Investigating the role of *Cited 1* in promoting *Wnt* induced tumorigenesis in intestines

Summary

Colorectal cancer is a significant health hazard of the 21st century, and GLOBOCAN predicts increasing cancer incidence in the coming decades. Though numerous studies have established the mechanism of colorectal cancer initiation and progression, further insights at the molecular levels are needed to establish novel treatment paradigms circumventing cancer. Recent research implicates that CBP/p300-interacting transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain (*Cited-1*) is associated with many cancers including Wills tumor, papillary thyroid carcinoma, and malignant melanomas. Furthermore, Cited-1 has also been linked with trophectoderm and placenta development in the embryo. Although Cited-1 is associated with both embryogenesis and tumorigenesis, its role in colorectal cancer is still unresolved. To this end, the present work aims to delineate the propensity of the functional role of *Cited-1* in promoting Wnt- induced tumorigenesis in intestines. State-of-the-art multi-omics-analysis will be employed for profiling and identification of *Cited-1* expression in different grades, stages of human colorectal cancer. Cited-1 knock-in/out cell models will be generated using CRISPR/ Cas9 genome editing tools. The functionality of *Cited-1* in proliferation, migration, and invasion will be determined by re-engineered molecular biological methods and applied on CRISPR/Cas9 knock-in/out cell models to analyze the activity on the level of single cells. To further evaluate the role of Cited-1 in colorectal carcinogenesis, transgenic murine models overexpressing the *Cited-1* gene will be generated using GONAD CRISPR/Cas9 approach. Characterization of these murine models will provide insights into understanding the specificities and dynamics of the disease and aid in the clinical paradigm for therapeutic intervention.

1. Introduction

Colorectal Cancer (CRC) is a heterogeneous disease-causing approximately 6,00,000 deaths annually worldwide (Siegel et al. 2020). While the survival rates of the CRC patients have increased to 56% for the first five years of the treatment, further understanding about the etiology of the disease is necessary to diagnose the disease early and procure the possible treatments (Rawla, Sunkara, and Barsouk 2019). The intestinal epithelium is constantly under wear and tear and its maintenance and self-renewal are orchestrated by the Wnt pathway. CRC development is a sequential process involving several genetic changes in normal epithelium, which results in the growth of pre-cancerous cells into an adenomatous polyp which in-turn develops into an invasive tumor (Armaghany et al. 2012). One of the hallmarks of CRC is the deregulation of the Wnt signaling pathway due to mutation in adenomatous polyposis coli (APC) gene, a tumor suppressor gene that controls β-catenin concentrations resulting in the formation of the polyps across the intestine (Zhang and Shay 2017). Though mechanistic evidence of Wnt signaling cascade in colon cancer has already been established,

further insights at genetic levels are needed for the establishment of novel treatment regimens circumventing cancer.

Recently CBP/p300-interacting transactivators with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain (*Cited-1*) have been implicated in many cancers including Wills tumor, papillary thyroid carcinoma, and malignant melanomas (Li et al. 2018; Lovvorn et al. 2007). *Cited-1* has also been reported to be upregulated in MMTV-CRWE/floxNeo NT mammary tumors by regulating oncogene EGR2 (Dillon et al. 2007). Similar studies have shown its association with Six2, a transcriptional factor that promotes the maintenance of self-renewing nephron progenitor cells in the kidney (Mugford et al. 2009). *Cited-1* gene also plays a significant role in regulating the trophoblast lineage specification via activation of bone morphogenetic protein (BMP) signaling pathway in embryogenesis. Cited-1 can enhance TGF-β signaling and inhibit Wnt signaling subject to the cellular milieu. The bifunctionality of transcription is reliant on the Cbp/P300 binding C-terminal transcription activation domain CR2, which is preserved all over the Cited family(Hendry et al. 2011). Although, these studies strongly suggest the dual role of *Cited-1* in embryogenesis and carcinogenesis, its role in Wnt induced intestinal tumorigenesis is largely unknown.

