and MDM4 and its activation following DNA damage to induce cell cycle arrest and cell death by apoptosis.

The tumor-suppressive function of p53 in colorectal cancer, however, can be severely compromised by significantly high gene mutations, which alter its structural conformation, prevent its critical sequence-specific binding to DNA and, thereby, inactivate the p53 signaling pathway. Moreover, specific mutations may allow gain-of-function phenotypes to emerge by permitting off-target DNA binding of mutant p53 to augment pro-survival pathways and accelerate tumor development [122]. Mutations of p53 in colorectal cancer appear to occur during the latter stages of adenoma-to-carcinoma progression [126, 127]. An extensive analysis involving 3583 colorectal cancer patients reveals that p53 mutations are more frequent

in distal colon and rectal tumors than in proximal colon tumors (45% vs. 34%) [128]. Majority (~80%) of these are missense mutations, largely in exons 4-8 and about a half of these mutations occur in the five hotspot regions at amino acid 175, 245, 248, 273 and 282 [126, 128, 129].

Resistance to therapeutic agents can also provide valuable information on the p53 pathway in colorectal cancer. Therapy of this disease involves a number of drugs, including small molecules 5-fluorouracil (5-FU), oxaliplatin, irinotecan (CPT-11) and capecitabine as doublet or triplet combinations [130]. Interestingly, these drugs are dependent on wild-type p53 [131, 132], and the negative impact of mutation on antitumor effects has been confirmed in colorectal cells in vitro [133, 134] and in patients [129]. In some studies, such as with 5-FU [135] or oxaliplatin [136], absence of a clear delineation in response between wild-type and mutant colorectal tumor cells has made it difficult to use p53 status as a prognostic indicator of treatment outcome. However, this may be due to either wild-type p53 in some tumors failing to become activated following exposure to therapeutic drugs, as has been exemplified by the NCI-747 colorectal tumor model, or that the mutation does not prevent drug-induced activation of p53 function, as seen in HCA7 colorectal cells [137]. That is, mutant p53 may retain significant function, and this has been reported for 50% of about 300 rare (non-hotspot) mutants in a variety of human cancers [138]. In agreement, data mining of two complimentary and closely-related reports on mutation and functional analysis of 1180 p53 mutants from the more extensive 3583 clinical cases of colorectal cancers indicates that 36.2% (105/290) of mutant p53 in proximal colon cancer, 37.3% (44/118) in distal colon cancer and 27.5% (212/772) in rectal cancer retain significant activity [128, 139].

As indicated above, mutant p53 is associated with loss of its transactivation function, and it may be anticipated that expression of downstream target gene could instead be used as a surrogate biomarker of p53 function. Indeed, downregulation of p21 expression has been correlated to poor overall and disease-free survival in colorectal patients [140]. Another important gene target of p53 transactivation is Bax, defects in expression of which have also been associated with reduced survival of patients with colorectal cancer metastatic to the liver [141]. In this particular study of Bax expression, the best outcome was with an intact p53/Bax pathway. However, low Bax expression in context with mutant p53 resulted in poor survival. Interestingly, low Bax expression was also observed in the presence of wild-type p53, and this led to an even worse outcome; in fact, over a two-fold reduction in survival rate due to low Bax expression was observed in patients expressing wild-type p53 in their cancers (median 23 vs. 54 months) [141]. This is consistent with the understanding that wild-type p53 must be transcriptionally activate to increase expression of downstream genes for antitumor response [142] and, thereby, strongly suggests that wild-type p53 can also be inactivated in colorectal cancer. Not surprisingly, other downstream targets of p53 are also likely to be disrupted with dysfunctional wild-type p53. For instance, wild-type p53 can induce cell cycle arrest and apoptosis by also regulating expression of miR-34a, miR-34b and miR-34c microRNA, and significance of the p53-miR-34 pathway in colorectal cancer is highlighted by the development of resistance when promoters of these microRNAs become silenced via CpG hypermethylation [122].

