Statin mediated regulation of SATB family of proteins and β-catenin in colorectal cancer

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

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स्नेहा त्रिपाठी/ Sneha Tripathi

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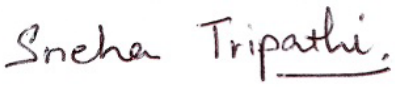
“Work is worship” - Nani

Dedicated to my family

Mumma, Daddy, Shreya, Baba, Nani and Nana

Declaration

I declare that this written submission represents my ideas in my own words and where others' ideas have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.



Sneha Tripathi

20152015

Date: October 2, 2023

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Statin mediated regulation of SATB family of proteins and β-catenin in colorectal cancer**" submitted by **Sneha Tripathi** was carried out by the candidate under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

Prof Sanjeev Galande
(Supervisor)

Date: October 2, 2023

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I hope to continue making all of them proud with my future endeavors.

Sneha Tripathi

Table of Contents

Abstract

Colorectal cancer (CRC) is the second highest cause for cancer-driven mortality worldwide, justifying the continued extensive research to delineate the tumorigenic mechanisms and new therapeutic regimes. The aberrant activation of Wnt/β-catenin signaling is one of the major mechanisms responsible for adenoma formation. However, current therapies do not entail targeting this pathway directly. Statins are family of drugs that target the mevalonate pathway leading to reduction in serum cholesterol levels. Here, we provided evidence of anti-tumor activity of statins *in vitro* and *in vivo* using a multi-pronged approach.

Through the integrative analysis of transcriptomics, proteomics and lipidomics data, we demonstrated that statins downregulate tumor promoting pathways including Wnt/β-catenin signaling, specifically targeting the chromatin organizer SATB1 and β-catenin for degradation. We further showed that statins alter the expression profile of SATB family of proteins by downregulating SATB1 and upregulating SATB2. Analysis of biopsy specimens from CRC patients using immunoblot and immunohistochemistry further suggested differential expression of SATB proteins as key in determining the tumorigenic outcome.

Mechanistically, we observed degradation of β-catenin upon statin treatment via a link between the cholesterol pathway and Wnt signaling. Strikingly, this degradation seemed to be phosphorylation-independent and occured specifically via the lysosomal route. Analysis of β-catenin interactome in statin treated cells revealed multiple novel partners including Farnesyl pyrophosphate synthase, an enzyme crucial for farnesylation of target proteins. The *in silico* analysis to assess the possibility of a farnesyl modification on β-catenin confirmed occurrence of the consensus motif ‘CAAX’. We delineated Cysteine 419 as a novel farnesyl modified residue in β-catenin, mutation of which resulted in the loss of nuclear localization of β-catenin.

Furthermore, we observed that statin treatment reduces the farnesylation modification as well. However, removal of the farnesyl modification is not enough for the statin mediated degradation of β-catenin. We found that another stabilization factor, PCAF, was also downregulated on statin treatment. Furthermore, the interaction between PCAF and β-catenin was lost upon statin treatment, suggesting a dual mechanism by which β-catenin is destabilized and degraded. Collectively, these results underscore the molecular mechanism of antitumor activity of statins, targeting the upstream players in a crucial tumorigenic pathway, potentially providing new therapeutic possibilities.

Chapter 1

Introduction

1.1 Colorectal cancer: The base and relevance of the study

Colorectal cancer is an aggressive malignancy which has been recorded as the second highest cause for cancer related mortality worldwide (1). In the Indian context, recent consensus indicates a notable increase in the incidence of colorectal cancer (CRC) cases. A recent analysis reveals a 20% rise in CRC cases from 2004 to 2014 (2). Furthermore, the five-year survival rate for CRC in India is among the lowest in globally, with less than 40% survival rate, largely due to the majority of cases being diagnosed in advanced stages (3, 4). The current treatment regime relies heavily on chemo-radiation and surgical removal of the adenomatous polyps depending on the stage of tumor progression (5). However, early detection offers the best chance of prolonged survival (6, 7). Hence, there is a growing body of literature unveiling novel molecular markers for disease progression and therapeutic targets, emphasizing the importance of early detection in improving outcomes.

Colon-rectal region of the intestinal system is most prone to wear and tear, and the production of stable epithelial cells is required due to constant sloughing off of the lumen lining. Therefore, occurrence of mutations in the newly formed cells is inevitable. Most of the incidences, these mutations are handled by the damage responsive pathways, including removal of the mutated cell by apoptosis. However, the failure or evasion of this defense system, causes accumulation of these harmful mutations and might trigger adenoma formation. If the mutations are pro-oncogenic, there is transformation of the adenoma into a carcinoma and it leads to tumorigenesis. Other precursors to the development of colon-rectal cancer (CRC) are inflammatory bowel disease (IBD) including ulcerative colitis and Crohn's disease (8), familial adenomatous polyposis harboring APC tumor suppressor mutation (9), genetic predisposition with hereditary germline mutations (10, 11).

The inevitable manifestation of CRC is metastasis to other distant organs, the most common site for which is liver. The hepatic metastasis is responsible for recurrences after therapy and has a 15-25% chance of prevalence at the time of diagnosis (12, 13). The major reason for high incidences of liver metastasis lies in the physiological and anatomical link between the large intestine and liver. The hepatic portal vein and the lymphatic system draining into the large

intestine from liver, provides a convenient route for the spread of the tumorigenic cells (14, 15). Therefore, the CRC therapeutics also target hepatic resection or the hepatic portal vein embolization to prevent metastasis and recurrence (16).

Additionally, the current treatment strategies are targeting the molecular players and foci mutations for immunotherapy and specific drug mode of action. The most widely adopted immunotherapeutic approaches include anti-EGFR (epidermal growth factor receptor) (17) and anti-VEGFR (vascular epidermal growth factor receptor) (18) antibodies that target cell proliferation and angiogenesis respectively. Immune checkpoint inhibitors or ICIs are a class of antibodies that majorly function by activating the effector T cells for tumor inhibition (19). One of the commonly used ICI is anti-cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) antibody that suppresses the regulatory T cells and promotes effector cells towards CRC adenomas (20). The anti-programmed cell death ligand 1 (PD-L1) antibody is another frequently prescribed ICI, generally in combination with other therapy regimes, for targeting advanced stages with PD-1 aberrant overexpression (21).

The main challenges associated with immune based treatment include limited penetration and efficacy in advanced stages (22), better yield usually in combination therapy (23, 24), side-effects could promote tumor invasiveness in some cases (25), and functionality specific to mutations in the tumor that make its success case-dependent (26, 27). Alternative treatment modalities are under development, focusing on designing small non-coding RNA molecules targeting oncogenes, delivered specifically to tumor cells via vector based or nano-particle delivery based methods (28). Progress in the microRNA (miRNA) system has shown promising results in initial phase studies being conducted in murine models as well as human subjects (29). Examples of the molecular targets of miRNA-based inhibition are VEGF (Clinical trial ID: NCT01158079), Eph2A (Clinical trial ID: NCT01591356), PLK1 (Clinical trial ID: NCT02191878), GSTP (Clinical trial ID: NCT03819387), and MYC (Clinical trial ID: NCT02110563) that have demonstrated positive outcomes in the Phase I of trial studies, indicating their potential in disease management (30). Many of these targets are integral to major molecular pathways implicated in tumor progression, highlighting the importance of understanding the perturbed and/or aberrantly upregulated signaling cascades.

There have been multiple studies suggesting varied pathways responsible for colorectal oncogenesis, including Notch, Hedgehog, TGF-β (transforming growth factor-β)/SMAD, Hippo signaling, phosphatidylinositol 3-kinase (PI3K)/AKT active proliferation cascade (31). However, one of the major pathways that has been observed to be aberrantly expressed and has been

studied for early adenoma formation, is Wnt/β-catenin signaling (32-34). It is widely recognized that aberrant Wnt activation, with loss of function mutation of APC (Adenomatous Polyposis Coli) protein, followed by subsequent genetic alterations such as KRAS activation and p53 inactivation mutations are the hallmarks of tumor progression in CRC (35). Additionally, CRC tumor tissues frequently exhibit chromosomal instability, leading to microsatellite expansion and/or contraction due to downregulation of the mismatch repair machinery (36). Recently, the Ca^{2+}/NFAT signaling pathway has emerged as another significant contributor to adenoma formation, alongside the canonical Wnt pathway. Nevertheless, the diagnosis and characterization of CRC primarily rely on molecular players targeted by the Wnt pathway.

1.2(i) Wnt/ β-catenin signaling: The critical pathway for colorectal homeostasis and tumorigenesis

Wnt signaling is a major cascade that involves multiple players, and is divided mainly into three broad pathways. The two pathways, the Planar Cell Polarity and the Wnt/Ca^{2+} signaling, comprise the non-canonical category, triggered by the binding of the Wnt ligand to the Frizzled receptors. The Planar cell polarity pathway plays a role in alignment and polarity of cells(37), whereas, the Wnt/ Ca^{2+} signaling focuses on intracellular calcium regulation and is involved in cell adhesion, cell migration, and tissue morphogenesis(38).

The third of the well elaborated pathways is the Wnt/ β-catenin signaling that has been characterized as the canonical signaling cascade. The major component of the pathway is the destruction complex that is central to the downstream signaling. It comprises of multiple players, namely, the APC (Adenomatous Polyposis Coli) scaffolding protein, AXIN1, another scaffolding protein for facilitating the recruitment of other components of the complex, GSK3-β and CK1α, the kinases that phosphorylate the target β-catenin and result in its degradation(39).

In the presence of the Wnt ligand, when the signaling is ‘ON’, the destruction complex is recruited to the cytosolic part of the transmembrane receptors Frizzled and LRP5/6 by Dishevelled (DVL). The cytoplasmic β-catenin is rendered free to enter the nucleus and upregulate the downstream Wnt target genes by binding to TCF/LEF transcription factors(39-41). However, in Wnt ‘OFF’ conditions, when the ligand is absent for stimulating the downstream signaling, the destruction complex gets sequestered from the transmembrane receptors and interacts with cytoplasmic β-catenin to lead it towards degradation. Since, the β-catenin is not available to enter the nucleus, the downstream signaling is hampered (Figure 1). Reports have suggested a significant role of Wnt/ β-catenin pathway in tumor initiation in multiple cancer types (42).

However, this Wnt pathway is not only involved in oncogenic tumor progression (43), but also important for maintaining the intestinal crypts homeostasis (44, 45). The base of the crypts harbors the stem cells which are required for the constant renewal of spent intestinal epithelium. These stem cells express Wnt/ β-catenin signaling and thereby contribute towards crypt homeostasis by maintaining their stemness. However, the aberrant expression of APC (46) or mutations in β-catenin in the upper epithelial layer lead to tumor initiation (47, 48). Multiple players have been since discovered for the past decade to better understand the intricacies of this complex pathway.

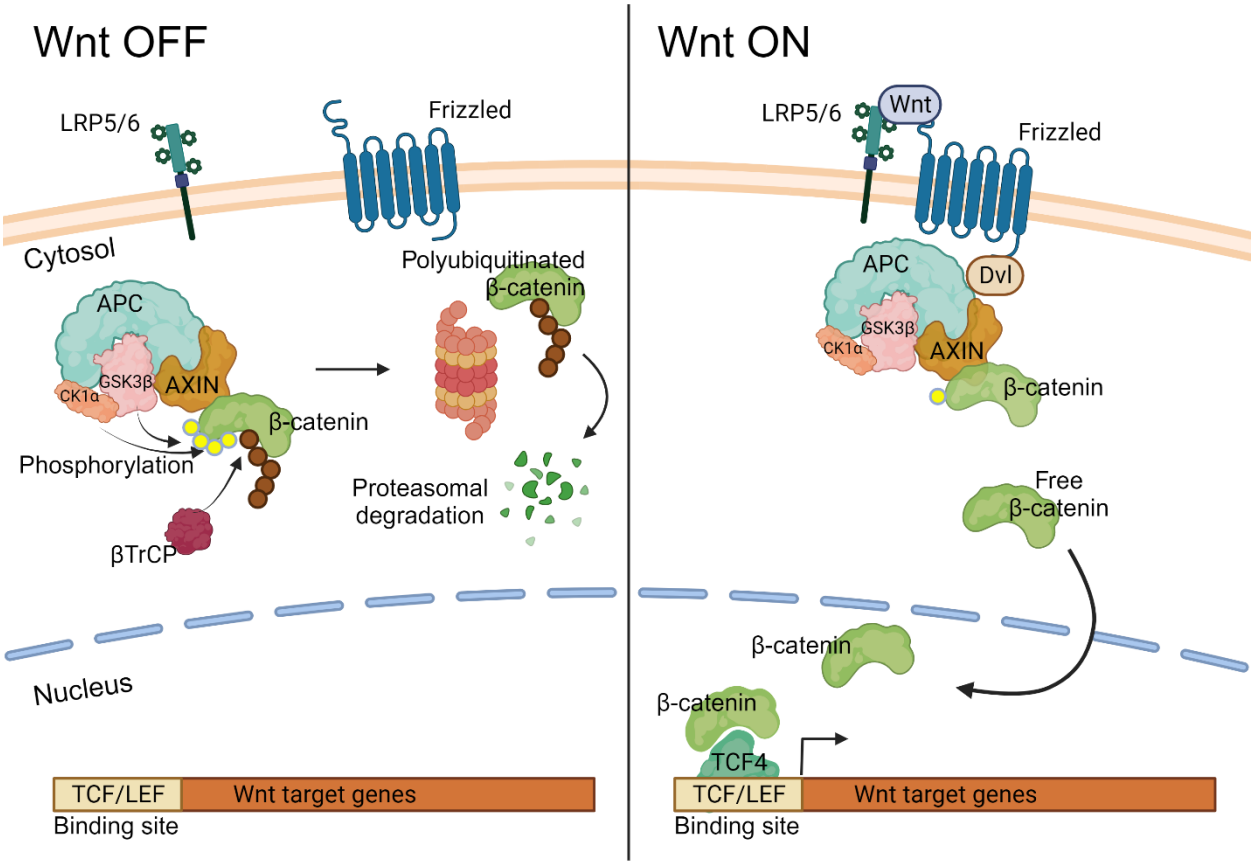

Figure 1: Schematic of the Wnt/ β-catenin signaling in the absence and presence of the Wnt ligand. In Wnt OFF conditions, the LRP5/6 and the Frizzled receptor are apart in the absence of the Wnt ligand. The destruction complex comprising of APC, AXIN, GSK3β, CK1α, recruit and sequentially phosphorylate β-catenin. This further signals βTrCP to ubiquitinate and lead the β-catenin protein to proteasomal degradation. In Wnt ON conditions, in the presence of the Wnt ligand, the two receptors come in proximity, and with the help of Dishevelled, recruit the destruction complex to the cytoplasmic base of the receptors. The β-catenin protein is then free to enter the nucleus and upregulate the Wnt responsive genes in association with TCF/LEF transcription factors.

1.2(ii) β-catenin as the major player in the canonical Wnt pathway

As mentioned above, the β-catenin player is a major upstream protein that drives the signaling cascade in the canonical Wnt pathway. The destruction complex is the prime regulatory machinery for the fate of β-catenin, in the presence and absence of the Wnt ligand. The degradation of β-catenin is a multi-step process which involves various kinases and ubiquitin ligases to target the protein for proteasomal degradation. Among the innumerable enzymes, the two conventional kinases that phosphorylate β-catenin are CK1α and GSK3-β. After recruitment to the destruction complex by APC and AXIN1, β-catenin is first phosphorylated at the serine 45 residue by CK1α, which further primes it for sequential phosphorylation by GSK3-β at threonine 41, serine 37 and serine 33 (49). These phosphorylation modifications signal the βTrCP E3 ubiquitin ligase to bind to β-catenin and target the protein for poly-ubiquitination at lysine residues 19 and 49 (Figure 2). The ubiquitination finally leads β-catenin towards proteasomal degradation (50).

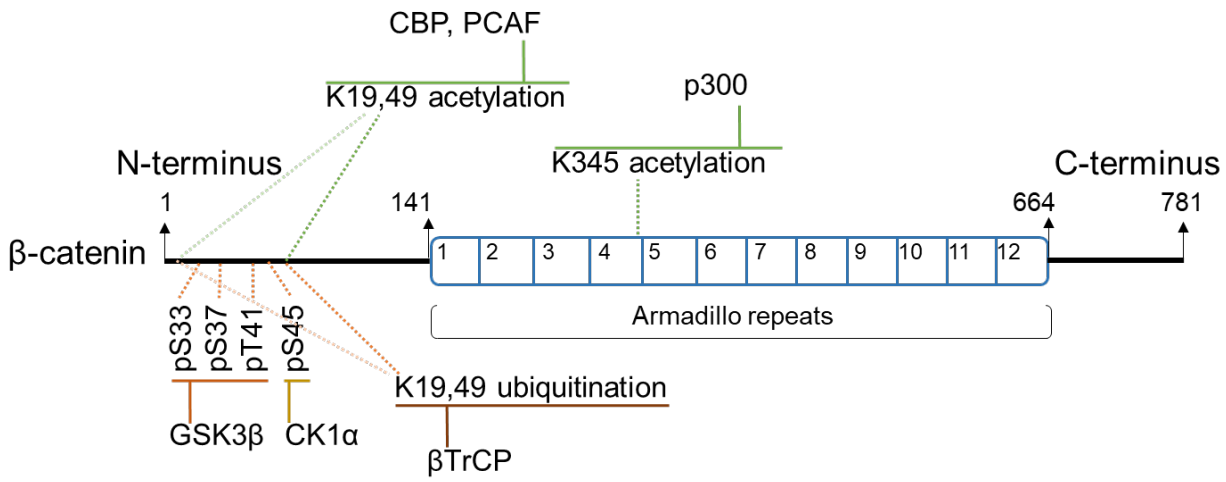

Figure 2: The schematic representation of β-catenin with the major post translational modifications. The PTMs on the residues that govern the stability (marked by green) or instability (marked by red, yellow and brown) of the protein have been highlighted. The phosphorylation by CK1α at serine 45, followed by phosphorylation by GSK3β on serine 33, 37 and threonine 41, lead to βTrCP mediated ubiquitination at lysine 19 and 49. This primes the protein for proteasomal degradation. Whereas, lysine 19, 49 acetylation by CBP and PCAF and lysine 345 acetylation by p300 stabilizes the protein and increases nuclear localization respectively.