Aside from the evidence that Cited-1 is a regulator of the Wnt pathway, Clarke et.al for the first time reported the deficiency of Cited-1 can suppress intestinal tumorigenesis in Apc^{min} models. In the end, they proposed a model where the loss of Cited-1, over-stimulated the Wnt pathway thereby inhibiting colorectal cancer. Microarray analysis of $Apc^{-/-}$ murine models also identified Cited-1 to be upregulated in colorectal carcinoma (Méniel et al. 2013). Hence, the current study is aimed to unravel the functional role of Cited-1 in the initiation and progression of colorectal cancer using genome editing tools. The present work will give a brief mechanistic framework of the role of Cited-1 in Wnt deregulated colon carcinoma leading to the formation of early lesions.

2. Review of Literature

Colorectal cancer (CRC) is the third most frequently observed cause for cancer-related deaths in both sexes in the United States, accounting for an approximate 147950 individual diagnosis and 53200 deaths in the year 2020 (Siegel et al. 2020). With an average survival rate of 2.5 years, about 15% of the CRCs are diagnosed in the later stages of metastasis. Moreover, more than 50% of CRCs diagnosed at lower TNM stages will gradually undergo liver metastasis (Balacescu et al. 2018). The survival rates are accustomed linked to various factors like the stage and grade of the diagnosed cancer tumor, the environmental factors, and the genetic factors. A substantial portion of the CRC related complications and deaths are attributed to potentially preventable hazardous factors like smoking, alcohol consumption, delicate diet, and excess body weight. The genetic contributions come from either a strong family history of autosomal dominant inheritance of CRC, presence of multiple primary cancers in the patient, early age diagnosis, or existence of tumors with a risk of inherited CRC among the patient's kindred (Marley and Nan 2016). Although the incidence rate has declined by 3.6%

every year from 2007 to 2016, and, the 5-year survival rate has shown a relieving 64%, the fact that the incidence rate among the cohort aged below 50 is shooting at a rate of 2% every year is alarming (Siegel et al. 2020). Even though the improvement in adjuvant therapies that includes surgery, chemo, and radiotherapies has enabled an overall increment in patients' survival rate in their earlier stages, there are no effective therapies for the treatment of CRC with metastasis. Therefore, a molecular-level understanding of cellular transition in CRC is imperative in developing optimized strategies to treat CRC and prevent incipient metastasis during adjuvant therapies.

There are three main routes of CRC carcinogenesis. The first one is known as the suppressor pathway or pathway of chromosomal instability and was proposed by Fearon et al. This involves the accumulation of mutations that lead to activation of an oncogene (KRAS) and inactivation of suppressor gene (DCC, APC, SMAD4, TP53). Regardless of the order in which the accumulation of these mutations takes place, they are responsible for neoplastic transformation (Tariq and Ghias 2016). The second one involves the accumulation of errors during DNA replication due to mutations in genes involved in repair (MSH2, MLH1, MSH6, PMS2, MLH3, MSH3, PMS1, and Exo1). This results in mutations in various target genes due to errors that predominantly accumulate in repetitive DNA fragments (microsatellites) scattered throughout the genome. This mutator pathway or microsatellite instability is involved in Lynch syndrome and 15-20% of sporadic CRCs (Armaghany et al. 2012). Histologically, these tumors are characterized by an increased lymphocyte infiltration (Crohn-like) and are mucinous, poorly differentiated tumors. The third route involves aberrant hypermethylation which is a mechanism to silence gene function. Dinucleotide methylation in the promoter region of many genes is referred to as the CpG island methylator phenotype (CIMP). The CIMP-positive tumor is the one that involves methylation of at least three of the following markers: CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 (Binefa et al. 2014). Clinical expression is observed when inherited mutation in one allele is followed by another hit mutation or deletion of the second APC allele.