Although the role of wild-type p53 in refractory colorectal cancers may seem counter-intuitive, it can be easily explained. Functional wild-typ3 is normally kept inhibited by regulated levels of MDM2 and MDM4, which can become deregulated, and reports indicate that 9% and 19% of colorectal cancers overexpress MDM2 [143] and MDM4 [144], respectively, and inhibit wild-type p53 function. Deregulation of post-translational phosphorylation is also an important process in inhibiting wild-type p53 activation [122], but reports examining the role of kinases in wild-type p53 deregulation in colorectal cancer are scant. However, in one study, poor expression of HIPK2, which phosphorylates p53 at Serine-46, has been associated with reduced survival rate [145]. Chk2 has also been reported to be downregulated in 50% of colon cancer [146], and it is conceivable that irinotecan-based therapy, which would be expected to activate Chk2 [131] and phosphorylate wild-type p53 at several sites, including

Serine-20, may be less effective at mediating p53-dependent antitumor effects in these specific colorectal cancer patients. To our knowledge, such an investigation has not been reported as yet.

Voltage-gated ion (sodium) channel expression in colorectal cancer:

The bioelectricity of cancer cells generally differs from normal cells in several different respects [147]. In particular, increasing evidence suggest that a variety of ion channels, including voltage-gated ion channels, play a significant, dynamic role in the pathophysiology of cancer, including CRC [148]. Virtually all types of ion channel are involved, contributing to different components / stages of the cancer process. These include voltage-gated Na^+, K^+, Ca^{2+} and Cl^- channels, ligand-gated ion channels and 'transient receptor potential' (Trp) channels [148]. Thus, cancer may even be regarded as a 'channelopathy' [148]. Here we focus on ion channels involved in metastasis since this is the main cause of death from cancer including CRC. In this regard, it is that has received the most attention [149]. According to the "Celex Hypothesis", acquisition of metastatic potential in cancer cells involves upregulation of functional voltage-gated Na^+ channels (VGSCs) activity and concomitant downregulation of outward currents, driven at least in part by voltage-gated K^+ channels (VGPCs) [150]. Overall, the possible electrical 'excitability' could form the basis of aggressive cancer cell behavior [150].