Apart from these PTMs, β-catenin is tightly regulated by other stabilizing and destabilizing post translational modifications, that govern the outcome of the downstream cascade. It is reported that phosphorylation at serine 191 and serine 605 by JNK2 (51), phosphorylation at serine 552 by AKT (52), and phosphorylation at 675 by PKA (53) result is stabilization of β-catenin. Acetylation at lysine 345 by p300 increases its interaction with TCF transcription factor(54), acetylation at lysine 49 by CBP enhances its transcription(55), and acetylation at lysine 19 and 49 by PCAF imparts stability to the protein(56). The degradation of β-catenin is also mediated variably by different E3 ubiquitin ligases. The SIAH1 E3 ubiquitin ligase has been shown to degrade β-catenin in a phosphorylation independent manner(57, 58). Another study also delineated a lysosomal degradation of β-catenin post lysine 48-linked polyubiqitination (59). Methylation of β-catenin lysine residues also determine the stability or instability of the protein, as it has been reported that lysine 180 mono-methylation by SET7/9 is primed for degradation(60), whereas, lysine 133 methylation by SMYD2 enhances nuclear localization of β-catenin(61).

This fine-tuning of the major player of Wnt signaling, ensures the proper functioning of the cascade. However, a perturbation, mutation, or aberrant expression of any of the components involved has the potential to wreak havoc in the regulatory mechanism, thereby leading to disorders like tumorigenesis. Therefore, a thorough study of all the players, and constant unravelling of novel partners is essential to better understand the missing links that might prove critical for determining tumor progression and ultimately therapeutics.

Our lab has also been interested in contributing towards understanding the pivotal role of Wnt/ β-catenin signaling in colorectal cancer. A previous report from our group has reported a novel player critical in the pathway. It was observed that SATB1 (Special AT rich binding protein 1) can bind to β-catenin and upregulate the expression of Wnt target genes, thereby acting as an upstream molecular player. SATB1 was also observed to upregulate its own expression by binding β-catenin and TCF7 to its promoter in Wnt ON conditions. The increased expression of SATB1 under the regulation of Wnt/ β-catenin signaling in CRC seemed to be crucial for tumorigenesis, as knockdown of SATB1 resulted in reduced tumor burden in mice(62).

1.3 SATB proteins: The dynamics and the tumorigenicity

SATB1 and SATB2 belong to the Special AT-rich Binding protein family containing two CUT domains and a homeodomain for DNA binding. These are chromatin remodelers that are responsible for regulation of gene expression in developmental (63) as well as oncogenic pathways(64). However, the expression and function of SATB1 and SATB2 has been reported to

be context dependent and non-overlapping with each other, even after structural homology of 61% (65). SATB1 has been observed to be important for T cell development(66-68), however, SATB2 has been majorly associated with neuronal development(69, 70).

In cancer progression, the role of both SATB1 and 2 has been documented however, their functions have not been observed to be redundant. SATB1 has majorly been observed to be highly upregulated in cancer types like breast (71), ovarian (72), prostate (73), colon-rectal (74, 75), whereas, SATB2 expression has been variable (76-78), mostly opposite to the SATB1 expression (79-81). Therefore, it has been proposed that SATB1 and 2 are dynamically expressed in a reciprocal manner in tumorigenesis (Figure 3). A report published by our lab also suggested the positive correlation between the poor survival rate and the high expression of SATB1 across multiple cancer types by analyzing TCGA datasets, including colorectal cancer (82).

Another study from our lab delt with the mechanistic regulation of SATB1 in CRC, where it was reported to be under the canonical Wnt/ β-catenin signaling (62). The upregulation of SATB1 by the dual method of recruiting β-catenin and TCF7L2 transcription factor on its promoter and augmenting its own expression by a feedback loop was observed to be pivotal for tumor progression. It also showed that SATB1 additionally acted as a transcription factor and promoted the expression of the downstream Wnt responsive genes, signifying the correlation between a crucial signaling cascade and SATB1 protein in tumor progression.

However, independent studies have reported a negative regulation of SATB2, by the canonical Wnt pathway. The miR 31 microRNA is known to promote the Wnt/ β-catenin signaling by targeting the Wnt antagonist Dkk1 gene for repression (83, 84). A recent article suggested that miR31 also targets SATB2 for downregulation and thereby promotes CRC tumorigenesis (79, 85). The above inferences suggest that both SATB proteins are regulated by the canonical Wnt pathway in opposing manner. Therefore, it is of utmost importance to further study the dynamics of SATB1 and SATB2 collectively in the CRC tumor progression as novel players under the Wnt signaling.

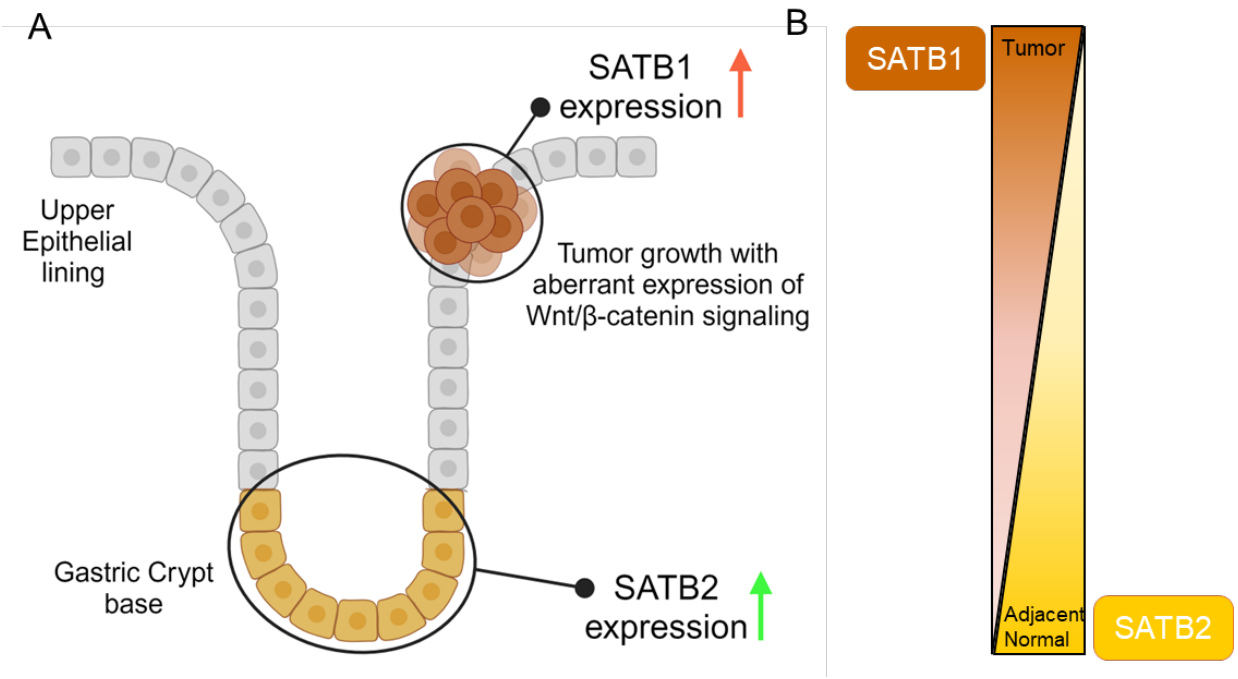

Figure 3: The reciprocal expression profile of SATB1 and SATB2 in the normal and tumor tissues. (A)The SATB1 expression is observed to be upregulated in tumorigenesis under the aberrant expression of Wnt/ β-catenin signaling in upper epithelial lining, whereas, the SATB2 is expressed inversely, with higher expression in the normal colon tissue at the base of the crypt. (B)The dynamic reciprocal expression of SATB1 and SATB2 in depicted in the form of a gradient from tumor tissue to the normal adjacent tissue in CRC.

1.4 Repurposing of drugs for cancer therapeutics: Statins as a potential candidate

Unearthing such novel players in a central pathway gives us an opportunity for deducing therapeutic targets as well. The current therapy regime for CRC does not involve a direct target on Wnt/ β-catenin signaling. Therefore, researchers have now directed their attention towards finding new drugs that can be easily administered and have least side-effects. One of the paradigms being adopted for cancer therapeutics is repurposing of drugs, already present in the treatment protocols (86). The mode of action of these drugs is well known as they act on a physiological pathway. The rational for using them in cancer therapeutics lies in the consideration that the physiological pathway they target is known to be dysregulated in tumorigenesis, as depicted in Figure 4A. Therefore, these repurposed drugs should be able to curtail tumor progression by targeting the physiological status of the tumor cells. Such drugs can then be used in combination with existing protocols to curb the advance of the cancer stages.

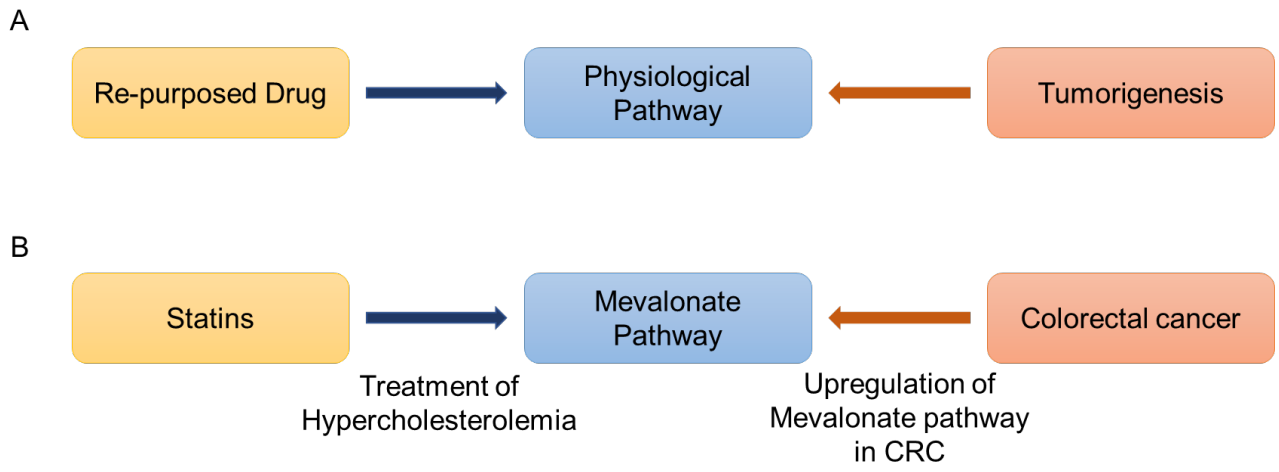

Figure 4: The paradigm of repurposing of drugs in cancer therapeutics. (A) Schematic for showing correlation between the repurposed drug used to target the physiological pathway that is known to be dysregulated in tumorigenesis. (B) Schematic to emphasize on the paradigm fit for statins as repurposed drug, that target mevalonate pathway or cholesterol biosynthesis pathway, which is known to be upregulated in colorectal cancer.

In the cases of colon-rectal diseases, dyslipidemia has been reported to be a major physiological disfunction which leads to inflammation in the epithelial lining (87, 88). High serum cholesterol levels due to hypercholesterolemia have been correlated with increased propensity of developing colorectal cancer (89) as well as liver metastasis (90). One drug that has been in the market for treatment of hypercholesterolemia since its discovery in 1970s is Statins (91). Statins are family of drugs that target the enzyme that catalyzes the rate limiting step of mevalonate pathway and inhibit the cholesterol biosynthesis. It acts on the enzyme HMGCoA reductase by competitive inhibition and halts the cascade at an upstream level by preventing the production of mevalonate (Figure 5A). This forces the cells to take up free cholesterol from serum to meet their lipid metabolism requisites, thereby lowering the cholesterol levels in blood as well as tissues (92).

The statins were first discovered by Arika Endo in 1970s, in the form of a substance obtained from a fungal extract of *Penicillim citrinum,* and was labelled as compactin or mevastatin (91, 93). Promising results regarding the specificity of inhibiting only the liver cholesterol biosynthesis, augmented the clinical trials. However, it did not prevent the complications arising from high or prolonged dosage. The upstream inhibition by statins and reduction in an important cellular component, cholesterol, was accounted for its pleotropic effects. Since cholesterol and its derivatives are involved in normal cellular functioning, it was speculated that inhibiting such an

essential cellular building block affected other non-target pathways and caused detrimental manifestation in the form of side effects. Some of them included gastro-intestinal discomfort, inflammation and even cataract. However, with extensive research by Brown and Goldstein (94, 95) in 1980s, and others in 1990s (96), it was concluded that the side effects were not because of drug toxicity but mechanism of action (97). Most of these effects were the property of the drug being metabolized in the body which were a function of the R group attached to the lactone ring (Figure 5B). This motivated further research into developing compounds with the lactone structure intact but slight variations in the R group for better drug management.

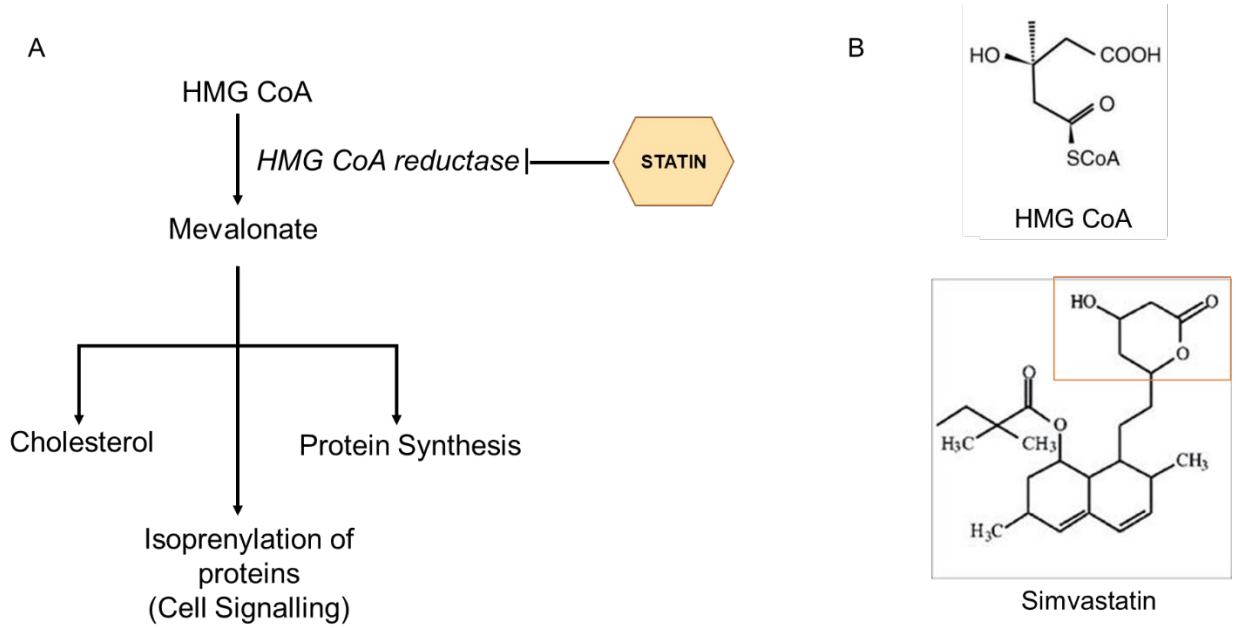

Figure 5: The mode of action of statins via targeting the mevalonate pathway. (A) The simplified schematic of mevalonate pathway showing the rate limiting step catalysed by HMG CoA reductase, that converts HMG CoA to Mevalonate. The three major downstream products of this pathway are cholesterol, isoprenylation of target proteins, and protein synthesis. Statin inhibition is shown at the top-most stage, affecting the HMG CoA reductase. Statin competes for the active site of the enzyme as the lactone ring structure (red box), (B) shown in bottom right example of Simvastatin, is similar to the structure of its substrate HMG CoA, top right. The rest of the simvastatin structure comprises of the R group attached to the lactone ring.

It was elucidated that the R groups augmented the drug delivery as well as the processivity of the statin inside the cell. Based on the R group attached to the lactone ring, statins have been classified into two types, hydrophobic and hydrophilic. Hydrophobic statins like simvastatin,

fluvastatin, pitavastatin, lovastatin, and atorvastatin are known to enter the cells easily, and hydrophilic statins like rosuvastatin and pravastatin are more effective after getting metabolized in the liver (98). The first commercially available statin, lovastatin, had side effects of nausea, fatigue and constipation, which were reduced consecutively in simvastatin and pravastatin. Furthermore, these side effects were tackled even better in the synthetically produced statins like rosuvastatin and atorvastatin, owing to the alteration in the R group (99). Therefore, the significance of the R group can be realized by the increased number of novel and improved variants of statins present in the market, which have shown better side-effect management than the early formulations (100).

The horizon for statin application widened beyond the cardiovascular diseases, when correlations were made between various types of cancers, including lymphoma (101), gastric cancers (88, 102, 103), breast cancer (104)and dyslipidemia (105). The role of cholesterol pathway became increasingly pivotal and extensively studied in tumorigenesis (106). It is now well established that tumor cells exploit the cholesterol biosynthesis pathway for their metabolic survival and proliferation (107, 108). Cholesterol is not only required for making cell membrane to match the extensive multiplication of tumor cells, but is also required for signaling. There are free fatty acids, oxysterols, prostaglandins, lipid modified oncogenic proteins that play a vital role in cellular signaling (109-111). Therefore, usage of statins to prevent the tumor progression by targeting the mevalonate pathway, became a subject of expedited research.

The anti-neoplastic effects of statin started taking the center stage in therapeutics with multiple reports establishing the mode of action, majorly focusing on apoptosis (112), cell proliferation inhibition via STAT3/SKP2 signaling (113), a more recent review of effect on YAP/CD44 growth axis (114). Moreover, there have been suggestions that statins might act as preventive drug, as it does not allow an excess of cholesterol or its derivatives to accentuate the tumor cell proliferation (99, 115, 116). However, a direct mechanism for the anti-cancer effects of statins has not been deduced yet. Therefore, with the current research, statins fit the paradigm for re-purposed drugs (Figure 4B) that serve the dual purpose of lowering the cholesterol as well as eliciting an anti-cancer mode of action.