Cited-1 is a transcription co-factor, which belongs to the CBP/p300 binding C-terminal activation domain CR2. It is expressed in progenitors of the heart, axial skeleton, limb, kidney, and placenta at the time of vertebrate development (Plisov et al. 2005). It also affects embryo growth and survival. Depending on the cellular context, it enhances TGF-β signaling while inhibiting Wnt signaling. Cited-1 has been implicated to be deregulated in various human cancers, including Wilm’s tumors, nephroblastoma, and melanomas (Li et al. 2018; Lovvorn et al. 2007). Cited-1 is found to be up-regulated in MMTVCre/FloxNeoNeuNT mammary tumors in the mouse and is mostly linked with the transcription factor EGR2 to regulate the oncogene ErbB2 (HER2, Neu) expression (Dillon et al. 2007). Cited-1 gene plays a key role during embryonic development, by regulating the expressing in extraembryonic tissues and in trophectoderm-derived cells of the placenta. Transcriptional regulation of Cited-1 gene is seen in exclusively in the nephrogenic progenitor cells (Hendry et al. 2011). It is detectable in the nuclear compartment of Wilms' tumor blastema, indicating that Cited-1 is a diagnostic

marker, which could give insights about Wilms' tumor initiation and pathogenesis (Lovvorn et al. 2007). Papillary thyroid carcinoma is found to have up-regulated Cited-1 expression after evaluation by tissue microarrays and immunohistochemistry. Recent research has shown that Cited-1 with another transcription factor Six2 expression, together, specify self-renewing nephron progenitor cells in kidney development and it is suggested that Cited proteins may contribute to the maintenance of the self-renewing capping mesenchyme in the developing kidney (Mugford et al. 2009). Thus, a plethora of literature has implicated Cited1 in both carcinogenesis and, embryogenesis its functional role in Wnt-induced intestinal tumorigenesis remains unanswered.

Recent advances in the genome editing technologies give impetus in better understanding the molecular interactions occurring inside the cellular level. Somatic mutations that instigate oncogenic elements, fosters the halt of differentiation or give rise to tumorigenesis by affecting a broad spectrum of cellular processes, which eventually leads to the constitutive activation of fundamental intracellular signaling pathways associated with the pathogenesis of colon cancer (Kwong and Dove 2009). CRISPR/ Cas9 system has revolutionized the genome editing research by its ability to induce specific targeting, ease of use, simplicity, and robustness (Zheng et al. 2014). CRISPR/Cas9, originally found as an acquired defense mechanism of bacteria in response to invading bacteriophages, have now been fine-tuned to edit any set of sequences. CRISPR/Cas9 makes double-strand breaks (DSBs) at a target gene locus by a robust and easily manipulated single guide RNA and the Cas9 enzyme. CRISPR/Cas9-associated DSBs can initiate one of two major pathways for DNA repair: (1) homology-independent repair, such as non-homologous end recombination (NHEJ), or (2) homology-directed repair (HDR) with an endogenous or exogenous homologous DNA template (Fang et al. 2019). Recent research has implicated that the homology-independent pathways can be exploited to insert bulky sequences *in vivo.* Although CRISPR/ Cas9 mediated in-*vivo* genome editing approaches has three major steps: super-ovulated females mating and segregation of zygotes, microinjection of genetic engineering components into the zygotes, and transference of microinjected transformed zygotes into the oviducts/ ovary/ fallopian tubes of pseudo-pregnant females. With the advent of i- GONAD CRISPR method, it has become easy to introduce to large segments of gene *in-vivo,* and its offers several advantages against its counterparts like does not require a highly skilled technician or sophisticated logistics, less than 40% of animals used when compared with the animals spent in conventional methods and females used for i-GONAD can be recycled; thus, creation of genome-edited animals can occur without loss of the female (Ohtsuka et al. 2018).

3. Hypothesis

The underlying hypothesis is that Cited-1 is a major detrimental non-DNA binding transcriptional factor in colorectal cancer initiation and progression. Transcriptional activation of the Cited-1 gene results in direct activation of the Wnt pathway through β-catenin levels that could lead to aberrant polyps' formation across intestines.

4. Objectives

1. **Determination of *Cited 1* expression in colorectal cancer patient samples/cell lines of different grades compared to healthy controls.**

   To understand the Cited-1 role in colorectal cancer, we first need to assess its expression in different grades and stages of colon cancer samples. This will help find and validate the relative abundance of Cited-1 in human tumor samples. Moreover, to the best of my knowledge, we will be the first to delineate the Cited-1 levels in different stages of human colorectal cancer. To achieve this objective, we will be doing extensive sampling and quantitative reverse transcription real-time PCR of CRC samples and appropriate controls.