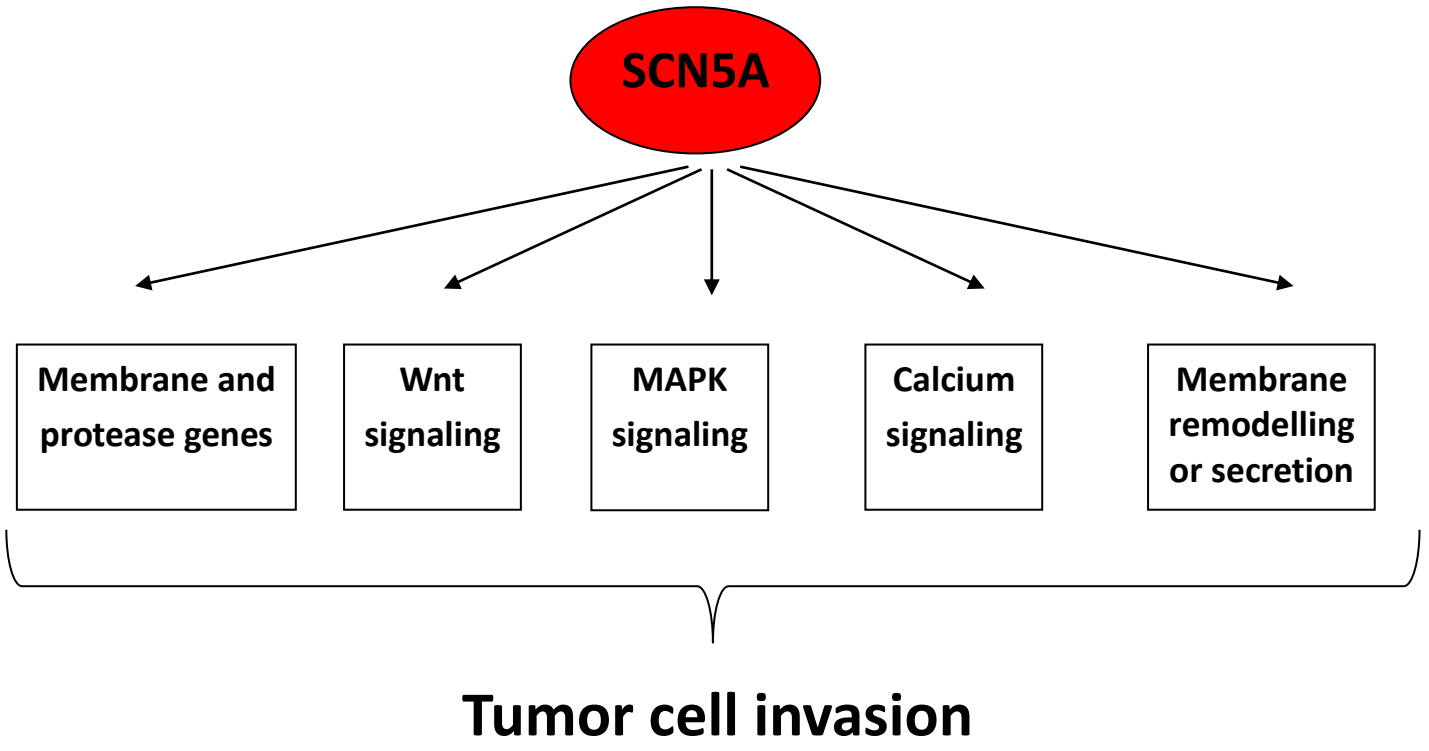

Figure 6. Schematic diagram showing that SCN (gene coding for Nav1.5) is upstream of several canonical signaling mechanisms known to be involved in CRC invasiveness. Evidence originally provided by House et al. (2010) [151].

House et al. (2010) initially investigated VGSC expression in human CRC and showed that the Nav1.5 subtype of VGSC occurred functionally in several cell lines [151]. Treating the cells with the highly specific VGSC blocker, tetrodotoxin (TTX) or silencing *SCN5A* (the gene encoding Nav1.5) by siRNA suppressed the cells' Matrigel invasiveness by $\geq$50 %. In biopsies, also, Nav1.5 protein expression was upregulated significantly compared with matching mucosa. Importantly, computational analysis revealed *SCN5A* to be an upstream "key regulator" of CRC invasiveness, driving a network of canonical genes including those for Ca^{2+} signaling, Wnt signaling, MAP kinase and proteases (Figure X). Subsequently, House et al. (2015) showed that the transcriptional effects caused by VGSC / Nav1.5 activity involved persistent activation of ERK1/2 MAPKs which was PKA-dependent [152].

Nav1.5 is developmentally regulated via alternative splicing of exon 6, giving rise to 'adult' (3') and 'neonatal' (5') forms that differ in the S3-S4 region of domain-I by several amino acids [149]. This difference enabled a polyclonal antibody (NESOpAb) specific for the neonatal splice form of Nav1.5 (nNav1.5) to be produced [153]. In human breast cancer, the functional VGSC was shown earlier to be nNav1.5 [149, 154]. This is in line with the expression being 'oncofoetal' [155]. Originally, House et al. (2010) stated that the Nav1.5 in CRC was the 'adult' form (aNav1.5). Subsequently, nNav1.5 mRNA was also shown to be expressed in CRC *in vitro* [156]. Finally, in a detailed study, Guzel et al. (2018) reported that functional expression of nNav1.5 was dominant in CRC (SW620) cells and was largely responsible for the invasiveness [157].

A variety of VGPCs have been discovered in human CRC cells and tissues. These include Kv1.1. Kv1.3, Kv1.5, Kv3.1, Kv3.4, Kv9.3 Kv10.1 (Eag1) and Kv11.1 (hERG). Their expression appears to be downregulated or upregulated in CRC, dependent probably on the stage of cancer. By far the bulk evidence, however, points to Kv's promoting proliferative activity [158]. As regards secondary tumourigenesis, both increased and decreased VGPC activity could promote the metastatic phenotype by promoting (i) accumulation of mutations and/or (ii) the VGSC activity itself, respectively. In addition, we should note that there is also evidence for major remodeling of Ca^{2+} signaling in CRC but this is outside the scope of the present review [159,160].

The discovery of functional nNav1.5 expression in human CRC cells (and tissues) offers several clinical possibilities. First, as suggested initially by House et al. (2010), it is upstream of several 'mainstream' mechanisms of invasiveness, so it can be expected to be expressed early in metastasis, consistent with its pathophysiology. Thus, the available evidence supports the case for nNav1.5 being a viable functional biomarker. Approximately 50% of CRC patients relapse, some with distant metastases, even after surgery and/or chemotherapy, so earlier diagnosis is vital [161]. In this regard, a recent study has reported that high Nav1.5 expression levels correlated with unfavorable disease-free survival in patients with non-metastatic CRC [162]. Second, as a neonatal splice variant, it has the potential to be 'cancer specific' in the adult body [163]. It can be targeted with antibody, therefore, both for diagnosis and therapy. Chioni et al. (2005) produced a blocking polyclonal antibody that had >100-fold selectivity for nNav1.5 over its 'nearest neighbour' (aNav1.5). Such an antibody could also form the basis of directed killing of CRC cells including micro-metastases [153]. Third, it is well known that an important characteristic of growing