1.5 Hypothesis and Key Questions

Our lab has been interested in studying colorectal tumorigenesis, not only in a mechanistic approach, but also in therapeutics. In a serendipitous study from our group, it was observed that statins downregulate the protein levels of SATB1 in CRC lines, in a dose and time dependent manner (117). Since, SATB1 has been shown to be under Wnt/ β-catenin signaling by our group in a previous study, we wanted to elucidate whether statins can affect Wnt/ β-catenin pathway via its major molecular players.

In this thesis study we have tried to delineate the effect of statins on CRC, by delving into the mechanism by which Statins might target the oncogenic pathway, specifically, the aberrant Wnt/ β-catenin signaling, as it has been established as one of the major tumor initiating pathway (Figure 6). We have also taken into consideration, the effect of Statins on the cholesterol biosynthesis pathway in CRC, and used the cholesterol levels as a read-out for the cellular responsiveness to statin treatment. The correlation between the mevalonate pathway and Wnt/ β-catenin signaling is pivotal, as it is known that cholesterol biosynthesis plays an important role in regulation of Wnt pathway via β-catenin (118-120).

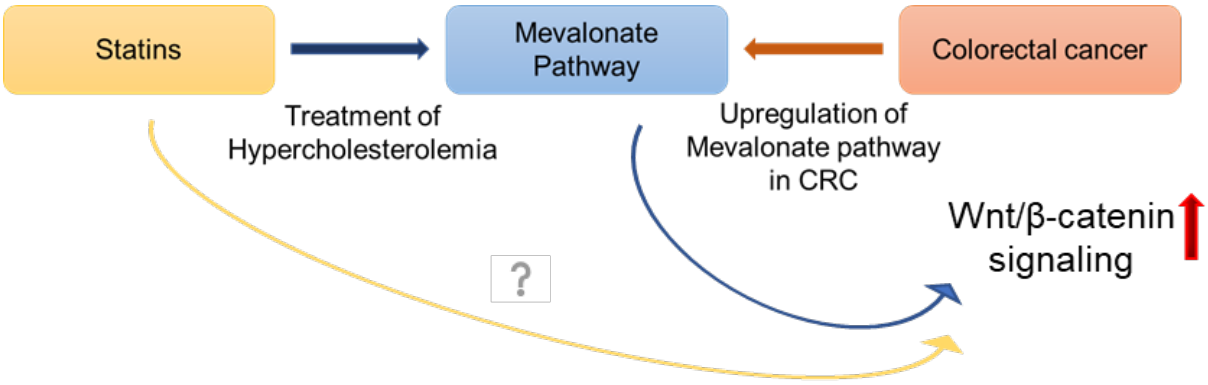

Figure 6: Schematic depicting the hypothesis for our study. Here we have attempted to delineate whether the anti-tumor effect of statins on colorectal tumorigenesis is via mevalonate pathway or its effect on a major pathway known to be upregulated in CRC, Wnt/ β-catenin signaling. We have also looked at the possibility of statin mediated regulation in both the mevalonate and Wnt pathways synergistically, as Wnt/ β-catenin signaling itself is also known to be regulated by the cholesterol biosynthesis.

Our study reveals the effect of statins on the major upstream molecular players of Wnt/ β-catenin signaling, SATB1 protein and β-catenin. We have also looked at SATB2 in correlation with SATB1

levels as it is imperative to observe the two proteins together because of their dynamic reciprocal expression in CRC.

We have divided our study into three major aims in the form of each chapter. The first aim deals with the multi-omics study, conducted in CRC lines on statin treatment, with a rationale to deduce the specificity of the mode of action of statins in CRC. We observed the lipidomic, transcriptome profile as well as proteome profile to provide us with a wholistic view of the effect of statins, focusing on the pathways getting affected. We report an overall tumor-suppressive phenotype on statin treatment, with a specific effect of downregulation of Wnt/ β-catenin signaling. Out of the oncogenic pathways getting downregulated, the canonical Wnt pathway was the major target. The cholesterol biosynthesis pathway genes are upregulated as expected, as it is already reported that statins kick-in the feedback mechanism at the transcript level (121).

In the second aim we elaborate on the effects of statins on SATB proteins in CRC, supplemented by our in vivo studies. The reciprocal expression profile of SATB1 and SATB2 proteins seems to be getting reversed on statin treatment. Our 3D spheroid model recapitulated the more aggressive tumorigenic phenotype with higher expression of SATB1 than SATB2. However, this profile was reversed on statin treatment, as we observed a lower expression of SATB1 and slightly higher SATB2 level. Therefore, justifying study of the two homologs in conjunction with each other. This observation not only provides us with a therapeutic target but also presents SATB proteins as reliable markers for CRC tumorigenesis.

The spheroid model, *in vivo* mice as well as human study analysis recapitulates the SATB1 expression in aggressive tumor samples and SATB2 expression in normal adjacent tissue biopsies. The effect of statin on SATB1 expression profile *in vivo* augmented our approach towards adding statins in the current therapy protocol for CRC patients. We report our observations of the phase II/III trial study where statin is being administered along with the neo-adjuvant therapy to the CRC patients. The human study data we have obtained so far seems to be corroborating with the *in vitro* results of the molecular markers, for both tumor progression as well as therapeutics.

The third aim describes the mechanistic regulation of the statin effect on β-catenin specifically. Since β-catenin is the major upstream molecular player of the Wnt/ β-catenin signaling, we have observed a significant downregulation on statin treatment. We have reported the mode of action of statin that targets the degradation of β-catenin via a novel post-translational modification. We observe a previously unreported isoprenylation modification on β-catenin, which might be

responsible for providing stability to the protein. Since isoprenylation of proteins falls downstream of the mevalonate cascade, it has been observed that statins result in reduction of this modification from the target proteins(105, 122, 123). This de-stabilizes the proteins and causes their degradation. We suggest statin degrades β-catenin by removal of this isoprenylation modification and also by affecting other stabilizing agents, in a dual mechanism towards destabilization of the β-catenin protein, making it a direct specific target.

Therefore, to summarize, we observe the tumor suppressive phenotype of the statin treatment in CRC lines *in cellulo* and *in vitro*, which is recapitulated in our *in vivo* observations. We validate the effects of statin treatment by a multi-pronged as well as multi-model approach and suggest a specific mode of action towards Wnt/ β-catenin signaling. We infer from our collective results that statins affect Wnt/ β-catenin pathway by targeting its two upstream major players, β-catenin and SATB1. We were also able to delineate the significance of studying SATB1 and SATB2 in conjunction with each other in CRC, making this protein family an important paradigm for characterizing CRC tumorigenesis markers. The clinical investigation in human subjects further augments our proposition towards inclusion of statins in CRC therapeutic protocols along with the existing regime, as the immunohistological analysis of biopsy samples stained for SATB proteins pre- and post-statin treatment corroborate our *in vitro* analysis.

Collectively, we have provided a regulatory mechanism for the effect of statins in CRC, via Wnt/ β-catenin pathway, making it a potent addition to the therapeutic regime.

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Chapter 2

To elucidate the specificity in the effect of Statins via multi-omics approach in colorectal cancer cell lines

2.1 Introduction

Since mevalonate pathway is known to be upregulated in cancer (1) and statins inhibit this signaling cascade, we wanted to elucidate the effect of statins on colorectal cancer (CRC) by delving into the expression profiles of the cell lines on statin treatment, in our first aim. The lipid species, mRNA transcripts and protein levels provided us with a more inclusive view of the effect of statins. The cell lines used were HCT116 and HCT15 colorectal cancer lines, treated with statin for 48 hours, and then harvested for the three aforementioned profiles.

The rationale for elucidating the effect of statin in CRC lies in the subjected pleotropic effect of statins. It has been widely propagated that statins have a wide range of effects, including various signaling players due to a broad spectrum of action (2, 3). However, it is imperative to observe whether the range of effects on statin treatment are pertaining to a particular category of molecular functions, or are varied. The reason behind the prevalence of the pleotropic effect of statins majorly is responsible because of its effect on mevalonate pathway. The mevalonate pathway is a major central cascade that governs a plethora of downstream players, involved in cell cycle, proliferation and differentiation. Downregulation of this central pathway would certainly affect the signaling circuitry of the cells.

The mevalonate pathway (MVA) plays a crucial role in metabolic homeostasis of cells as it is closely linked with glycolysis (4). Three molecules of acetyl CoA derived from the glycolytic pathway combine to form 3-Hydroxy 3-methylglutaryl CoA (HMG CoA), the precursor for mevalonate (5, 6). The conversion of HMG CoA to mevalonate, catalyzed by HMGCoA reductase, represents the rate limiting and pivotal step of the pathway. Mevalonate production not only regulates the synthesis of downstream intermediates but also modulates the regulation of MVA pathway-responsive genes through a feedback mechanism. Thus, mevalonate acts as a sensor for maintaining cholesterol homeostasis (7). When cellular cholesterol levels decrease due to inhibition, mevalonate concentration serves as a direct signal to activate the master transcriptional regulator Sterol Regulatory Element Binding Protein (SREBP), leading to the upregulation of cholesterol-responsive genes, including HMGR (8, 9).

The primary output of the mevalonate pathway is cholesterol, which serves as a key component in maintaining the structural integrity cell membranes. Intermediate molecules generated between mevalonate and cholesterol, such as squalene, lanosterol, and isopentenyl pyrophosphate, participate in numerous biological processes (10, 11). An important consequence of this pathway is the lipid post-translational modification (PTM) called isoprenylation, facilitated by the production of farnesyl pyrophosphate and geranylgeranyl pyrophosphate. These modifications are critical for modulating the activity of Rho and Ras GTPases, pivotal for signal transduction (12, 13). Furthermore, the production of free fatty acids (FFAs) plays a significant role not only in normal cellular signaling but also in dysregulated pathways associated with various disorders (14-16).

Given that inhibit HMG CoA reductase, the key enzyme in the mevalonate (MVA) pathway, they effectively reduce the levels of several intermediates, including mevalonate, isopentenyl pyrophosphate, lanosterol, squalene, and ultimately cholesterol. This reduction in isopentenyl pyrophosphate further lowers the farnesylation and geranylgeranylation of target proteins, thereby affecting cellular signaling (17). At the genetic level, inhibition of mevalonate production triggers a feedback loop leading to the upregulation of responsive genes. Additionally, the cell compensates for the decreased cholesterol synthesis by increasing the expression of LDL receptors, facilitating the uptake of circulating cholesterol from the bloodstream and consequently lowering the serum cholesterol levels (5, 18). Therefore, statins have emerged as potent drugs for managing hypercholesterolemia and are extensively used for treatment of associated conditions such as coronary heart disease, atherosclerosis (19), and diabetes mellitus (20). However anti-tumor effects of statins have not been solely attributed to their canonical mode of action. There are numerous reports suggesting that statins exert cholesterol-independent effects on apoptosis (21), cell proliferation (22, 23) and cell cycle (24) in tumor cells.

Therefore, we wished to begin with validating whether the mode of action of statins is intact in CRC lines, for which we first monitored the targeted lipid profile, followed by mRNA and protein levels. One of the views prevalent for statin use also pertains to the general effect on transcriptional and translational machinery. Therefore, with our mRNA and protein profiles, we wanted to observe the specificity of the mode of action. The molecular, biological, as well as functional pathways were closely studied to observe the specific cluster of genes getting either repressed or over-expressed, as well as the protein clusters translated specifically on statin treatment. Interestingly, we did not observe any alteration in the house-keeping genes expression of the statin treated cell lines, indicating the non-pleotropic effect of statins. We observed some effect on the cellular response to stress, however, that is expected due to the drug response in

the cell lines. The cell cycle and DNA damage response machinery is slightly upregulated, again pertaining to the drug response by the cells *in cellulo*.

Strikingly, we observed a specific cluster of pathways which were getting either downregulated or upregulated. However, not all transcriptional profile was translated. We observed that only a fraction of proteins were getting significantly affected by statin treatment, and interestingly, all of them showed downregulation. There were no significantly upregulated proteins, except myosin 9, while most of the proteins which showed their respective transcripts as upregulated in our transcriptome data, were unaltered. This further ascertained the strict regulation of the transcriptional and translational machinery on statin treatment.

Interestingly, all the pathways affected were promoting tumor suppression as we observed a downregulation of oncogenic signaling players. However, we also observed an interesting effect on Wnt/β-catenin signaling. The Wnt target genes were significantly reduced at both RNA and protein level, however, the upstream players, β-catenin and SATB proteins were only affected at the protein level. The transcript levels of these upstream molecules are not altered. Their protein levels however, seem to determine the downstream expression of Wnt targets.

We further validated the dependence of the expression profiles on mevalonate pathway by supplementing the cells with mevalonate in the culture media, separately and along with statin treatment. We observe a rescue in the proteins level of the affected Wnt upstream players, whereas, transcript levels were unchanged. This further strengthened our observations with respect to the specificity of the statin effects in CRC lines, which seem to be sensitive to the mevalonate concentrations and the statin treatment.

We also performed an inverse experiment, where we depleted the cellular cholesterol by Methyl-β-Cyclodextrin. This compound chemically removes the cholesterol from cells, independent of the effect on mevalonate signaling. The removal of cholesterol did not replicate the observations made on statin treatment. Thereby, again validating that the effect of statin treatment in CRC lines is not responsible because of the shear removal of cholesterol, but is governed by the regulation on the signaling pathway. This further ascertained our hypothesis, that statins affect CRC lines at molecular and functional level, specifically in a tumor-suppressive manner and one of its major targets seem to be Wnt/ β-catenin signaling.

2.2 Results

2.2(i) Lipid profile on statin treatment shows a tumor suppressive phenotype

Our rationale to look at the lipid, transcript and protein profile in CRC on statin treatment was to give us a wholistic preview of the physiological and tumorigenic pathways in conjunction with each other. It was important to look at the lipid profile first because the mode of action of statins is known to affect the cholesterol biosynthesis pathway. Therefore, it was imperative to observe the status of lipids on statin treatment in CRC lines, which are known to play a role in tumor progression as well (14, 15, 25, 26).

Statin treatment was given in CRC cell line HCT116 and cells were harvested for lipid extraction. Chloroform and methanol (2:1) were used for processing the samples and the lipids were run in both negative and positive modes in LC-MS method, keeping Free Fatty Acid (FFA 17:1, 1 nano mole final conc.) as internal standard.

The primary observation was the level of cholesterol on statin treatment, which was significantly reduced (Figure 1). The inference was in confirmation with the mode of action of statins. The use of statins has been accepted as a potent drug for hypercholesterolemia as it directly targets the cholesterol synthesis pathway and inhibits the production. Thereby, forcing the cells to take up free cholesterol circulating in blood and using it up. This ingenious method of allowing the cells to lower the serum cholesterol levels has made statins a comparatively safe and reliable drug for hypercholesterolemia therapy. Our data also infered a reduction in oxysterols, most of which are derivatives of cholesterol. Therefore, statin mediated reduction in cholesterol resulted in lowering of these species as well.

There is literature available as early as 1970s, which has characterized different species of lipids, and their respective role in physiology. Oxysterols and prostaglandins are essential for signaling cascades, as they play a crucial role in relaying information, not only in metabolic pathways (8, 27-29) but also in cell division and differentiation (30). Therefore, we specifically looked at species that are imperative for signaling in the context of tumorigenesis. We observed a reduction in oxysterols; however, no significant change is observed in the levels of prostaglandins (Figure 1A).

The other set of lipids crucial for cell signaling are Free Fatty Acids (FFAs). Dysregulation of FFAs also play a role in tumorigenesis by either acting as mediators of tumor progression or by remodeling of microenvironment (31-33). In our data we observed no significant alteration in the levels of most of the FFAs, except three.

The species like (24:1) cis-15-tetracosanoic acid, (24:0) Tetracosanoic acid, (22:6) all cis 4,7,10,13,16,19-Docosahexenoic acid, (22:4) all cis 7,10,13,16-Docosatetraenoic acid, (22:1) cis-13-Docosenoic acid, (22:0) Docosanoic acid, (18:1) Oleic acid, (20:1) cis-11-Eicosenoic acid, (18:2) Linoleic acid, (12:0) Lauric acid (Dodecanoic acid), (20:0) Arachidic acid, observed are not altered significantly. However, a sharp reduction in the levels of (18:0) Stearic acid, (16:0) Palmitic acid, (14:0) Myristic acid (Tetradecanoic acid) is observed (Figure 1A). There is limited analysis regarding the specific significance of these three FFAs; however, some reports have indicated a potential tumorigenic role for palmitic acid, stearic acid and tetradecanoic acid, as their levels were elevated in tumor cells (34-36). The reduction in the levels of these FFA species mediated by statins may imply a tumor-suppressive effect in CRC cell lines. Most strikingly, we recorded a significant increase in the levels of Arachidonic acid (20:4), which has an established role in inflammation (Figure 1B). Inflammatory pathway is regulated by a cascade of players including prostaglandins (37), cytokines and NF-κB pathway. Statin treatment has been reported of causing reduction in inflammation by downregulation of cytokines like IL-6 and IL-8 (38). In our targeted lipidomics data, slight upregulation of arachidonic acid might not be enough for indicating upregulation of inflammatory pathway, as other components responsible for inflammation are not affected. We observe no alteration of prostaglandins which are major mediators of inflammation and our transcriptomics data, discussed further, shows reduced levels of NF-κB as well.

Our overall lipidomics study suggested the canonical function of statin, by lowering of cholesterol in CRC cell line and thereby, reduction in derivative oxysterols. In oncogenic studies, there have been mentions of a surge and accumulation of lipids in tumor cells. Myristic acid has been known to be correlated with higher risk of breast cancer (16). Cholesterol is high in tumorigenic cells to provide enough building material for membranes while uncontrolled cell division takes place (18, 39). Statin mediated reduction in the harmful levels of the known tumor promoting lipids might be suggestive of a tumor-suppressive lipid profile along with its known mode of action.