2. **Generation of knock-in/out Cited-1 cancer cell models by CRISPR/Cas9 systems.**

   After the Cited-1 expression is analyzed in human tumor tissues/ cell lines, we will generate Cited-1 deletion and insertion mutants in normal and cancer cell lines by using the CRISPR/Cas9 system. This would be carried out by the construction of Cas9 cassettes and determining guide RNA sequence. The CRISPR/ Cas9 system will be then transfected into the cell lines using lipofectamine. Then, confirmation of knock-in and knockout can be achieved PCR and sequencing respectively and it can be further validated by using either immunoblots or quantitative PCR.

3. **Deciphering the functional role of *Cited 1* knock-in/out cell models in proliferation, migration, and invasion**

   State-of-the-art multi-omics-analysis will be used for the identification of the functional role of Cited-1 cell lines models in proliferation, migration, and invasion. Different Wnt inhibitors and inducers will be used to access the expression of Cited-1 to Wnt pathway activation and deletion. CRISPR/Cas9 systems will be re-engineered by molecular biological methods and applied to transfection/infection models to analyze phenotypical bioactivity on the level of single cells. For exploring Cited-1 role in proliferation, MTT, and cell cycle assay can be used. Migration and invasion can be investigated by the Boyden chamber, wound healing assay and the expression of various migratory markers will be evaluated to validate the results.

4. **Development and characterization of *Cited 1* overexpression transgenic murine models using i-GONAD CRISPR/ Cas9 approach**

   To understand the functional role of Cited-1 in intestinal tumorigenesis, transgenic murine models were created using i-GONAD (Genome-editing via Oviductal Nucleic Acids Delivery) CRISPR systems. To simplify germline genome editing, we will be using this technique which does not require ex vivo handling of zygotes or their isolation for microinjection and it readily edit the genome in the recipient females with significant transfection efficiency. We will be using a villin promoter (constitutive) with the Cited-1

gene to specifically induce its expression in intestines. GONAD is performed on pregnant mouse females bearing E1.5 (2-cell stage) embryos. Through incision at a dorsolateral position, the ovaries are surgically exposed and there the genome editing soup containing the reagents are injected into the oviductal lumen using a glass capillary pipette. After the injection of the reagents, the entire oviduct is electroporated using tweezer-type electrodes and placed back in the appropriate places. After the birth of the T0 generation (founder), we will use immunohistochemistry to access the localization and quantification of Cited-1 and activation of other Wnt target genes and its changes in the gut.

5. Methodology

1. Determination of *Cited 1* expression in colorectal cancer patient samples/cell lines of different grades compared to healthy controls.

- **Collection of Human Colon cancer tissues**

  Human colon cancer tissues will be collected from one hundred patients each having different grades and stages of cancer reported at Government Medical Hospitals. All procedures will be executed following the declaration of Helsinki, after obtaining written informed consent from the patients. Colon cancer tissues and their adjacent normal will be taken from patients who underwent surgical resection of tumors without prior treatments. Fresh samples obtained will be stored in RNA Later for further analysis.

- **Quantitative PCR**

  Total RNA will be isolated from a total of 100 colon samples along with healthy controls using a kit as per the manufacturer's instructions. The purity and quantity of RNA will be measured using NanoDrop ND 1000 spectrophotometer and integrity will be checked by determining RNA integrity number (RIN) using an Agilent Bioanalyzer (2100). Only those RNA samples with RIN number >7 will be taken for this study. Cited-1 and c-Myc specific primers will be constructed using Primer 3.0 software. The first-strand cDNA will be synthesized from total RNA (1 µg) using High capacity cDNA synthesis reverse transcription kit according to the manufacturer's instructions (Nair et al. 2020).