tumors is development of internal hypoxia which promotes their aggressiveness (e.g. [157,164]. Independently, hypoxia has been shown to enhance the VGSC 'persistent current' (I_{NaP}) component of which most work has been done on Nav1.5 expressed in cardiomyocytes [e.g. 165]. Importantly, I_{NaP} can be blocked by the anti-anginal drug ranolazine with little effect on the regenerative 'transient current' component responsible for nerve/muscle excitation/conduction [e.g. 166]. Indeed, clinical doses (<10 μM) of ranolazine blocked the hypoxia-induced increase in invasiveness, consistent with the involvement of I_{NaP} [157]. Ranolazine was found previously also to reduce metastatic dissemination in a breast cancer xenograft model [167]. Thus, ranolazine and other available VGSC/I_{NaP} blockers could be 'repurposed' as novel anti-metastatic drugs in clinical management of CRC [168-171].

Conclusion:

Colorectal cancer (CRC) is therapeutically challenging and increasing list of ground-breaking discoveries has helped us to improve our understanding about colorectal cancer to a previously unprecedented extent. In this review, we have provided detailed list of pathways which played key role in CRC development and progression. Oncogenic mutations in the Wnt pathway are prevalent in CRC and potentially a therapeutic target. However, as outlined here, experimental data is needed to: (a) determine whether the pathway is indeed a viable target for therapeutic intervention in colorectal carcinogenesis and (b) identify essential signaling nodes regulating the oncogenic form of the pathway that are therapeutically tractable targets in CRC. Two recent studies have demonstrated intriguing avenues for inhibiting oncogenic Wnt pathway activity by targeting newly characterized pathway regulators localized to the cell surface. The Rohatgi laboratory has demonstrated that RSPO2 and RSPO3 but not RSPO1 and RSPO4 can potentiate Wnt pathway activity independently of expression of their cognate LGR receptors in CRC cell lines with APC mutations (172). The development of neutralizing antibodies against RSPO2/3 may ultimately show therapeutic promise for a broad range of CRCs.

A second study demonstrates that RNF43 can interact with TCF7L2, tethering it to the nuclear membrane and inhibiting Wnt pathway activity (173). The direct link between a plasma membrane localized factor, RNF43, and the key transcription factor in the Wnt pathway that potentially short circuits oncogenic mutations in APC and β-catenin is a particularly compelling target – the possibility of increasing RNF43 expression levels or its TCF7L2 binding activity will enable direct modulation of the oncogenic Wnt pathway transcriptional programme. Further work will be required for the development of therapeutic strategies that either inhibit RSPO2/3 function or enhance RNF43-TCF7L2 interactions. However, in light of the molecular tractability of these plasma membrane-localized Wnt pathway regulators, further development may provide a fruitful avenue for targeting a broad spectrum of CRCs. JAK-STAT signaling pathway needs to be effectively targeted. Additionally there is a need to identify synthetic and natural products which can target STAT proteins and simultaneously shut down non-canonical STAT pathway. TGF/SMAD signaling has been shown to drive CRC, however, we still have to indentify the positive and negative regulators of TGF/SMAD pathway in CRC. SMURF and NEDD ubiquitin ligases have been studied individually but it needs to be seen how these proteins behaved in colorectal cancer cells. Detailed study of regulation of TGF/SMAD pathway by SMURF and NEDD proteins will surely provide a clear snapshot of the proteins which can be exploited for an effective treatment of CRC. TRAIL pathway has been extensively studied in different cancers and there has been an exponential growth in the publications related to role of TRAIL pathway in different cancers and how it can be re-activated. However, in CRC, TRAIL pathway is insufficiently studied. We still have to see how death receptors can be activated and decoy receptors can be inhibited in different stages of CRC. Additionally, there is a need to focus on the identification of agents which can increase cell surface expression of death receptors. There are various situations in which death receptors are internalized and sorted for degradation. Therefore, molecular biologists have yet to fully decode the underlying mechanism which cause reduction in the number of death receptors on surface of colorectal cancer cells. Epigenetic inactivation of death receptors, overexpression of decoy receptors may also severely compromise TRAIL-induced apoptosis. Better

understanding of these aspects will pave the way for effective translation of TRAIL-based therapeutics and death receptor targeting antibodies from bench-top to the bedside.

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