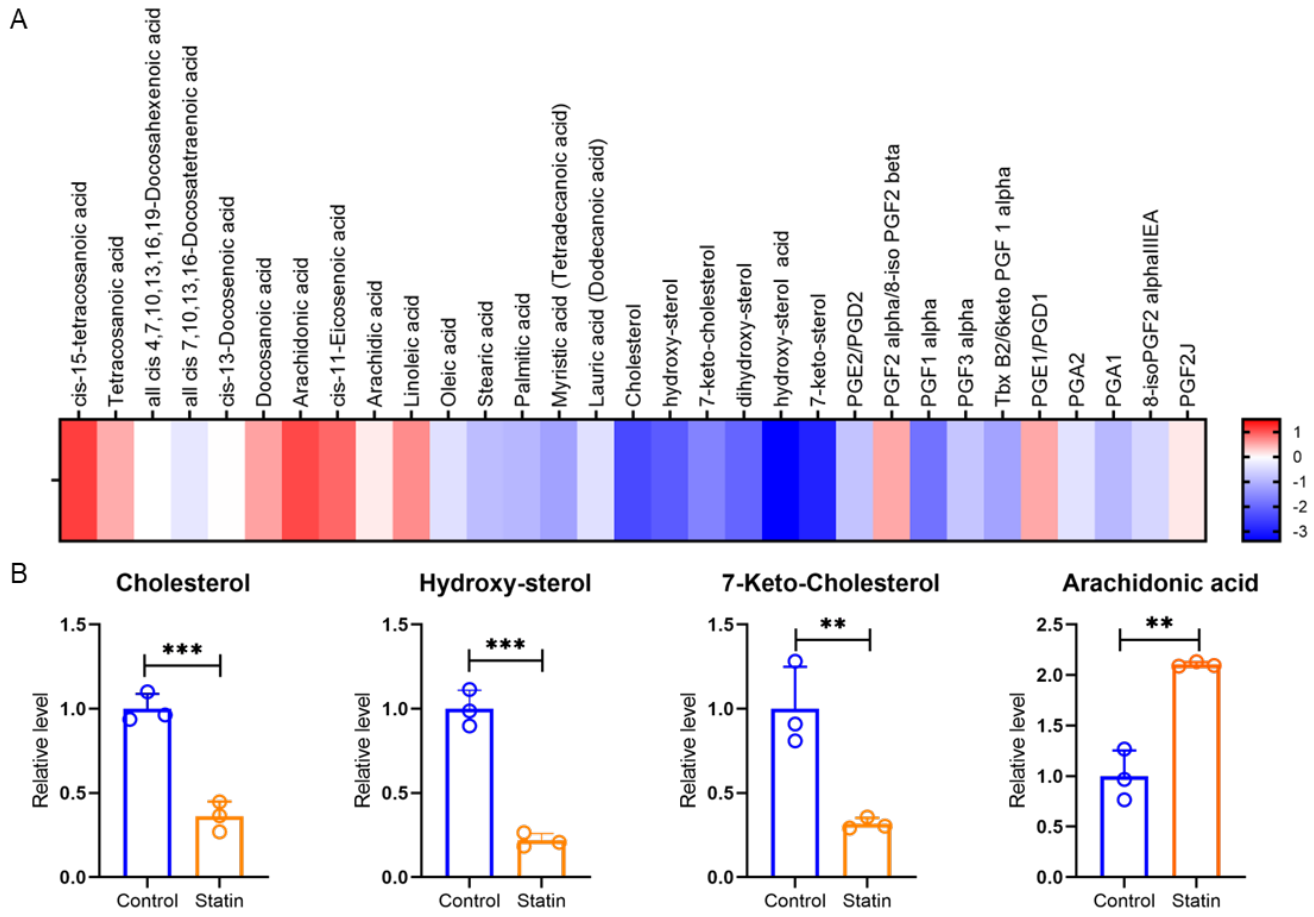

Figure 1: Targeted lipidomics study to show that statins lower the levels of cholesterol, its derivatives oxysterols and a few FFAs in CRC line HCT116. (A) Heatmap to represent the Log 2 fold change levels of Free Fatty Acids (FFAs), Cholesterol, Oxysterols and Prostaglandins in statin treated CRC line HCT116. (B) Graphs to plot the Relative levels of Cholesterol, Hydroxy-sterol, 7-Keto-Cholesterol and Arachidonic acid on statin treatment. Biological replicates n=3, Students t-test was performed for statistical analysis **p<0.001, ***p<0.0001.

2.2(ii) Statin treatment affects other tumorigenic pathways like Wnt/β-catenin signaling in Transcriptome data

The significance of studying the entire genetic transcript levels on statin treatment rested upon its effect on cholesterol biosynthesis pathway responsive genes. It has been well established that cholesterol reduction causes the cholesterol biosynthesis genes to get upregulated. The major transcription factor SREBP2 is sensitive to the cell cholesterol levels, via mevalonate and oxysterols. When the cholesterol levels are high, the mevalonate pathway genes respond by getting downregulated. The reduced level of cholesterol signals the genes to get upregulated via

SREBP2, to replenish the cholesterol by either biosynthesis or uptake from blood (8). We see the same effect being projected in our transcriptome study on statin treatment in CRC line HCT116. Statin responsive reduction in cholesterol caused the mevalonate pathway gene to get significantly upregulated (Figure 2 A,D). Our qPCR validation for HMGR (gene for coding HMC CoA Reductase) and SREBF2 (gene coding for SREBP2) showed the increase in transcript levels as well (Figure 2C).

The other upregulated set of genes (Figure 2A, Appendix, Section 1, Table 1) as observed in our Gene Ontology term analysis, were related to fatty acid synthesis, miR33 activity, Omega 9 fatty acid synthesis, Interleukin 2 family signaling. Interestingly, the activity of the transcription factors predicted to be upregulated, like KLF15, AP2, have been reported to play a tumor suppressive role (40, 41).

On the other hand, the GO terms for the downregulated genes were plotted separately and the transcription factor (TF) activity prediction showed a reduction in tumorigenic factors (Figure 2B, Appendix, Section 1, Table 2). The most distinct GO term highlighted in this set was Gastric cancer network which included adenocarcinoma genotype. Given the reported similarities in the genetic profiles of gastric and colorectal cancer adenocarcinomas (42), we proceeded to examine the transcriptional regulatory factors that are affected. The transcription factors highlighted as downregulated were c-Myc, p300, E2F1/2, TCF-1, which are known as tumor-promoting. Interestingly, it is known that these TFs are downstream of major molecular players of Wnt/β-catenin signaling (42). A camparitive heatmap has been plotted to observe the cholesterol as well as Wnt responsive genes expression collectively (Figure 2D).

Wnt/ β-catenin signaling is central to the colorectal cancer ontology because previous reports have suggested a pivotal role in aberrant expression and initiation of CRC. The APC and β-catenin mutations are the two major dysregulations in Wnt/ β-catenin pathway and approximately 75% of the CRC patients harbor these mutations (43, 44). Therefore, if statin treatment seems to be showing a reduction in downstream effectors of this pathway, there is a possibility of statins targeting the Wnt/β-catenin signaling.

Since β-catenin regulates the expression of the above-mentioned TFs, we wanted to observe the effect of statin treatment on the transcript levels of this major upstream player. Interestingly there was no alteration for β-catenin at the transcript level on statin treatment. The same was validated by qPCR and there is no significant change (Figure 2 C).

We further validated the expression of another essential upstream factor, SATB1 (Special AT rich Binding Protein 1) which is known to have a tumorigenic effect. The role of SATB1 in Wnt/ β-catenin signaling has been previously established by our lab in a major finding where we elucidated that SATB1 interacts with β-catenin and causes upregulation of Wnt target genes. SATB1, being a chromatin remodeler, also has the capability to augment its own production and thereby, result in an upregulation in Wnt/ β-catenin pathway. Our transcriptome data and our validation qPCR both showed no alteration of SATB1 on statin treatment again. SATB2, a homolog of SATB1, and known for its anti-tumorigenic effects and reciprocal expression to SATB1, was also observed to have no change (Figure 2C).

Therefore, our findings with transcriptome analysis pointed towards the observations that statins result into an upregulation of cholesterol biosynthesis genes and downregulation of oncogenic genes, focused on the Wnt responsive genes. Nevertheless, the principal molecular components of the Wnt pathway, including β-catenin, SATB1, and its homolog SATB2, showed no alterations at the transcript level. Despite this, there was a decrease in the transcript levels of Wnt-responsive genes. This implies that the impact of statins might occur at the protein level for these upstream molecules, potentially influencing downstream gene expression without affecting their own transcript levels.

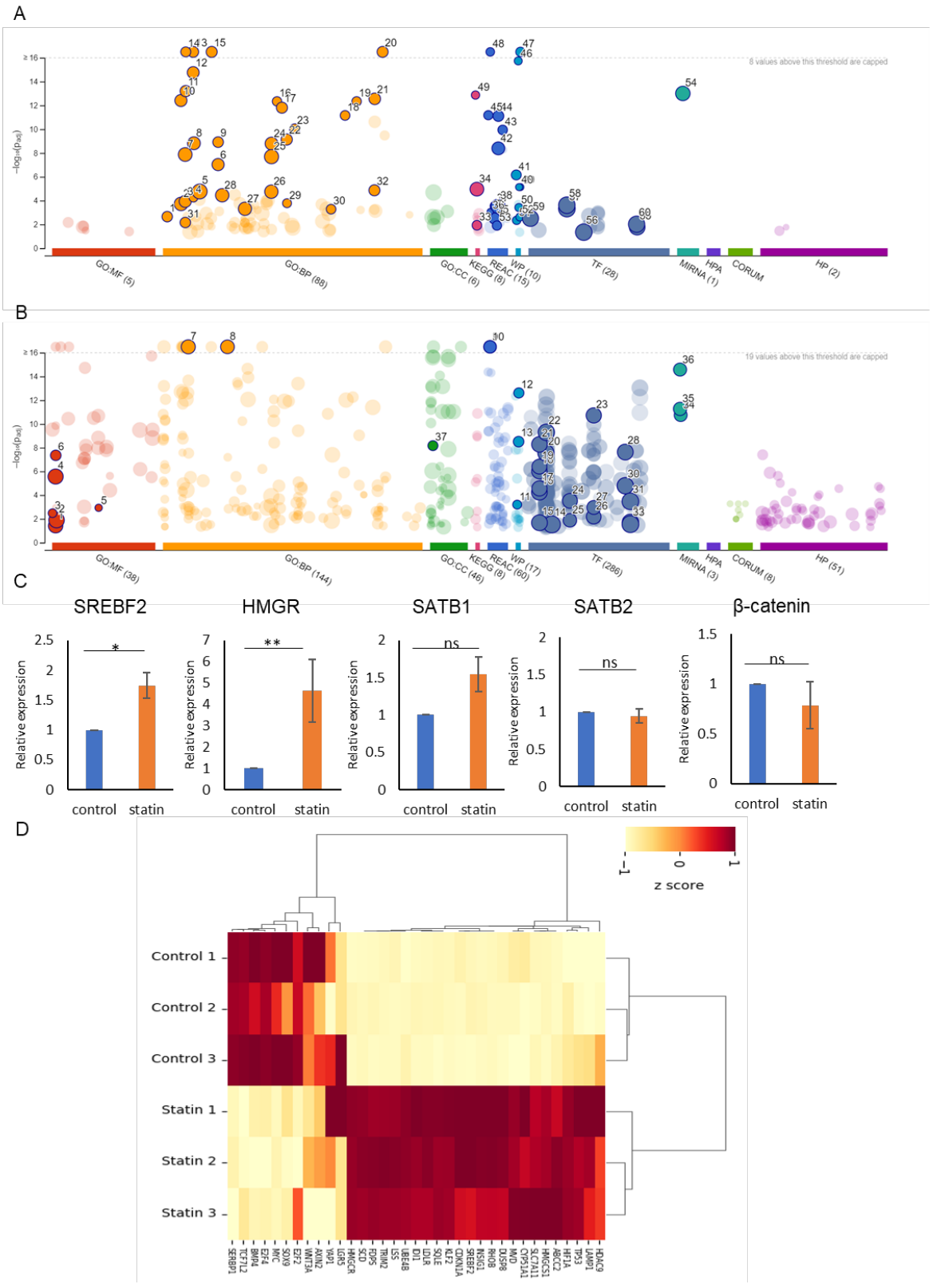

Figure 2: Transcriptome analysis on statin treatment and validation by qPCR. GO term for the genes observed to be upregulated (A) and downregulated (B) on statin treatment in HCT116

cell line. (C) Validation for transcript level alteration of SREBF2, HMGR, SATB1, SATB2 and β-catenin. (N=3, $p<0.05$) (D) Heatmap for the DE genes significantly expressed in Control vs statin treated sets. The heatmap has been made selecting a few representative genes of cholesterol pathway and Wnt target genes using FLASKI online portal. [Iqbal, A., Duitama, C., Metge, F., Rosskopp, D., Boucas, J. Flaski. (2021). doi:10.5281/zenodo.4849515] (Biological replicates n=3,*$p<0.05$, **$p<0.005$ according to Students' t test analysis)

2.2(iii) Proteome analysis for Statin treated CRC cell line affirms downregulation of Wnt/ β-catenin signaling

To further validate the effect of statin we performed a whole cell lysate MS-MS in CRC cell line HCT15 on statin treatment. This proteome data gave us a compiled view of the pathways getting affected and their direct implication translated, in the form of the GO term analysis. Interestingly, most of the proteins were not affected on statin treatment, contributing to the building evidence against the off-target effects of the drug often categorized as pleotropic or side effects. The only significantly altered proteins were the ones which were getting downregulated. Therefore, a Gene Ontology term analysis was plotted for the downregulated proteins to observe the pathways getting affected (Appendix, Section 1, Table 3).

The most striking result was downregulation of Wnt/ β-catenin signaling, along with EGFR signaling and TGF-β signaling, all of which play a role in tumorigenesis. The transcription factor activity was also predicted to be reduced for E2F1/2, p300, Sp1 (Figure 3A). We plotted the peptide counts for major players, β-catenin, YAP and CUL-3 E3 ubiquitin ligase, also known to play a role in Wnt/ β-catenin pathway. The relative expression of these proteins was significantly downregulated on statin treatment, whereas, the expression of housekeeping genes like actin and GAPDH was not altered (Figure 3C).

We further validated the effect of statin by probing immunoblots for Wnt/ β-catenin players, β-catenin, AXIN 2, TCF4, PCAF (Figure 3B, Appendix section 3). We observe a significant downregulation for all the targets. This corroborated with our inference that the transcript level alteration of the downstream Wnt target genes goes in hand with the protein level, however, the upstream players like β-catenin only seem to be getting affected at the protein level. Since SATB1 has been reported to be an integral part of the Wnt/ β-catenin signaling, we probed an immunoblot to observe the levels of both SATB1 and SATB2 (Figure 4A). Interestingly, SATB1, which is reported to be critical for tumorigenesis, gets downregulated, and SATB2, which has been reported to be opposite to its homolog, gets slightly upregulated or unaltered. There have been reports which have suggested the opposing roles of both SATB family proteins independently,

however, an inclusive study with both these proteins, in context of one another, has not been conducted. Therefore, the dynamics of the expression of SATB1 and 2 needs to be delineated to understand the therapeutic target, which has been delt in the next aim.

The above observation provided us with an insight towards the specificity of the anti-tumor mode of action of statin in CRC. The cholesterol biosynthesis pathway does get affected canonically, however, the tumorigenic pathway getting downregulated is focused on Wnt/ β-catenin signaling. This observation is of significance because of the importance of Wnt pathway as one of the initial aberrant expressions responsible for adenoma formation in CRC. Statins affecting a pathway critical for tumor initiation can help establish these family of drugs as a potential therapy in combination with existing regime.

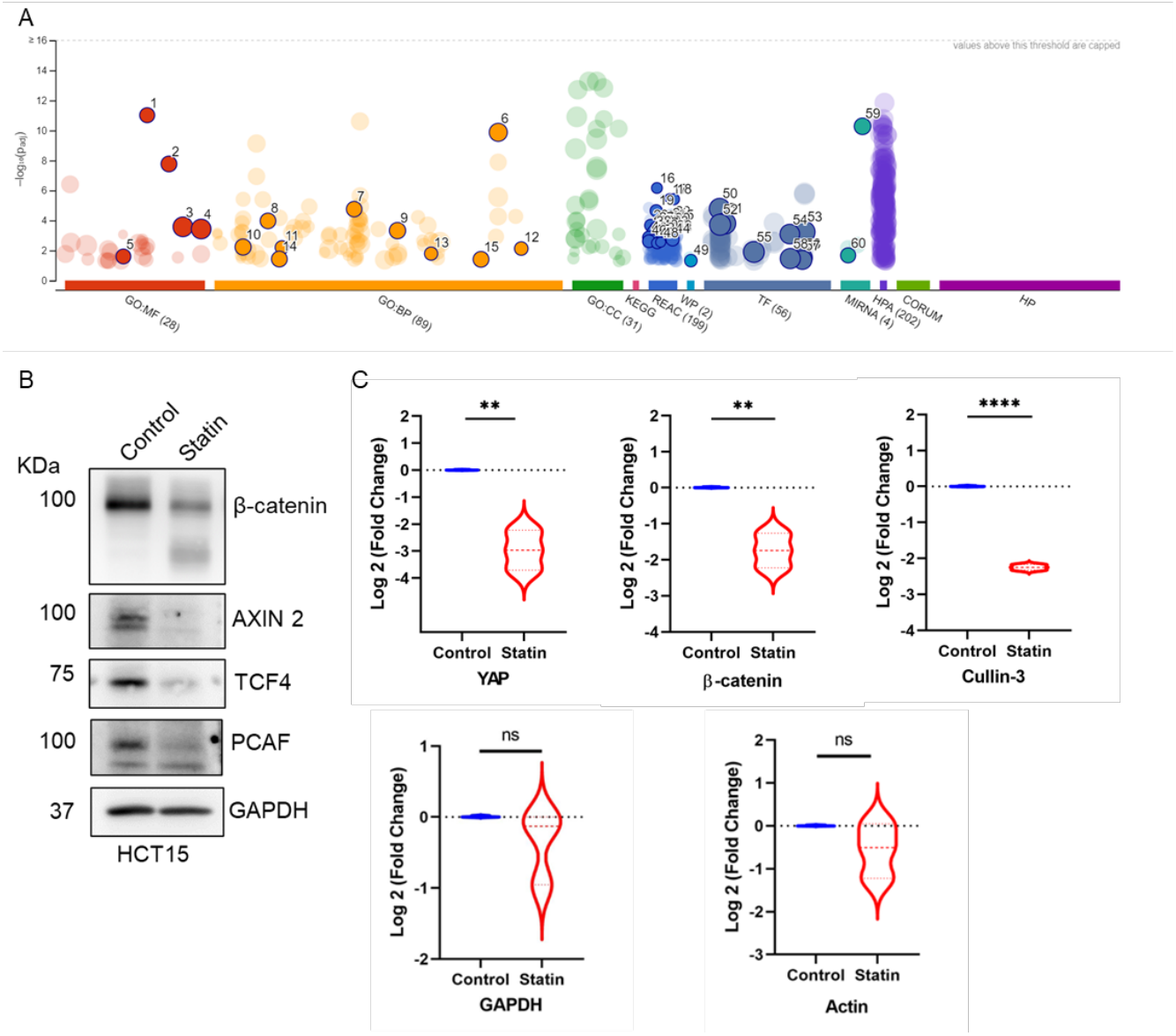

Figure 3: Whole lysate proteomics of the statin treated HCT15 CRC cell line indicate a specific effect on Wnt/ β-catenin signaling (A) Downregulated proteins depicted in GO term (g:Profiler) on statin treatment in HCT15 cell. (B) Validation of effect of statin treatment on Wnt target proteins by immunoblotting. (C)Log fold change was observed in the peptide counts of YAP, β-catenin, and Cullin-3 in control vs statin treated proteomics analysis, whereas, the housekeeping proteins like GAPDH and Actin did not show any alteration in peptide counts. Biological replicates=3 with statistical significance of **p<0.005, ****p<0.00005 according to Students' t test analysis.