2. Generation of knock-in/out *Cited-1* cancer cell models by CRISPR/Cas9 systems.

- **Cell lines and Culture**

  Human cell lines such as HEK293, SW480, and HCT116 will be obtained from American Type Culture Collection ATCC. The cell lines will be maintained routinely and checked regularly for the presence of mycoplasma. HEK293 and HCT-116 will be grown in DMEM containing 5% fetal bovine serum. The cultures will be maintained in sterile irradiated 25/75 cm^2 culture flasks at a concentration of 1x106 cells/cm^2 with suitable media for each cell line. The cells will be regularly passaged once they attain 80-85% of confluency. All the cell lines will be maintained strictly at 37°C and 5% CO2 in DMEM medium

supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100ng/ml) (Tangudu et al. 2015).

- **SgRNAs, plasmids, and cloning**

  For knockout cell models, CRISPR/Cas9 vectors will be designed for specific targeting of the selected sites and areas within the Cited-1 gene in HCT-116 and SW-480 cells. Cited-1 gene will be mined from the NCBI database. All the guide RNAs will be constructed using CRISPRdirect (http://crispr.dbcls.jp/). The single guide RNA (sgRNA) oligomers will be formulated and cloned into the vector pSpCas9(BB)-2A-GFP. The sgRNAs will be cloned into a BbiI-digested vector by annealing two DNA oligos (Cong et al. 2013). To increase the promoter efficiency and activity, we will be taking all the sgRNAs that did not start with a 5' G and adding an extra 5' G nucleotide (Van Treuren and Vishwanatha 2018). For knock-in models, CRISPR/Cas9 vectors will be designed in the selected regions within the AAVS (Adeno associated virus integrated site) in the HEK293 cells. The donor plasmid containing the fusion construct with the GFP will be prepared and supplied with the CRISPR/Cas9 plasmid. 17 sgRNA vectors will be taken which targets four specific sites and different regions within the Cited-1 gene will be designed and built (Kanwal et al. 2018). The plasmid containing the CRISPR/Cas9 target site for each target mutation will be confirmed by plasmid DNA sequencing. A mixture of 1 µg of plasmid DNA containing each target sgRNA sequence will be used for cultured cell transfection.

- **Vector transfection**

  Cultured cell models will be first seeded in 6-well plates and then transfected when they reached a density of 70% using Lipofectamine 3000 following the manufacturer's instructions. Two different plasmids (pSpCas9(BB)-2A-GFP expressing green fluorescence and Phage-to-dCas9-3XmCherry expressing red fluorescence) will be used to compute the transfection efficiency (Kanwal et al. 2018). All the cell lines will be transfected with both plasmids separately. The cells will then be incubated for an additional 48 hours, after which GFP positive cells will be sorted by FACS on a Sony SH800 Cell Sorter. Single-cell clones will be grown and further screened via PCR and immunoblotting for Cited-1 knock-in/out models (Martinez-Lage et al. 2020). Two different cancer cell lines will be transfected with varied vectors and will be screened to select the cells that might have a high transfection rate. The empty vectors will be used as an internal control.

- **Sequencing**

  Regions around the sgRNA target/off-target sites within the Cited-1 genes will be amplified by PCR. PCR reactions will be cleaned using a PCR Purification Kit. Amplicons will then be analyzed by Sanger sequencing (Takeda et al. 2019).

- **Immunoblotting**

Protein from selected clones will be extracted using ice-cold lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 1% NP40, 1 mM EGTA, 50 mM NaF, 40 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 8 mM PMSF, pH 7.5) supplemented with protease inhibitor cocktail. Total protein will be quantified using Bradford Reagent. Typically, ~25 µg of protein (controls and genome-edited cohorts) of tissue lysates will be separated by SDS-PAGE on a 10 – 12% acrylamide gel, and proteins will be electro-transferred onto Hybond nitrocellulose membrane. Immunoblotting will be performed as described previously with antibodies specific to Cited-1. Signals will be detected using the ECL substrate in an enhanced chemiluminescence system. The membrane will be re-probed for β-actin to confirm the equivalent loading and transfer of protein (Chakraborty et al. 2019).

3. Deciphering the functional role of *Cited 1* knock-in/out cell models in proliferation, migration, and invasion

- **Cell proliferation assay**

  Cell proliferation will be analyzed using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 24, 48, and 72 hr of treatment with Wnt inducers, the MTT reagent will be added, and the mixture will be incubated for 3 hr at 37°C. Dimethyl sulfoxide will be then added, and the absorbance will be measured at 595 nm by a microplate reader (Van Treuren and Vishwanatha 2018).

- **Immunocytochemistry**

  Human colon cancer cell lines like HCT 116, SW480 cells, and HEK293 were cultured on a coverslip. The cultured cells were then transfected with WNT inducers (ΔNLRP, Flag –AX, Δbcat, -VP-16-TDB, Wnt 3a, Si RNA A, LiCl), CITED 1 shRNA, scrambled shRNA, and CITED 1 cDNA. The cells were further fixed with 4% paraformaldehyde followed by treatment of 0.5% TritonX – 100 and blocked with 10% normal goat serum. To validate the expression of genes in the cultured cells, cells were incubated primarily by mouse monoclonal antibodies overnight at 4°C. Mouse monoclonal antibodies are specific to β- catenin, c-MYC, and Cited-1 (1:50). Later the cells were exposed to anti-mouse Alexa 488 conjugated secondary antibody (1:200) for 60 minutes at room temperature (RT). The immunostaining results were analyzed and recorded under a confocal laser scanning microscope (Chakraborty et al. 2019).

- **Wound healing migration assay**

  Cited-1 knock-in/out models of density 1 x 105 cells/well will be plated on 24 well plates and transfected with WNT inducers (ΔNLRP, Flag –AX, Δbcat, -VP-16-TDB, Wnt 3a, LiCl). After 24 hours of incubation, a thin strip of cells (1.5mm2) will be scratched and removed from the well with the help of a yellow tip pipette. The scratched area will be monitored at different time points as 0, 12, and 24 for respectively each experimental group and later

calculated the percentage of wound closure at given different time points. Analyzation and monitoring of wound closure at different time points due to migration of cells will be done through pictures taken from phase-contrast microscope Zeiss Axiovert 200M with the magnification at 10X.

- **Boyden chamber invasion assay**
  A modified Boyden chamber assay will be performed using 48-well chambers using polyvinylpyrrolidone-free polycarbonate membranes with 8 µm pores. The bottom chamber will be filled with 25 µL of DMEM supplemented with 10% FBS as a chemoattractant. Cited-1 knock-in/out cell models will be seeded into the upper compartment with appropriate controls. The cells will be allowed to migrate for 4 hours. The cells will be counted in five individual high-powered fields for each membrane and migration of cells across the membrane will be quantified under a light microscope (Chakraborty et al. 2019).

4. Development and characterization of Cited 1 overexpression transgenic murine models using i-GONAD CRISPR/ Cas9 approach

- **Animals**
  Mice will be maintained at the appropriate animal facility in the research institute. Adult Jcl: MCH(ICR), C57BL/6JJcl (inbred strain), and BALB/cAJcl will be taken for in-vivo experiments. All the animal studies will be performed under the Institutional Animal Ethical Committee and the guidelines will be approved by the Institutional Animal Care and Use Committee (Ohtsuka et al. 2018).

- **Preparation of CRISPR electroporation solutions**
  The solution containing *in-vitro* synthesized CITED-1 sgRNAs (or commercially procured crRNA/tracrRNA mixes) and the commercially procured Cas9 protein will be procured from the respective manufacturers. When the donor DNAs are included in the electroporation solutions, they will be either commercially synthesized ssODNs or ivTRT synthesized long ssDNA containing the Cited-1 gene. Lyophilized crRNA and tracrRNA will be first re-suspended in RNase-free Duplex Buffer to a concentration of 200 µM. The electroporation solution will be slowly diluted using Opti-MEM to re-adjust the volume to 1.5 µl/oviduct per injection (Takahashi et al. 2015).

- **GONAD procedure**
  Female mice that are not super-ovulated will be used for the GONAD procedure except for the C57BL strain. For most of the strains except C57BL, female mice in the estrus cycle will be mated with stud males. Mating will be set up in the evening time, and by next morning (9:00–10:00) copulation plugs will be confirmed by visual inspection of the cage. Day 0 will be used as a designated gestation time at 0:00 (midnight) according to

Manipulating the Mouse Embryo: A Laboratory Manual, and the female mice with plugs at day 0.4 of gestation day 0.7 of gestation will then be further taken ahead for the electroporation experiments.