2.2(iv) Mevalonate supplementation rescues the effect of statin at the protein level

As described previously, statins target the mevalonate pathway by inhibiting the rate limiting step catalyzed by HMG CoA Reductase. The lactone ring of statin, being similar in structure to the substrate of the enzyme it targets, HMG CoA, competes with it for the active site of the enzyme and thus prevents the formation of mevalonate. This results in inhibition of cholesterol, as mevalonate is an important precursor of downstream cholesterol production. Therefore, we wanted to observe the effect of mevalonate supplement along with statin treatment in the CRC lines. By supplementing mevalonate, we provided the substrate for proceeding the downstream cascade and production of cholesterol. The presence of mevalonate seemed to negate the effect of statin, as the protein level of β-catenin and SATB1 were rescued, whereas, the SATB2 levels seemed to be unchanged (Figure 4B, C, Appendix section 3). The transcript levels were unaltered as seen in the previous results (Figure 4D, E).

Additionally, we observed the levels of cholesterol in the mevalonate and statin treated conditions as a readout for validation of the intact mode of action of statins. The mevalonate plus statin set of cells exhibited rescued levels as compared to the control untreated set (Figure 4F). However, the only mevalonate supplemented set of cells exhibited reduced cholesterol levels relative to control. This might be because of the feedback mechanism which inhibits the transcription of the genes responsible for cholesterol biosynthesis. The mevalonate supplementation causes an increase in cholesterol biosynthesis, which shuts down the responsive genes. However, since no new cholesterol is getting produced thereafter and the one formed as a result of transient mevalonate supplementation is quickly consumed by cells, the cholesterol levels in lipidomic analysis is observed to be reduced. Our assay lasted for 48 hours, therefore we speculate that it might have been enough to deplete the existing cholesterol and since the gene machinery is inhibited, there is no new cholesterol being formed (Figure 4F). Therefore, this observation might be a result of the transient supplementation of mevalonate and the time point at which the cells have been harvested. However, we have performed all assays at the same time point throughout our study for consistent results. Though, this observation may open a few avenues for further exploring the cholesterol dynamics with respect to mevalonate supplementation.

Collectively, these results indicated the effect of statin on CRC to be specific and not an artifact of the general drug stress. Statins indeed seem to be affecting the Wnt/ β-catenin signaling by downregulation of major molecular players at protein level specifically, which was reversed on mevalonate supplementation.

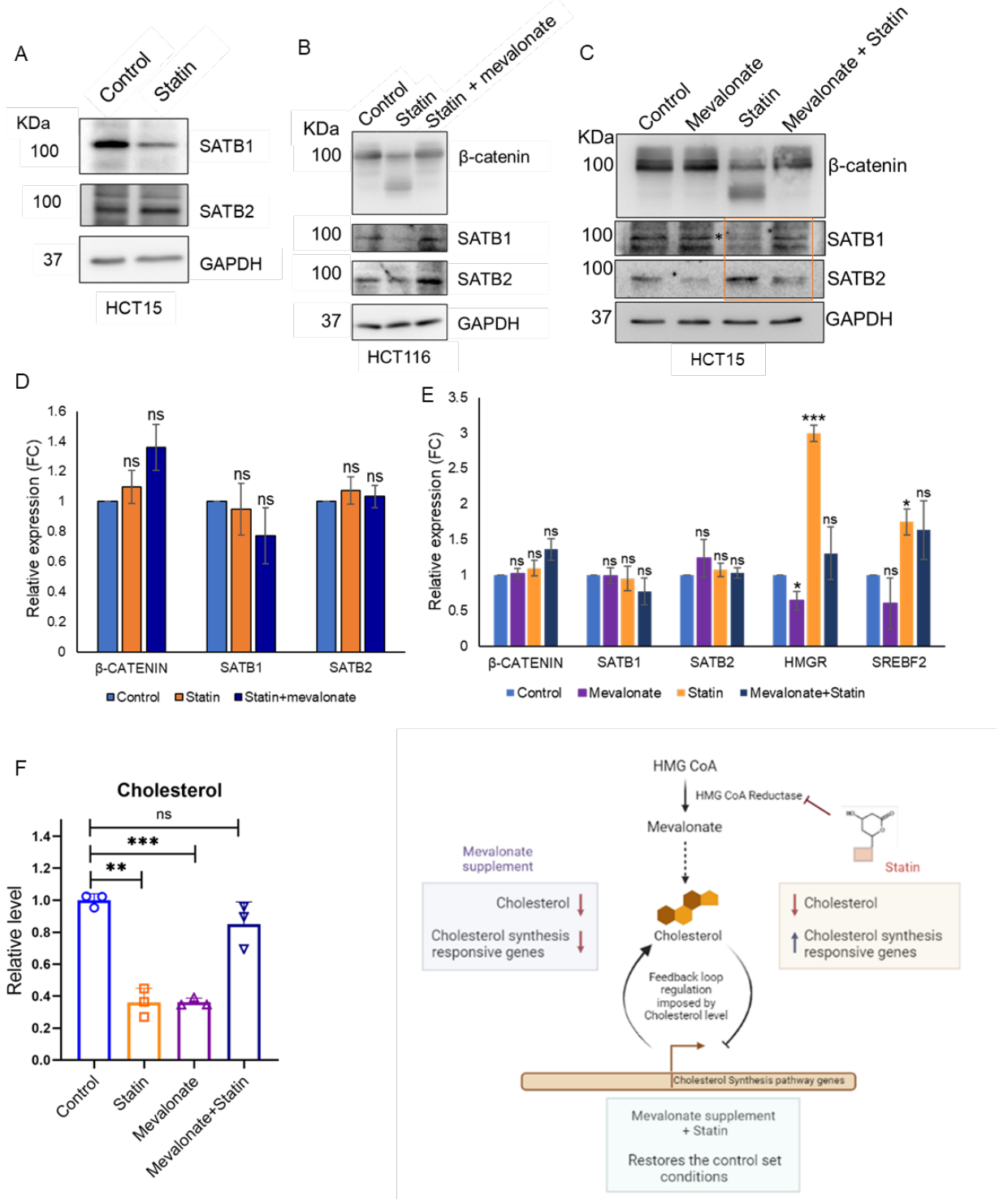

Figure 4: Mevalonate supplementation along with statin treatment rescues the reduction in protein levels of Wnt upstream players, β-catenin and SATB proteins. (A) SATB1 and SATB2 protein levels in HCT15 cell line on statin treatment. n=3 (B & C) Immunoblots for protein level alterations of β-catenin, SATB1 and SATB2 on statin treatment and mevalonate supplementation in HCT116 and HCT15 cell lines respectively. The red box in (C) depicts the

reciprocal expression of SATB1 and SATB2 on statin only and statin+mevalonate set. The asterisk in the SATB1 blot depicts the upper band to be the right molecular weight of SATB1. (D & E) Relative expression of β-catenin, SATB1 and SATB2, HMGR, SREBF2 to observe transcript level changes in HCT116 and HCT15 cell lines respectively. n=3 (F) Relative levels of Cholesterol on mevalonate supplementation and statin treatment in HCT15 cell line, supported by an illustration depicting the effect of the two conditions on the feedback mechanism for the cholesterol responsive genes. **p<0.05, ***p<0.005, ns is non-significant as per Students' t-test analysis.

2.2(v) MβCD mediated cholesterol depletion does not recapitulate effect of statin on β-catenin and SATB proteins

Cholesterol levels on statin treatment and mevalonate supplementation have shown a consistent trend in our observations with respect to the effect on mevalonate signaling. Therefore, we used cholesterol as a constant read out for determining the efficiency of statin mediated effects in CRC. Since, removal of cholesterol itself can alter transmembrane signaling cascades, including Wnt/ β-catenin signaling, we wanted to check whether the effect of statins on Wnt players is a result of cholesterol depletion in CRC lines. To this effect we used Methyl-β-Cyclodextrin, a chemical compound that extrudes cholesterol out of the membranes. The lattice structure of MβCD physically binds to cholesterol and pulls it out of the membranes, thereby depleting cholesterol levels in the cells chemically (45). The mechanism of cholesterol lowering is not metabolic or affects the pathway as compared to the effect of statins which inhibits cholesterol biosynthesis at the signaling level. However, we wanted to infer if the cholesterol depletion itself is sufficient and responsible for the effect on major Wnt players, β-catenin and SATB proteins.

We first validated the effect of MβCD on cholesterol levels and its derivatives by performing a targeted LC-MS. We observed a significant decrease in all the specified species in a dose-dependent manner as expected (Figure 5A). However, when we observed the β-catenin and SATB proteins levels to infer whether the effect on Wnt players is dependent on cholesterol depletion alone, we observed no effect by MβCD on the protein as well as transcript levels (Figure 5B,C). We used three different concentrations of MβCD (2 mM, 4 mM, 6 mM) in CRC line HCT15 and despite of the cholesterol depletion in all the concentrations, we did not observe any alterations on Wnt players. Therefore, we suggest that effect of statin treatment which seem to target the Wnt pathway might indicate a signaling regulation mechanism.

Furthermore, we conclude that only cholesterol depletion is not responsible for the effect observed on statin treatment. The reduction of β-catenin and SATB1 protein mediated by statin is a result

of a metabolic intervention. This sheds light on a mechanism of statins, exerting its effect on major molecular players of Wnt/ β-catenin signaling, suggesting specificity in its mode of action via a signaling regulation.

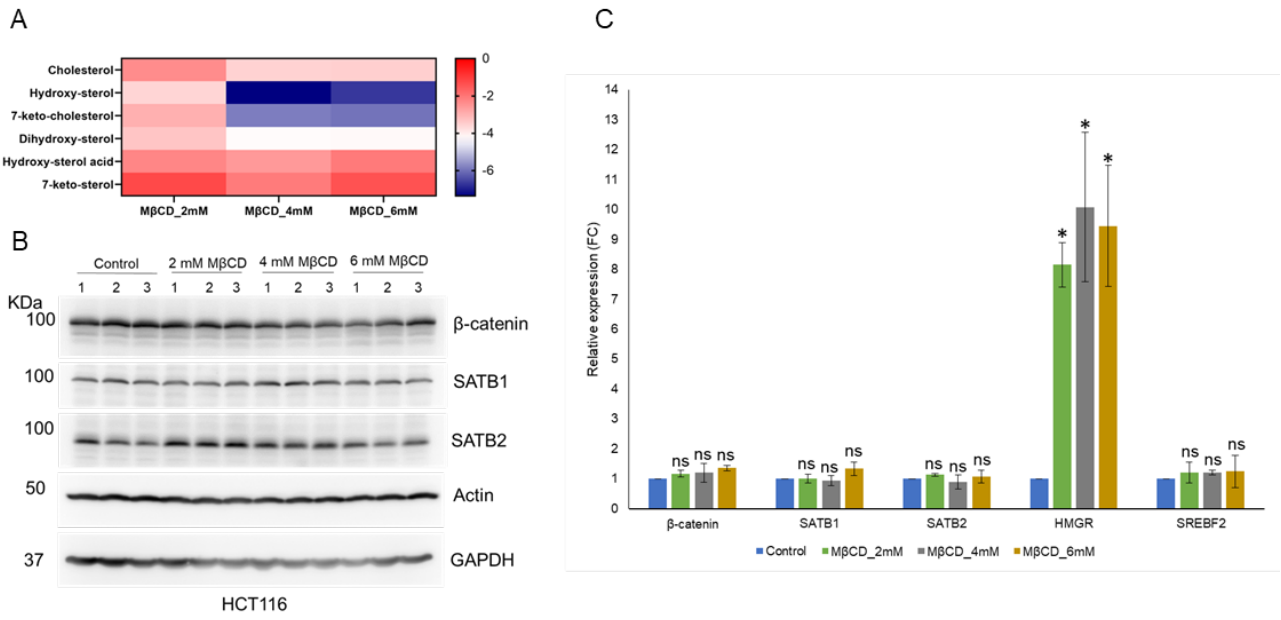

Figure 5: Cholesterol depletion by MβCD, independent of the effect on cholesterol biosynthesis pathway, seems to fail to recapitulate the effect induced by statin treatment in CRC lines. (A) Targeted lipidomics LC-MS analysis for cholesterol and oxysterols to validate the reduction in cholesterol levels on MβCD. Log 2 fold change is plotted in a heatmap and since there is a significant reduction in levels, all the values fall below zero. (B) Immunoblots to observe the effect of MβCD on β-catenin, SATB1 and SATB2 at the protein level. No change in the levels is observed for the Wnt players. (C) Relative expression profile for β-catenin, SATB1 and SATB2 indicate, no alteration at the transcript level, whereas, the cholesterol responsive gene HMGR seems to be significantly upregulated due to cholesterol depletion. However, SREBF2 is not significantly altered. Biological replicates n=3, **p<0.05, ***p<0.005, ns is non-significant as per Students' t-test.

2.3 Discussion

Since the effect of statins is known to reduce the cholesterol levels, we wanted to validate whether we observe the same effect in CRC lines as well. Our targeted lipid profile study helped us confirm the mode of action of statins in colorectal tumorigenesis and thereby use cholesterol levels as a reliable read-out for our further study. We observed a significant reduction in cholesterol and its derivatives, oxysterols, and also reported a significant reduction in a few species of Free Fatty Acids (FFAs) that are known to be critical for tumorigenesis. It is well reported that Acetyl-CoA is

converted to palmitic acid by FA synthase (FASN), that is further converted to stearic acid, both of which are observed to be accumulated in CRC tumor tissue (46). We observed a reduction in the levels of these FFA species on statin treatment in our results, indicating that statin might also help in tumor suppression via two ways, first, cutting off the cholesterol biosynthesis and depriving the tumor cells of cholesterol and oxysterols for cellular building, and second, by affecting the signaling in tumor cells through downregulation of oxysterols and FFAs, important for tumorigenesis.

We were able to link the above discussed lipid profile with our transcriptome data as well. With our transcript level results, we validated the effect on cholesterol biosynthesis machinery *in cellulo*. The cholesterol responsive genes were observed to be upregulated as a result of the cholesterol depletion, mediated by statin treatment in CRC lines. This feedback mechanism is well documented and further corroborates a functional canonical mode of action. We also observe a significant reduction in FASN, the enzyme that is responsible for production of palmitic acid observed to be reduced on statin treatment in our lipidomics data. These results corroborate with the lipidomic outcome in CRC lines and indicate towards a tumor suppressive phenotype.

Our proteome results further illuminate the effect of statin on oncogenic pathways and help in establishing the specific targets for statin mediated regulation. We observed major oncogenic signaling players getting significantly downregulated at both transcript as well as protein level in our omics data. One of the major targets observed was Wnt/ β-catenin signaling, as its upstream molecular players, β-catenin and SATB1 were significantly downregulated at protein level specifically. This affected the expression of downstream Wnt targets, thereby, causing a significant reduction in their levels. Interestingly, the effect on β-catenin, SATB proteins was restricted to the protein levels, and not at transcript levels, which suggests a mechanistic regulation implemented by statins. The effect was specific to the statin mediated downregulation, as we observed a reversal in expression profiles on mevalonate supplementation. The mevalonate substrate when present along with statin treatment, negated the effects imposed by statins and thereby reversed the effects.

To further emphasize on the specificity of mode of action of statin in CRC, cholesterol depletion independent of the effect on mevalonate pathway, was also performed. We chemically depleted the cells off cholesterol by treating them with MβCD (Methyl-β-cyclodextrin), without inhibiting the biosynthesis. The rationale was to observe if cholesterol reduction alone is responsible for the effects observed on Wnt players. However, we observe no effect on β-catenin, and SATB proteins

on MβCD treatment, thereby suggesting that statins might affect Wnt/β-catenin signaling specifically via a mechanistic regulation.

Collectively, we observed a tumor suppressive phenotype on statin treatment in CRC lines and Wnt/ β-catenin signaling as a potential target. The effect on downregulation of Wnt major players defines the specificity of statins as well as the potential therapeutic for CRC.

2.4 Materials and Methods

Lipidomics

All the samples for lipidomics analysis were prepared and analyzed using established protocols previously described (47-49) on a Sciex X500R QTOF mass spectrometer, fitted with an Exion series UHPLC. Briefly, the cells were washed with PBS and then resuspended in methanol:chloroform (1:2) mixture. The organic layer was collected and washed with chloroform to remove any contaminants. This organic layer has all lipid species which is then used for LC-MS.

Transcriptomics sample preparation and analysis

RNA isolation was performed using Trizol reagent (Taraka RNAiso Plus, Code: 9109) from the cells. Briefly, after washing the cells with 1X PBS, 1 ml Trizol reagent was added and cells were homogenized completely before washing the upper aqueous layer with chloroform. Isopropanol was used to precipitate the RNA and the pellet was washed with 75% ethanol, followed by drying it and dissolving in nuclease free water. The RNA was then quantified and integrity was monitored using Qubit 4.0 (ThermoFisher Scientific) and Bioanalyzer (2100 Agilent Bioanalyzer Systems) respectively.