Anesthetic female mice at day 0.7 of pregnancy (corresponding to late 1-cell stage zygotes, at 16:00 of the same day when the plugs were confirmed) will be used for carrying out surgical experiments under observation using a dissecting microscope. After making an incision at the dorsal skin the oviduct will be exposed. Around 1.0 µl of electroporation solution (pre-warmed at 37°C for 10 min) will be injected into the oviduct lumen from upstream of the ampulla using a micropipette. The micropipette apparatus consisted of a glass capillary needle with a mouthpiece attached to the needle. Instantly after the injection of the solution, the oviduct regions will be covered with a piece of wet paper soaked in phosphate-buffered saline (PBS) and then grasped in tweezer-type electrodes. The electroporation will be performed using a square-wave pulse generator T820. After the electroporation, the oviducts will be returned to their original position, after suturing the incision. The animals will be monitored for anesthesia recovery by IR lamps and will be housed for further downstream experiments (Ohtsuka et al. 2018; Takahashi et al. 2015).

- **Analysis of CRISPR/Cas9-induced mutations and insertions**

  Genomic DNA will be isolated from the mid-gestational fetuses' limb or tail of the neonatal mice using the NucleoSpin® Tissue DNA extraction kit. The PCR for amplification of target loci was carried out using a specific set of primers for the gene (half villin and half Cited-1) to confirm the transgenics using EmeraldAmp® GT PCR Master Mix (Takara). Direct sequencing will be performed using the PCR products and further segregated and transfection efficiency will be calculated. The positive mice will be kept for housing for 6 months for colon cancer to initiate and then used for further dissections and downstream analysis.

- **Tissue fixation and Immunohistochemistry/Immunofluorescence**

  The mice will be humanly culled by cervical dislocation and cardiac perfusion will be performed with 4% paraformaldehyde (PFA). Intestine from the stomach to colon end will be dissected out, cleaned by flushing with 1X PBS opened up, and placed in a 3mm Whatman filter paper fixed with methacam (Methanol: Chloroform: Acetic acid – 6:3:1) for 48 hours. After 48 hours of incubation of dissected gut in methacam, the gut will be rolled up with stomach end inwards and the tissue fixed in 10% formalin. The incubated tissue from formalin will be then subjected to further tissue processing steps to form paraffin blocks.

  Sections will be deparaffinized in xylene, washed in gradients of ethanol, and rehydrated. Heat-induced epitope(antigen) retrieval will be performed by boiling slides with two different buffers, 10mM Tris base, 1mM EDTA pH 9.0 for Ki67, BrdU and c-myc, and 10 mM sodium citrate buffer pH 6.0 for β- catenin, Cited-1, and lysozyme for 30 min each at

100 °C. Slides will be then cooled for 15 min before washing with 1X TBS. Blocking of endogenous protein will be done by using 10% BSA in 1X TBS for 2hrs at room temperature. Slides will be incubated with primary antibodies β-catenin, c-myc, Cited-1, Ki67, BrdU, and lysozyme, all at 1:100 dilutions at 4 °C overnight in a humid chamber. The next day slides will be washed three times with 1X TBST (0.1 % Triton- X) for 15 min and blocked with 3% H2O2 in methanol for 30 min to quench the endogenous peroxidase activity of the sections. Slides will be washed first with water and then with TBS and are incubated with anti-rabbit HRP conjugated secondary IgG for 2hrs at room temperature in a humid chamber. After incubation slides will be again washed three times with 1X TBST for 15 min and diaminobenzidine will be used (1 min) as a chromogenic agent for color development. After water wash, slides will be stained with Gills Hematoxylin Mod III and will be mounted. Negative controls of IHCs will be prepared for each set of sections by replacing the primary antibody with TBS (Chakraborty et al. 2019).

- **Data analysis**
  Data will be analyzed using GraphPad 8.0 software. The data will be tested for statistical significance using a two-tailed student t-test. All numerical data will be expressed as mean, SD from different experiments.