Total RNA (500 ng) was subjected to mRNA purification using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, USA) according to the manufacturer's instructions. The purified mRNA was used for library preparation using NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB, US) using the protocol provided in the kit. The final libraries were purified using HighPrep PCR Clean-up System (MagBio Genomics, USA) and were quantified using Qubit 1X HS DNA system (Thermo Fisher Scientific). All the libraries were pooled in equimolar ratios and subjected to 75bp PE chemistry on Nextseq550, Illumina.

RNA seq analysis

Paired-end sequencing was performed of RNA samples on Illumina platform (Macrogen Inc, Korea). In brief, after performing quality control (QC), qualified samples were processed for library construction. Sequencing library was prepared by random fragmentation of cDNA followed by adapter ligation. Adapter ligated fragments were PCR amplified and gel purified. The libraries were loaded into a flow cell and each fragment was clonally amplified through bridge amplification. The sequencing data was converted into raw data for analysis.

The quality of sequencing reads was checked on FASTQC (v 0.10.1) [Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/]. The sequence alignment was performed on the human genome (version hg38) [https://www.gencodegenes.org/] using HiSAT2 (v 2.05) [https://daehwankimlab.github.io/hisat2/manual/]. The resulting bam files were used to generate a count matrix (50) followed by differential expression analysis (v 1.18.1) (51). Ontology analysis was carried out using clusterProfiler (v 3.6.0) (52, 53).

Proteomics sample preparation and analysis

Whole cell lysates were resolved on a 10% SDS-PAGE gel and subsequently processed for proteomics using standard in-gel sample preparation protocols (54). Briefly, the gel bands of interest were excised, destained, reductively alkylated and digested using MS-grade trypsin. Subsequently, the peptides were desalted and processed for proteomics analysis on Sciex TripleTOF6600 mass spectrometer interfaced with an Eksigent nano-LC 425 system using established protocols previously reported (55, 56). All raw data was analyzed using the ProteinPilot software from Sciex using search parameters previously described (56). The GO term for the differential peptides were plotted using g:Profiler. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD041210.

Reagents and antibodies

Simvastatin and Rosuvastatin used in the assays were obtained from Sigma (Simvastatin Cat No. #79902-63-9, Rosuvastatin Cat. No. #SML-1264). Simvastatin (Sigma Cat No. #79902-63-9) was used for all assays at a final concentration of 30 μM in activated form. Briefly, for activation of statin, it was dissolved in NaOH and 100% ethanol and heated at 50 °C for 2 hr. The pH was adjusted to 7.4 with HCl and volume made up to 1 ml with Milli Q water. The final drug was filtered and stored in aliquots at -20°C. The activation of simvastatin is important to break the lactone ring that is the competitive inhibitor substrate for its target enzyme HMG CoA reductase. Mevalonate was purchased from Sigma (Cat no. M4667) and Methyl-β-Cyclodextrin was from Sigma (Cat. no 332615). The antibodies used were as follows, β-catenin (BD Bioscience Cat No. 610153), SATB1_L745 (Cell Signaling Tech, Cat No. 3650), SATB2 (AbCam, Cat No. Ab92447), GAPDH (AbCam, Cat No. G041), TCF4 (CST #2569S), AXIN2 (CST #76G6), PCAF (Santa Cruz #13124).

Cell culture

HCT116 and HCT15 CRC cell lines were obtained from the European Collection of Cell Cultures (ECACC) and HT29 cell line was obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). HCT116 was cultured in DMEM media (Gibco, Waltham, MA, USA) with 10% FBS whereas HCT15 and HT29 were cultured in RPMI media (Gibco, Grand Island, NY, USA) with 10% FBS. FBS (Heat-inactivated #REF- RM10409-500 ml) and anti-bacterial, anti-fungal-100X (#REF- A002A- 20 ml) were obtained from HiMedia. The culture grade Trypsin-EDTA solution 1X (#TCL099) was also obtained from HiMedia.

Immunoblotting

The protein lysates were prepared in RIPA buffer (20mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% NP-40, 1% Sodium deoxycholate, protease inhibitor cocktail (PIC, Thermo Fisher Cat No. A32963). The cells were pelleted after 1X PBS washes and resuspended in RIPA buffer+ PIC (Protease Inhibitor cocktail) + phosphatase inhibitor PhosSTOP (Roche #4906845001), stored in ice and allowed to lyse for 1 hr. The lysate was centrifuged at 14,000X rpm, 4°C for 30 min. The supernatant was collected and used for loading on SDS-PAGE gels after protein quantification using BCA assay kit (Pierce #23250). The 10% SDS-PAGE gels were freshly prepared and electrophoresed using the 1X SDS-PAGE running buffer. Signals on PVDF membranes were developed using clarity Western ECL substrate (Biorad #1705061), and LAS 4000 was used for visualizing the HRP based chemiluminescence chemistry.

Quantitative RT-PCR

Total RNA was extracted from cells grown under 2D culture conditions and vehicle control and statin treated sets using Trizol reagent (Taraka RNAiso Plus, Code: 9109). Extracted RNA was either subjected for library preparation for sequencing or PCR based gene expression analysis. For qPCR based gene expression analysis, cDNA was synthesized using ABi (Applied Biosystems) High Capacity cDNA Reverse Transcription kit (Cat No.: Ref 4368814). The cDNA was utilized for relative gene expression analysis using gene specific primers, Table 1, with 18srRNA as endogenous control.

S.No.	Gene name	qPCR Primer sequence
1	SATB1	Forward- AACTCAGGGCTTGCTTTC Reverse- CCTGGTATATTCGGTCTCTTTC
2	SATB2	Forward- AGAGATGAACCAGAGCACATTAG Reverse- GTTGCTGACACATTGGCATAAT
3	β-catenin	Forward- AAGGTGTGGCGACATATGCA Reverse- GTAATCTTGTGGCTTGTCCTCAGA
4	18srRNA	Forward- CGCCGCTAGAGGTGAAATTCT Reverse- CGAACCTCCGACTTTCGTTCT
5	SREBF2	Forward- GCTGCCAAGGAGAGTCTAT Reverse- AGGTTTCACCAAGGACTCTAT
6	HMGR	Forward- ACAAGAATTTAGTGGGCTCTG Reverse- TCCTGTCCACAGGCAAT
7	CDH1	Forward- GGCTGGACCGAGAGAGTTTC Reverse- CCTGACCCTTGTACGTGGTG
8	Vimentin	Forward- GCGAGGAGAGCAGGATTTCT Reverse- TGGGTATCAACCAGAGGGAGT
9	E-cadherin	Forward- GTCCTGGGCAGAGTGAAT Reverse- GGGTTATGAAACCGTAGAGGC

Table 1: The list of primers used for the validation by qRT-PCR.

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Chapter 3

To delineate the effect of Statins on the dynamic balance of expression of SATB proteins in CRC

3.1 Introduction

In the previous chapter, we elaborated on the effect of statins by emphasizing the compiled outcome of lipidomics, transcriptomics, and proteomics in CRC cell lines. We identified a novel target of statins, the Wnt/β-catenin signaling, via its major molecular players getting affected. We suggested the significance of studying them, namely β-catenin and SATB1 protein, as the mechanism of this specific mode of action of statins is not known in CRC. In consecutive sections, we would eventually elaborate on the mechanistic regulation of statin on each player separately. In this chapter, we would first explore the effect of statins on SATB1 and particularly study the dynamic balance of expression between SATB1 and SATB2 proteins together.

The previously published data from our lab suggested the significant role of SATB1 in Wnt/ β-catenin signaling (1). However, the importance of studying both SATB proteins in the context of colorectal cancer lies in the reports that have established distinct roles of SATB1 and SATB2 in CRC tumor progression. It has been suggested that SATB1 plays a tumorigenic role specifically (2-8), whereas there are variable reports for SATB2 (9-14). However, no such study has been conducted which explores the combined effect on SATB proteins in CRC and the dynamics between these two homologs with respect to each other.

SATB proteins are chromatin organizers that help in regulation of target gene expression. These proteins generally bind to other regulatory proteins via its N-terminal PDZ-like domain and interact with the promoter region of target genes via CUT domains and C-terminal homeodomain (15-17). There are an increasing number of studies where SATB1 overexpression has been shown to play a pivotal role in tumor progression. Galande group has contributed towards furthering this understanding in an earlier report showing the interaction of SATB1 with β-catenin upon canonical Wnt signaling (1). Furthermore, it was observed that both SATB1 and β-catenin are responsible for the upregulation of SATB1 expression in a Wnt-dependent manner as well as the upregulation of downstream Wnt target genes. This report shed light on SATB1 as an upstream molecular player along with β-catenin in Wnt pathway. Since SATB1 expression and role is established in tumorigenesis, and SATB2 expression is suggestive of tumor suppression, studying the effect of

statins on the Wnt player, SATB1 makes it critical to study the effect on its homolog, SATB2 as well. The significance of this correlative study is primarily because they are not redundant in their roles in CRC.

Thus, this dynamic expression intrigues the field of colorectal oncology even today, not only because of the different profiles but also the effect of SATB1 and SATB2 in one cancer type with respect to each other. Validating the expression profile, we ectopically over-expressed SATB1 in CRC lines and reported the levels of SATB2. We observed a striking downregulation of SATB2 on the upregulation of SATB1. We also recapitulated the results in a more tumorigenic model system of 3D spheroids, and observed that spheroids had higher endogenous levels of SATB1 than SATB2.

The role of SATB1 in Wnt/ β-catenin signaling as an upstream player and the reciprocal expression of SATB1 and SATB2, makes looking at the effect of statins on these two homologs collectively, a major requisite. In the following chapter we would first try to establish the tumorigenic model system, recapitulating our preliminary human study results for the expression of SATB1 and SATB2. We developed spheroid cultures with CRC lines and validated the tumorigenicity by observing the EMT-MET marker expression. We further explored the effect of statins on both SATB1 and SATB2, along with the putative impact on the mesenchymal to epithelial switch. Our clinical investigation in human subjects additionally helped us recapitulate the molecular expression of SATB proteins in statin pre-treatment and post-treatment biopsies. We observed a reduced tumor burden in our murine as well as clinical studies on statin treatment, suggesting a possible use of statins as an anti-neoplastic drug.

3.2 Results

3.2(i) Reciprocal expression of SATB proteins in CRC

We validated the dynamic expression of SATB1 and SATB2 in CRC in two ways. The first was an *in cellulo* method by ectopically over-expressing SATB1 in HCT116 cell line. The second, an *in vivo* method, by looking at the adjacent normal vs the tumor tissue biopsy samples of patients with CRC. In the first method we used CRC cell line HCT116, in which we transiently transfected a FLAG tagged SATB1 over-expression construct and thus ectopically over-expressed SATB1. We probed for the protein levels of SATB1 to validate the overexpression. SATB2 levels were significantly downregulated after ectopic over-expression of SATB1 (Figure 1A).

In the second approach, we obtained the adjacent normal colon/rectal tissue and the tumor tissue of the same patient during the routine biopsy procedure and probed for the protein level expression profile of both SATB proteins. The patient samples obtained were a part of the randomized trial study conducted in collaboration with the Tata Memorial Hospital (TMH), Mumbai (Ref no.: IECHR/VB/2018/014). As part of this phase II/III trial study, we have tried to characterize SATB proteins as CRC markers for tumor prognosis as well as include statins in the treatment protocol as drugs that target these markers for tumor suppression.

We observed 9 patients who underwent a routine biopsy to determine the progression stage of the adenomatous polyps and the status of the adjacent normal colon/rectal tissue. The molecular signature of these tissue samples was observed to be in corroboration with our *in cellulo* results. The SATB1 protein was highly expressed in tumor samples of these patients, as compared to the SATB2 level, which was higher in the adjacent normal tissue (Figure 1C, Appendix Section 3). This further recapitulated the reciprocal expression profile of SATB1 and SATB2 in CRC (Figure 1B) and proposed the probable use of SATB proteins as both markers as well as targets for colon and rectal tumor progression. Considering separate findings indicating a comparable expression pattern for SATB proteins, with SATB1 detected in tumor tissues (4) and SATB2 in adjacent normal colonic crypt (18), we hypothesized that a dynamic reciprocal inverse expression could distinguish between adenoma and normal tissue. However, exploring whether SATB1 and SATB2 mutually regulate each other's expression in their respective tissues would be an intriguing avenue for further investigation into these reciprocal dynamics.

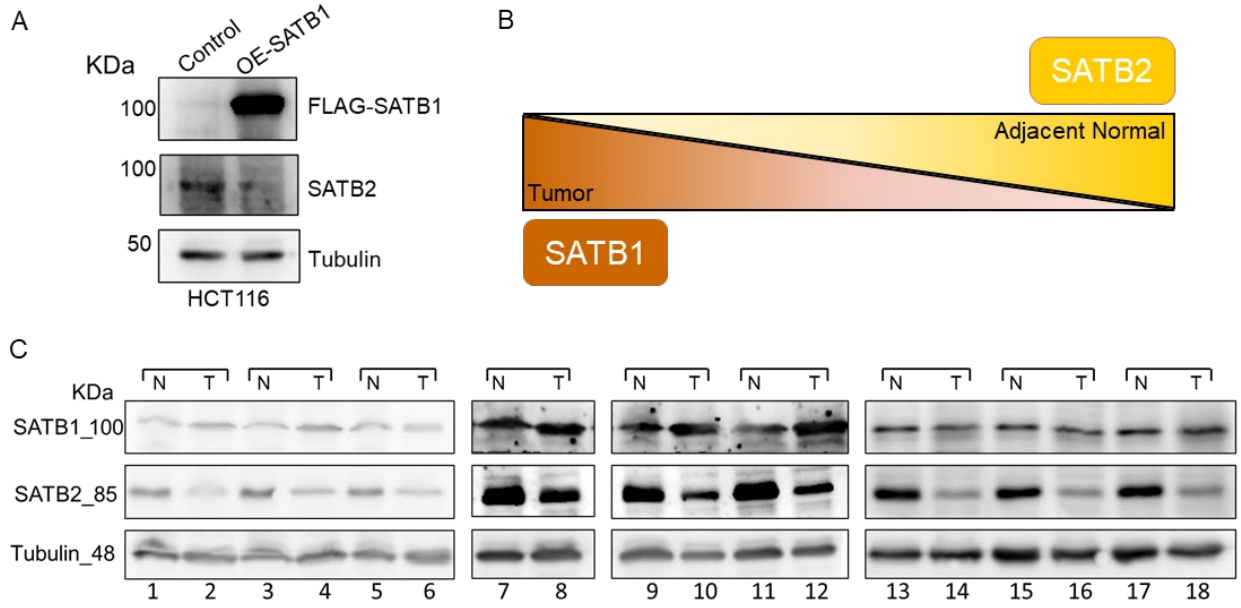


Figure 1: Dynamic expression of SATB proteins in tumor progression and Statin treatment. (A) Ectopic over-expression of SATB1 in the HCT116 cell line shows a reciprocal effect on SATB2 expression at the protein level. As SATB1 expression was increased, SATB2 protein level was significantly reduced. (B) Schematic representation of the opposite expression profile of SATB proteins in tumorigenesis and adjacent normal tissue. SATB1 was higher in tumor tissue as compared to SATB2 expression, which was higher in adjacent normal. (C) We validated the reciprocal expression profile of the SATB proteins in the tumor and normal adjacent biopsy tissue samples. A total of 9 patients, with paired samples of adjacent normal (N) and tumor tissue (T) were probed for SATB1 and SATB2 immunoblots. The tumor samples showed higher expression of SATB1 than SATB2. Whereas the SATB2 expression was higher in the adjacent normal tissue samples.

3.2(ii) 3D spheroids for CRC exhibit distinct expression of SATB1 and SATB2

After validating the reciprocal SATB proteins expression profile in CRC cell line and patient tissue, we further studied the effect of statins on these proteins. We wanted to set up a model system that recapitulates the tumorigenic properties better than the 2D cell lines. Therefore, we developed spheroids to recapitulate the reciprocal expression of SATB1 and SATB2 as well as study the effect of statins (Figure 2A). The significance of the spheroids has been well established in the field as a model system that is better than 2D cell lines in reproducing comparable phenotypes of tumorigenesis (19). However, we first validated them for EMT-MET markers as compared to the 2D cells.

The 3D spheroids had a better EMT (Epithelial to mesenchymal) phenotype as the transcript level of its marker, Vimentin, was significantly high in spheroids and the MET (mesenchymal to epithelial) marker like CDH1/ E-cadherin was low (Figure 2B). We then checked for the expression of SATB1 and SATB2 in 2D cells vs the spheroids. The transcript, (Figure 2B) and the protein levels of SATB1, (Figure 2C) were endogenously higher in 3D spheroids than their respective 2D cell line. Whereas, SATB2 levels were downregulated (Figure 2B,C). This observation is essential for establishing the spheroid model system to study the dynamics between SATB proteins and tumor progression. Since we observed enhanced expressions of SATB1 as well as the EMT marker Vimentin in spheroids, we noticed a possible correlation of SATB proteins and EMT-MET markers. SATB2 expression corresponded to lowered levels of MET marker E-cadherin as well. Therefore, we could suggest that the expression of SATB1 might go in hand with EMT phenotype and the expression of SATB2 might correlate with MET phenotype.

With the 3D model system established, we further wanted to observe the effect of statin on spheroids. However, before switching to the spheroid model system, we first tested for the statin effect on one of the tumorigenesis parameters of clonogenicity by performing the colony formation assay. The significance of studying the clonogenic capacity of the CRC lines in the presence of an inert matrix, lies in determining the clonogenic capacity of spheroids when provided with matrigel matrix. Therefore, when statin treatment was given to the colonies formed on the soft-agar plates, the number of colonies showed a direct effect of statin on the clonogenic capacity. We observe a reduction in the colonies on statin treatment, reiterating the anti-tumor effect imposed by this drug on one of the major oncogenic factors (Figure 2D).

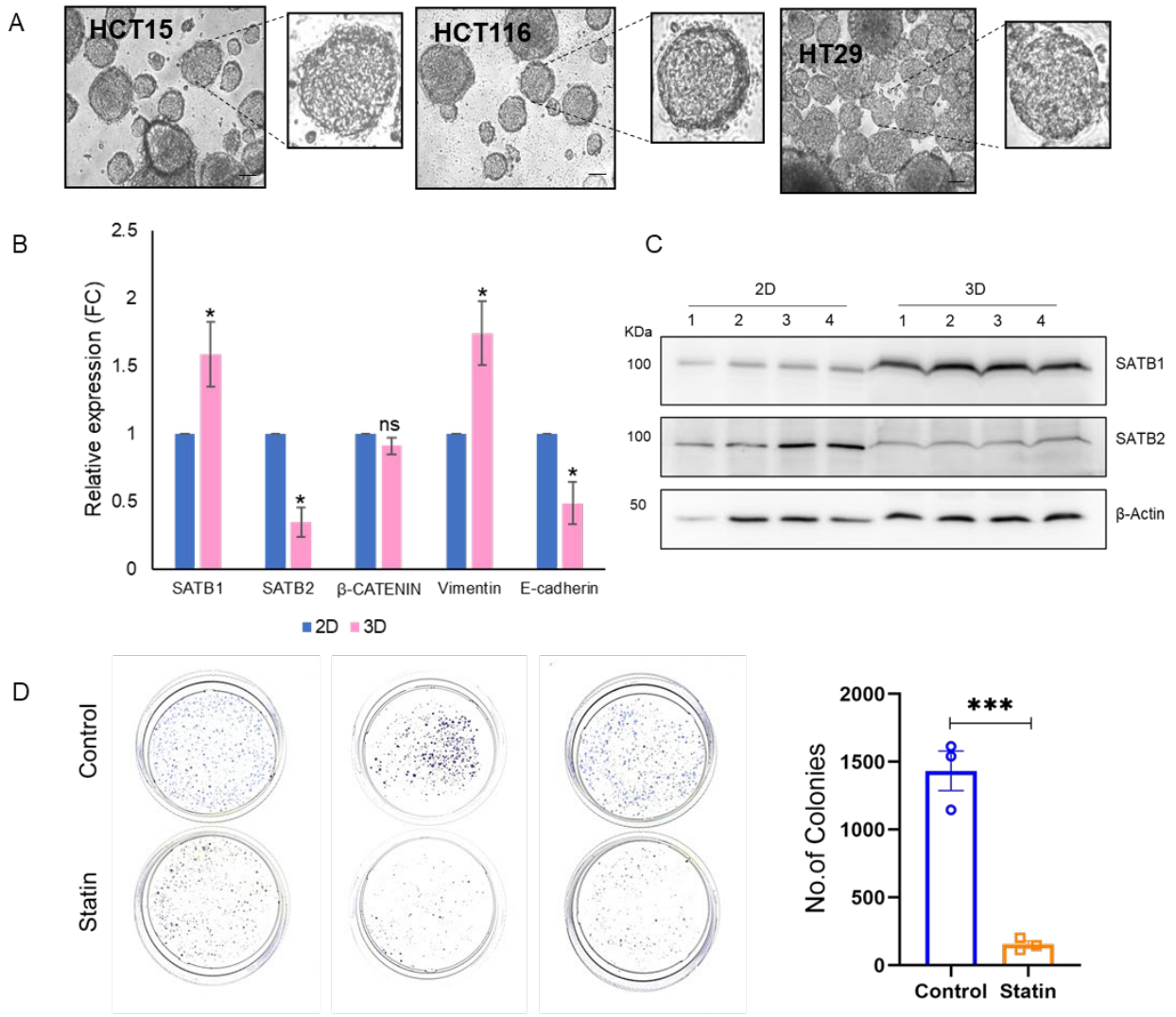


Figure 2: 3D spheroids of CRC lines represent EMT phenotype better than 2D lines. (A) Images of spheroids for CRC lines HCT15, HCT116, HT29 that were allowed to form in matrigel for 4 days. Phase contrast images of spheroids, scale bar 50 µm, with zoomed in image in

adjacent box. (B) Relative gene expression of SATB1, SATB2, β-catenin and EMT-MET markers, Vimentin and E-cadherin, respectively. The spheroids showed higher expression of EMT marker Vimentin along with higher expression of SATB1 than SATB2. β-catenin did not show any alteration. (C) Immunoblot to observe the protein levels of SATB1 and 2 in 2D and 3D spheroids recapitulating the tumorigenic phenotype of spheroids. (D) Colony formation assay of HCT15 cells on statin treatment and a graph representing no. of colonies in control vs treated sets. The colonies were allowed to form on soft agar for 5 days and then treated with statin for 48 hrs. The no. of colonies were significantly reduced upon statin treatment. Biological replicates n=3, *p< 0.05, **p<0.005 as per Students' t-test.

3.2(iii) Statin treatment of 3D spheroids reciprocally regulates SATB1 and SATB2

Furthermore, the spheroids were treated with statin and the first observation was made on the morphology of these spheroids. Interestingly, the statin-treated spheroids seemed to be disintegrated in both the HCT116 and HCT15 cell lines 3D cultures (Figure 3A). We checked for the apoptosis of the spheroids to rule out any excess cell death on statin treatment. We used annexin V staining to observe any cell apoptosis in vehicle control vs statin-treated set of spheroids. Annexin V binds to the phosphatidylserine that gets exposed when the cell membrane is turned inside out due to rupture or necrosis. Higher staining for Annexin V protein quantified by either flow cytometry or immunofluorescence assay depicts cell apoptosis. In our statin-treated spheroids we found no excess cell death after FACS sorting the cells (Figure 3D) suggesting that the spheroids did not undergo apoptosis on statin treatment but probably disintegrated.

The intriguing effect of statin on the colonies and the 3D spheroids led us to pursue the investigation of the transcript and protein alteration, especially concerning the SATB1 and SATB2 dynamic expression in the spheroid model system. However, we first checked for the EMT-MET markers in these spheroids as we observed a dissociation of cells from spheroid formation on statin treatment. We did not report any alteration of the EMT marker, Vimentin, however, we observed a significant upregulation in the transcript levels of the MET marker E-cadherin on statin treatment (Figure 3B). This suggested a possible reversion of phenotype from mesenchymal to epithelial on statin, which was observed by partial dissociation of the spheroids onto the matrix.

To further delve into the balance between the two SATB proteins and the effect of statin on them, we checked for their protein levels on statin treatment in spheroids. The immunoblots for the two cell line 3D spheroids recapitulated the reciprocal effect. Statin treatment lowered the protein expression of SATB1, however, the SATB2 expression was slightly upregulated (Figure 3E,F), corroborating our 2D cell line data. We monitored the alterations in transcript levels, and as

observed earlier, levels of SATB1 and SATB2 did not change upon statin treatment (Figure 3B). Collectively observed, the expression profile of the EMT-MET markers and the SATB proteins appeared to be correlative. Therefore, there appears to be a strong possibility of the statin-mediated effect on SATB proteins via the EMT-MET expression reversal (Figure 3C). Further investigation would be required to finally conclude whether the effect of statin on the SATB proteins is because of the effect on the EMT-MET phenotype or vice versa. However, the evidence of a correlation between the SATB proteins and EMT-MET markers seemed to be undeniable. It would be interesting to explore the statin-mediated regulation of these proposed CRC markers as well as the MET marker.

Collectively, with the above results we observed a critical role played by the upstream molecular proteins in maintaining the tumorigenicity. SATB1, being an integral part of Wnt/β-catenin signaling, promoted the tumorigenic property, as augmented by EMT marker expression in our spheroid model system. Whereas, SATB2, seemed to be playing an inverse role as supported by the MET markers expression. On Statin treatment, the SATB2 protein expression was slightly upregulated, along with transcript level upregulation of MET marker, whereas, SATB1 protein expression is downregulated. Therefore, Statin seemed to be disrupting the balance required for tumor progression and might be tipping it towards tumor suppression, by mediating the reciprocal expression of the markers.

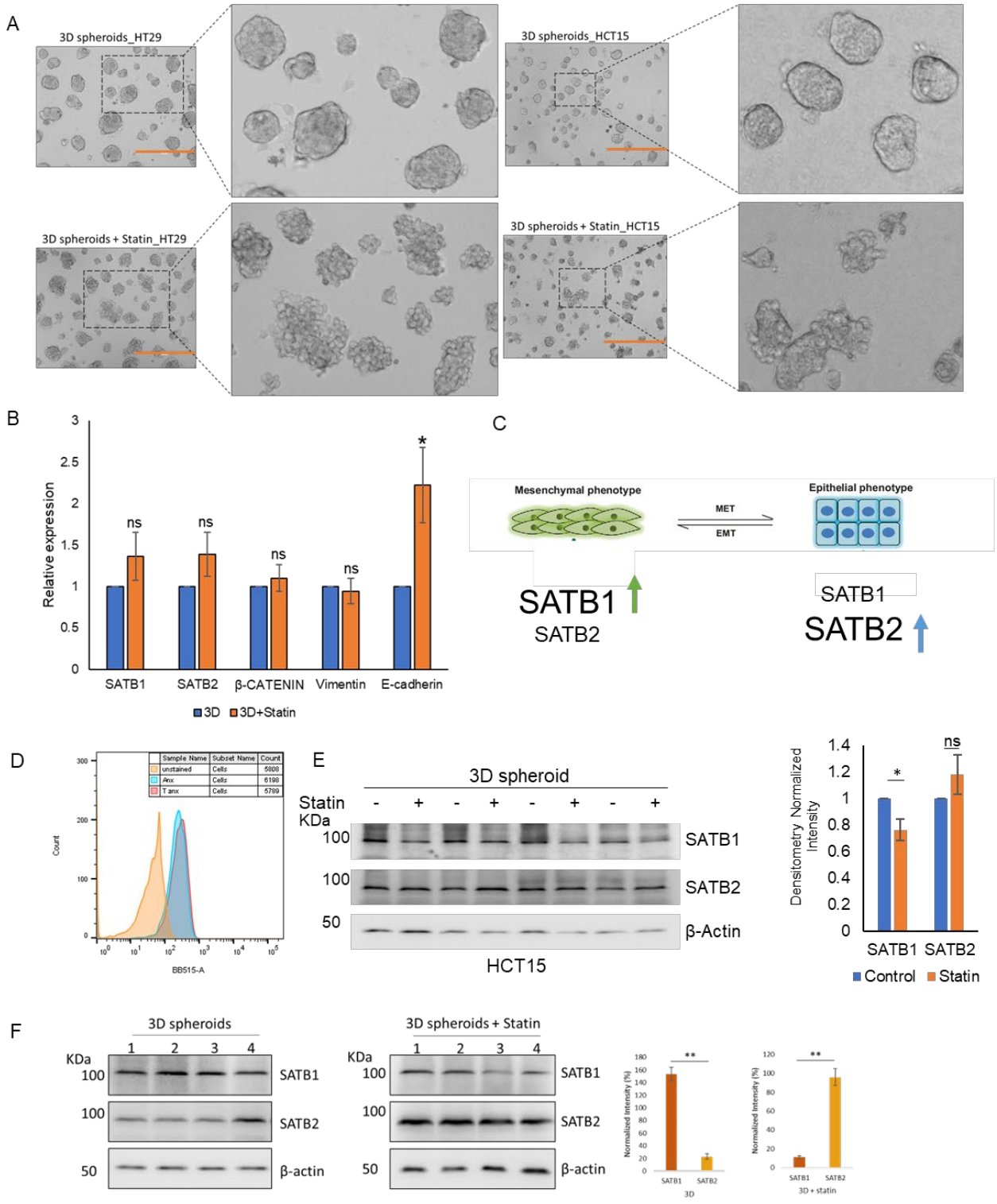


Figure 3: Statin treatment of spheroids causes reduced SATB1 protein expression and increased SATB2 expression. (A) Phase contrast images to show the effect of statin on HT29 and HCT15 spheroids, where, in the treated set there was disintegration of spheroids visible. Phase contrast images, scale bar 400 μm (orange) with zoomed in image in adjacent square box.

The spheroids were allowed to form for 4 days, thereafter statin treatment was given for additional 48 hrs. (B) Relative expression at transcript levels of SATB1, SATB2, β-catenin, Vimentin, E-cadherin in spheroids with and without statin treatment recapitulated the 2D cell line results of no alteration at transcript level. (C) Illustration to show the reverse correlation of SATB proteins and EMT-MET factors. Higher expression of SATB1 correlated with EMT marker Vimentin in tumorigenic tissue, whereas, increased expression of SATB2 correlated with MET marker, E-cadherin in epithelial tissue type. (D) Flow cytometry analysis for Annexin V stained spheroids to check for apoptosis. The statin-treated spheroid labeled 'T Anx' in the graph did not show any annexin V staining increase depicting no excess cell death compared to the untreated spheroids labeled 'Anx'. The unstained spheroids were used as FACS analysis control. (E) Immunoblot to monitor the protein levels of SATB1 and SATB2 in statin-treated spheroids of HCT15. The densitometry graphs on right depict the normalized intensities of SATB1 and SATB2. SATB1 levels were significantly reduced, whereas, SATB2 levels were not significantly altered (F) Immunoblot to monitor the protein levels of SATB1 and SATB2 in statin-treated spheroids of HCT116. The densitometry graphs on right depict the normalized intensities of SATB1 and SATB2. SATB1 levels were significantly reduced, whereas, SATB2 levels were slightly upregulated. Biological replicates n=4, *$p<0.05$, ns is non-significant as per Student's t-test analysis.

3.2(iv) Statins reduce tumor burden *in vivo* and recapitulate the effect on SATB proteins

To further strengthen the potential role of statins as an anti-neoplastic drug that can be repurposed for CRC therapeutics, we extended our study to *in vivo* analysis by adopting murine experimental set up as well as human study. For establishing the murine study, we injected the NOD-SCID immunocompromised mice with the CRC lines subcutaneously, and observed the tumor burden at the end of 45 days of injection. We administered statin orally at 40 mg/kg/day, after 7 days of the injection of cells. The tumor weight was taken at the end of the experiment regime and the tissue was utilized for transcript and protein analysis (Figure 4A).

We first used HCT116, an aggressive CRC cell line, to induce tumor in these mice and administered statin orally following the same regime mentioned above (Figure 4B). The statin treated mice developed a negligible tumor as compared to the ones being administered the vehicle control. The same experimental regime was conducted with mice injected with SW480 cell line but with FLAG tagged overexpression construct of SATB1 stably incorporated (Figure 4C). This allowed the ectopic over-expression of SATB1 in otherwise low expressing SW480 cell line. The tumor burden induced by this transformed cell line was again observed to be significantly reduced in the mice receiving statin treatment. The tumor tissues from the sacrificed mice were isolated and probed for SATB1 and 2 at transcript and protein levels for the molecular signature analysis. The transcripts did not show any change as seen previously in our *in cellulo* results of

both 2D and 3D spheroid systems (Figure 4E). However, there seemed to be a slight downregulation of SATB1 in these tumor tissues and slight upregulation of SATB2 at the protein level (Figure 4D). Therefore, we suggested that the SATB family of proteins have the potential to recapitulate not only the *in cellulo* results but also the preliminary human biopsy analysis discussed in the Figure 1C. The statin-mediated effect also seemed to be consistent in depicting a tumor-suppressive phenotype across simple and complex model systems.

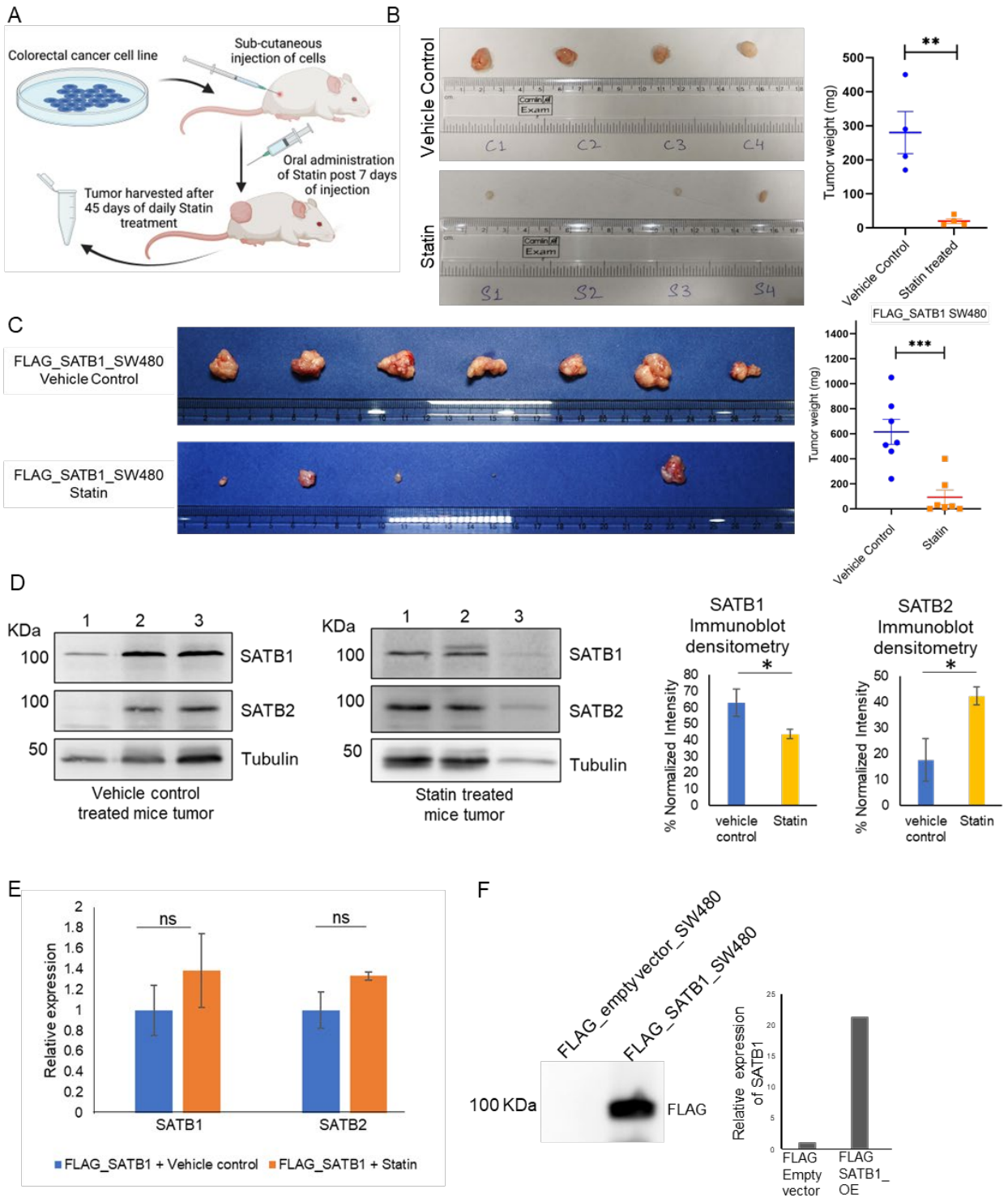


Figure 4: Reduction in tumor burden upon downregulation of SATB1 in the murine model system. (A) Schematic presentation of the experimental strategy using NOD-SCID mice. (B) Images and graph to show a reduction in tumor burden on statin treatment in mice injected with HCT116 cell line, biological replicates n=4) (C) FLAG-SATB1 overexpressed SW480 cells were

injected in NOD-SCID mice, and tumor burden was observed (n=7) **p<0.005, ***p<0.0005 as per Student's t-test analysis. (D) Immunoblots for probing SATB1 and SATB2 in the isolated tumor tissues of the SATB1 over-expressed SW480 injected cell line NOD-SCID mice, n=3. There seemed to be a reduction in SATB1 expression on statin treatment and a slight upregulation of SATB2 in these tumor tissues, as validated by the densitometry graph on the right. (E) Transcript level alteration of SATB1 and 2 in these isolated tissues. No change was observed at the transcript level on statin treatment. (F) Validation of the over-expression of SATB1 in SW480 cell lines post stable incorporation, both at the protein and transcript level. The immunoblot was probed with FLAG antibody, whereas, the transcript level was checked by using the qPCR primers for SATB1.