6. Timelines

	0th	12th	24th	36th	48th	60th
1. Determination of Cited-1 expression in colorectal cancer patient samples/cell lines of different grades compared to healthy controls.						
Sample collection, Reagents procurement, Optimization of RNA isolation, Total RNA isolation, cDNA synthesis						
TaqMan individual assay validation compared to adjacent normal tissues						
2. Generation of knock-in/out Cited-1 cancer cell models by CRISPR/Cas 9 systems.						
Sequence search, SgRNA design, and cloning, Cas9 constructs, donor DNA design						
Vector construction and ligation, Transfection, FACS sorting, PCR and Sequencing, Establishment of new knock-in/out Cited-1 cell lines models						

3. Deciphering the functional role of Cited-1 knock-in/out cell models in proliferation, migration, and invasion						
Determination of % viability by using MTT assay, Immunocytochemistry of the knock-in/ out cell models						
Determination transfection efficiency by invasion and migration by wound healing assay and Boyden chamber approach						
Analyzing the gene expression by qPCR and immunoblots						
4. Development and characterization of Cited-1 overexpression transgenic murine models using i-GONAD CRISPR/ Cas9 approach						
Design of single-stranded donor DNA, tracRNA and gRNA design and construction, Optimization of CRISPR/ Cas9 genome editing solutions, and Technical skill development in surgical methods						
Generation of transgenic using GONAD CRISPR/Cas9 procedure, Assessment of Knock-in efficiency, Dissections, Tissue Processing, Embedding, Sectioning, and Immunohistochemistry						

7. Expected outcome

We wanted to explore the pattern of expression of CITED-1 in different stages and grades of human colon cancer tissues and the adjacent normal tissue by using real-time PCR. It is already a known fact that c-Myc is deregulated in most cancers and CITED-1 induction is dependent on c-Myc; so, we wished to determine this interdependency in various stages and grades of human colon cancer tissues. The real-time PCR will indicate that the upregulation of CITED-1 is in concordance to increase in c-Myc levels in colon cancer without any fluctuations respective to the stages and grades.

To confirm that the Cited-1 gene could induce colorectal cancer initiation and progression, we focused on generating Cited-1 knock-in/out cell models by using CRISPR/ Cas9. The transfection was successfully achieved with a few off-target effects. PCR and sequencing data clearly showed the gene integration/ deletion in the subsequent cell models. Interestingly, sequencing data will be able to reveal that some mutations at specific sites and regions were produced as off-targets.

We have conducted the effect Cited-1 knock-in/ out models on the expression of various Wnt signaling components. Cited-1 knock-in/ out cells were transfected with respective plasmids

expressing various Wnt mutants such as Δβcat, FlagAx ΔNLRP, VP16, and Wnt3a. Transfection of knock-in cell lines with Wnt mutants led to gradual nuclear translocation of β-catenin. In converse, knock out cell lines caused the receding of β-catenin back to cytoplasm, thus confirming the inducing effect of CITED-1 on wnt signaling components. Cell invasion was considerably more knock-in cell lines as compared to the controls. Also, Cited-1 gene knock-out cancer cell lines have a decrease in the number of cells invading through the membrane indicating the vital importance of Cited-1 in promoting migration and invasion in malignant tumors.

The role of CITED 1 in colorectal cancer has not been known in CRC, thus overexpression of the Cited-1 gene under villin promoter using the GONAD procedure revealed the association of this gene in colorectal cancer leading to early lesions due to deregulation of Wnt signaling pathway. Polyp formation will be observed in the transgenics in the stereomicroscope Characterization of transgenic mice by differential staining and immunohistochemistry reveal the role of Cited-1 in phenotypically changing the architecture of the gut.

8. Deliverables

The research outcome will be published continuously for the benefit of all those working in this area. Any breakthrough in the work will be patented and collaborated with the specialized personnel and any other organizations to bring out the utility of the study. Furthermore, this study can be given clinical relevance by evaluating the therapeutic possibility of CITED 1 in colon carcinoma where shRNA against Cited-1 can be conjugated to appropriate nanoparticles and delivered orally in *Apc* knockout murine models.

9. References

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