3.2(v) The clinical trial involving the colon-rectal patients being administered Statin along with neo-adjuvant therapy for tumor suppression

We have conducted a human study (Ref no.: IECHR/VB/2018/014) in collaboration with the Tata Memorial Hospital, Mumbai, where we proposed a protocol to include statins in the therapeutic regime of patients admitted for colon and rectal cancer. The trial is in the phase II/III stage currently, where molecular and pathological data has been acquired from the patients after their consent to the trial terms and conditions. Only those patients were included in this randomized trial study who were not on prior medication of statins or aspirin. The patients aged between 18 to 70 years were categorized thoroughly concerning their colon and rectal cancer stages. After the initial histopathological characterization of the adenomatous tumor tissue biopsies, they were confirmed for progressive adenoma and divided into two therapy arms.

The patients in arm 'A' received rosuvastatin for 12-16 weeks at the dosage of 20 mg/day and then checked again for tumor progression. These patients resumed neoadjuvant chemotherapy after that for further treatment. Whereas arm 'B' received only the neoadjuvant chemotherapy without any statin administration.

We analyzed a total of 60 patients, with 30 patients in arm 'A' and 30 in arm 'B' (Figure 5A). The arm 'A' patients showed a better response to the therapy regime and showed a partial or near-complete regression of tumor. Out of the 30 patients in arm 'A', we obtained biopsies of 10 patients with paired samples from pre-treatment and post-treatment of statin. Morphologically, the tumors were reported to be scanty and highly reduced in post-treatment samples.

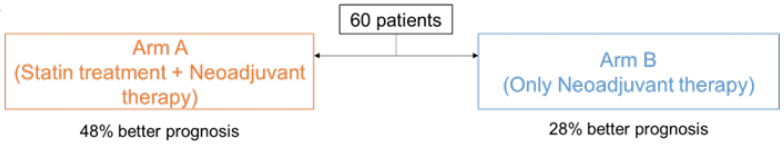
Further, the molecular studies for the expression profiles of SATB1 and SATB2, in the paired samples of the 10 patients, was conducted by Immunohistochemistry (IHC) analysis. The IHC signal intensity score for SATB1 showed a significant reduction in post-treatment samples as

compared to its respective paired pre-treatment samples (Figure 5A). This observation suggested that there was a significant downregulation of SATB1 protein on statin treatment in these patients. Whereas, SATB2 expression was varied in the two conditions (Figure 5A). The representative images of the IHC sections of three different patients showed the signal intensity of the molecular markers clearly (Figure 6). One of the patient's pre- and post-treatment expression profile of SATB1/2 (Figure 6A) showed the protein expression which corroborated well with all our above data. The adenomatous tissue marked with the yellow arrow in the H&E section confirmed the location of the tumor cells expressing SATB1. Whereas, the normal lumen section (middle section of upper panel) had higher expression of SATB2 at the base of the crypt which consisted of the non-tumorigenic cells. Therefore, the expression of SATB1 in tumor tissue and SATB2 in normal tissue corroborated with our preliminary protein profile (Figure1C).

Also, in the lower two representative images from two more patients out of the set of 10, the lower panels of (Figure 6B,C), showed a negligible signal of SATB1 on statin treatment, however, SATB2 expression was lowered as well. These set of two additional representative IHC images from patients showed a variable expression of SATB2 as shown in the table for IHC score, however, SATB1 is consistently observed to be lowered in statin treated samples.

Therefore, our data corroborates well throughout and shows astounding reproducibility and consistency throughout the different model systems, from simpler cell lines to a more complex, spheroids, murine as well as human systems. The results with respect to the expression dynamics of SATB proteins, the effect on the EMT-MET markers, the phenotypic alteration and the morphological changes, all indicate towards a tumor suppressive property of statins (Figure 5B). The significant reduction in the tumor burden in murine as well as human studies and the intact molecular signature pre- and post-statin treatment further strengthen our hypothesis of anti-neoplastic effects of statin and put forward a pivotal proposition of adding statins to the existing therapeutic protocol of CRC management.

A



Pre-Treatment	SATB1 Score	SATB2 Score	Post-Treatment	SATB1 Score	SATB2 Score
Patient 1	1	2	Patient 1	1	1
Patient 2	0	0	Patient 2	0	0
Patient 3	1	0	Patient 3	1	2
Patient 4	1	2	Patient 4	0	6
Patient 5	0	0	Patient 5	0	3
Patient 6	4	9	Patient 6	0	0
Patient 7	2	1	Patient 7	0	0
Patient 8	0	6	Patient 8	0	1
Patient 9	1	0	Patient 9	0	1
Patient 10	0	0	Patient 10	0	0

B

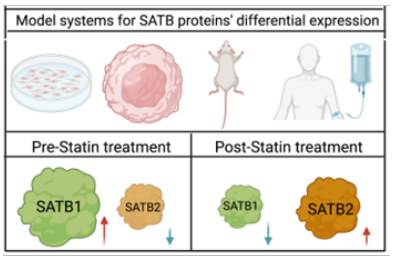


Figure 5: The phase II/III clinical trial for inclusion of statins in the treatment protocol for CRC as it shows anti-tumor effects. (A) The 60 patients were documented with a follow-up history for a year, after completion of their treatment regime. They belonged to two arms, 30 patients to arm 'A', in which statin was administered at a dosage of 20 mg/day for 12-16 weeks, along with the neo-adjuvant chemo-radiotherapy. The other 30 patients belonged to arm 'B', who received only the neo-adjuvant chemo-radiotherapy. Out of the 30 patients in arm 'A', we obtained paired samples of 10 patients, pre- and post-statin treatment. Molecular analysis of these paired samples was performed to check for the expression of our proposed prognosis markers SATB1 and SATB2. We observed a recapitulation of the *in cellulo* and murine data, as we recorded a significant reduction in SATB1 signal intensity in post-statin treatment sections. SATB2 expression in the tissue sections post-treatment were variable. (B) Graphical abstract to show our collective results, in multi-model system of cell lines, spheroids, murine as well as human samples. We observed a reproducible result regarding the expression profile of SATB proteins in normal tissue, tumor tissue and statin treated set of samples.

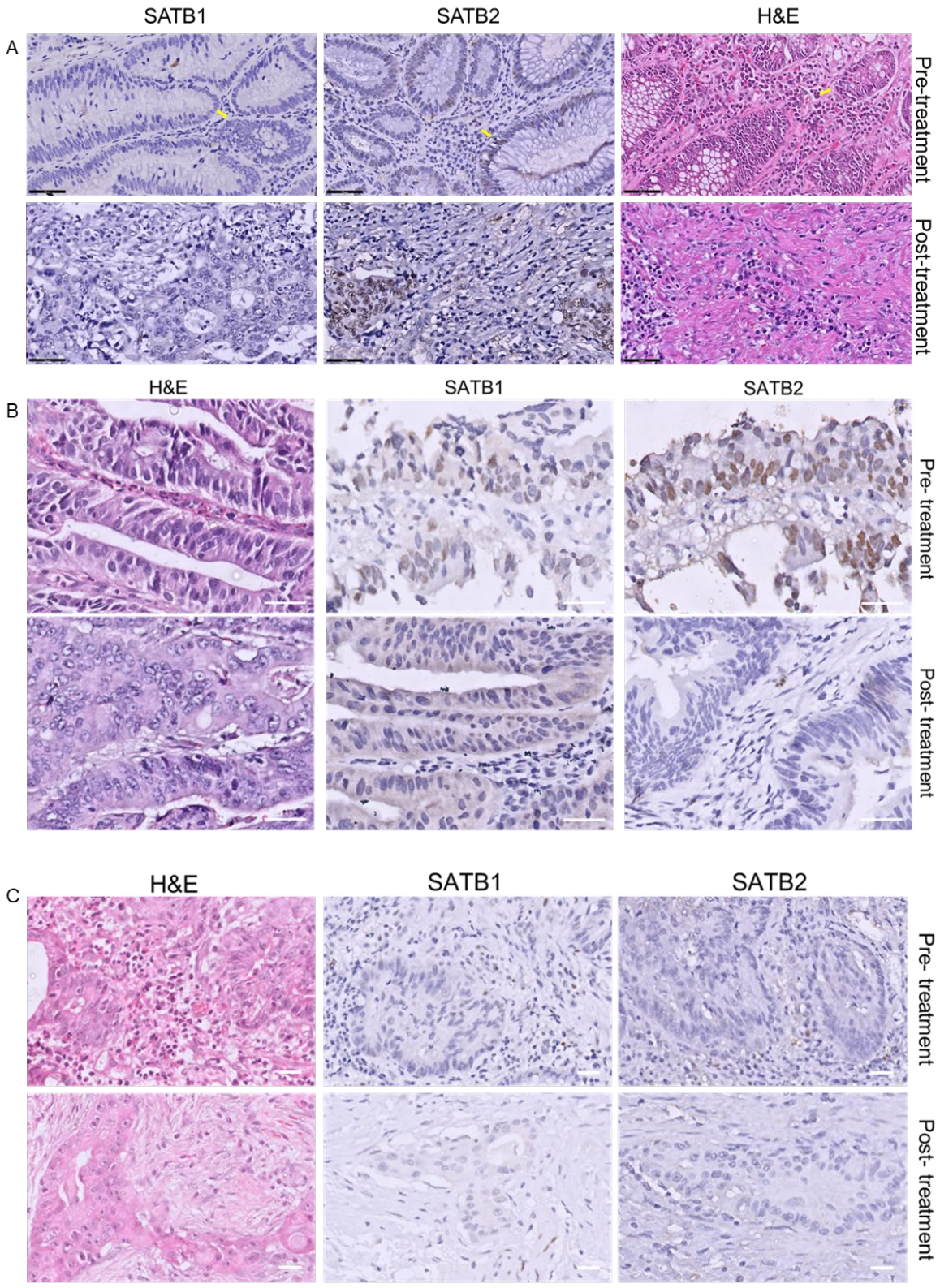


Figure 6: Representative images of the immunohistochemistry analysis of 3 patient samples with a set of pre- and post-statin treatment biopsy sections. (A) Representative

image of Immunohistochemistry for patient #1, SATB1 and SATB2 expression in a paired pre-treatment and post-treatment biopsy section (scale bar = 50 μm). The yellow arrows in the upper panel, i.e., pre-treatment samples point at the location of the expression signal. For SATB1, the expression was observed at the adenomatous section, whereas the SATB2 expression was marked at the normal crypt section. The H&E for the sample showed tumorigenic growth. The lower panel i.e., post-treatment showed mostly stroma with scattered cells as the tumor was scanty and reduced. SATB2 expression seemed to be higher in the post-treatment section. (B,C) Representative images from patient #2 and #3 respectively. In both patient #2 and #3, the SATB1 and SATB2 signal intensity was lowered post-statin treatment, therefore, both depicted downregulation (lower panel).

3.3 Discussion

In this chapter, we discussed the effects of statins on one of the molecular players of Wnt/ β-catenin signaling, SATB1, and also the impact of statin on the dynamic expression of SATB1 and its homolog SATB2 in multiple model systems. We also observed a correlation with EMT-MET markers, Vimentin and E-cadherin, respectively, where SATB1 expression correlated with Vimentin upregulation, suggesting a mesenchymal phenotype. On the other hand, SATB2 expression correlated with E-cadherin transcript level in spheroid culture. This correlation was reversed in statin-treated spheroids, which showed an epithelial phenotype based on the transcript levels of the MET marker. Morphologically, we observed the disintegration of spheroids on statin treatment, which we checked for apoptosis for confirmation of dissociation of cells only. We used Annexin V staining to check apoptosis in the statin-treated spheroids. However, it showed no extra apoptosis compared to the vehicle control spheroid sample, suggesting that statin treatment was not responsible for apoptosis in the spheroids. Therefore, the morphological alteration observed on statin treatment seemed to be a disintegration event.

This corroborated with the expression profile of the EMT-MET markers, as we observed a significant upregulation in the expression of E-cadherin on statin treatment. With a higher transcript level of E-cadherin, an MET marker, we proposed that statins might be affecting the balance between EMT-MET phenotype via either SATB protein dynamic expression or vice versa. However, it would be interesting to elucidate whether the statin-mediated reversal of SATB1 and SATB2 expression depends on the EMT-MET expression profile or the other way round as the correlation between SATB proteins and the EMT-MET markers was evident with and without statin treatment.

Furthermore, our results were recapitulated in *in vivo* model systems of mice as well as human subjects. We observed a significant reduction in tumor burden on statin treatment in mice and a lowered expression of SATB1 protein. The patient biopsies from pre-statin treated and post-treated sets showed a clear molecular marker profile as well. Recapitulating the *in cellulo* data, we observed a significant reduction in SATB1 expression on statin treatment in the 10 patients from arm 'A', whereas SATB2 expression was variable. However, the patients who were given statin along with the neo-adjuvant therapy in arm 'A' showed a better response and had lower relapse incidences in the follow-up history of approximately one year. We recorded a 48% recovery in patients with statin administration compared to the 28% recovery in patients without statin. The relapse rate was also higher in patients who did not receive statin. The cumulative results of the phase II/III trial would provide us with inevitable evidence for the inclusion of statins in the therapeutic regime of CRC patients.

Collectively, we can affirmatively suggest SATB1 and SATB2 as the molecular markers for tumorigenesis in CRC as well as targets for statin therapeutics. These novel targets would not only be able to help better classify the tumor progression in patients but also allow to be monitored for response towards therapy. Currently, the therapeutics followed for CRC do not include a direct effect on Wnt/ β-catenin signaling (20). The effect of statins on this pivotal pathway via its molecular players embarks a new direction in which the therapy regime can be improved. Our murine data well established the anti-neoplastic effect of statins, which could be extrapolated to the human studies, as we observed the same expression profile of the tumorigenic marker SATB1 protein and its homolog SATB2 in patient samples as well. The addition of statins in the treatment regime of the patients showed a regressed tumor with these molecular markers intact. Therefore, we believe that statin treatment in CRC patients will open a new avenue for repurposing drugs, as they seem to target a crucial pathway like Wnt/ β-catenin signaling via its molecular players, one of them being the SATB proteins.

3.4 Materials and methods

Immunoblotting

The protein lysates were prepared in RIPA buffer (20 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% Sodium deoxycholate, protease inhibitor cocktail (PIC, Thermo Fisher Cat No. A32963). The cells were pelleted after 1X PBS washes and resuspended in RIPA buffer+ PIC (Protease Inhibitor cocktail) + phosphatase inhibitor PhosSTOP (Roche #4906845001), kept in ice and allowed to lyse for 1 hr. For spheroids, the RIPA buffer +PIC cocktail mix was directly added to the 24 well plate and freeze-thaw cycle was repeated for at least 5 times, followed by incubation of the lysate on ice for 1 hr. This was done for spheroids because of the matrigel base that needs to be broken down as well. We standardized the freeze-thaw cycles to be the best and effective method of spheroid lysate preparation with efficient matrigel degradation. The lysate was then collected and centrifuged at high speed 14,000 rpm, 4°C for 30 min. The supernatant was collected and used for loading on SDS-PAGE gels after protein quantification using BCA assay kit (Pierce #23250). The 10% SDS-PAGE gels were made fresh and run in the SDS running buffer. For probing the protein bands on PVDF membranes, clarity Western ECL substrate (Biorad #1705061) was using and LAS 4000 was used for visualizing the HRP based chemiluminescence chemistry.

Cell culture

HCT116 and HCT15 CRC cell lines were obtained from the European Collection of Cell Cultures (ECACC) and HT29 cell line was obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). HCT116 was cultured in DMEM media (Gibco, Waltham, MA, USA) with 10% FBS whereas HCT15 and HT29 were cultured in RPMI media (Gibco, Grand Island, NY, USA) with 10% FBS. Spheroid formation was performed according to the protocol, as described (21). Briefly, the 24-well dish was coated with matrigel bed and allowed to solidify for 30 min at 37°C. Single-cell suspension of the cell line was made and 10^4 cells were seeded per well. On-top matrigel was added in media and spheroids were allowed to form for 4 days. The colony formation assay was also performed as described (22). Briefly, 1.2% low melting point agarose was used as the base with media. 10^4 cells were seeded in a 60 mm dish and topped with 0.6% agarose + media. The colonies were allowed to grow for 7 days. Matrigel was obtained from Corning (Cat. No. 356231).

Reagents and antibodies

Simvastatin and Rosuvastatin used in all assays were obtained from Sigma (Simvastatin Cat No. #79902-63-9, Rosuvastatin Cat. No. #SML-1264). Simvastatin (Sigma Cat No. #79902-63-9) was used for all assays at a final concentration of 30 μM in activated form. Briefly, for activation of statin, it was dissolved in NaOH and 100% ethanol and heated at 50 °C for 2 hrs. The pH was adjusted to 7.4 with HCl and volume made up to 1 ml with Milli Q water. The final drug was filtered and stored in aliquots at -20°C. The activation of simvastatin is important to break the lactone ring that is the competitive inhibitor substrate for its target enzyme HMG CoA reductase. The antibodies used were as follows, β-catenin (BD Bioscience Cat No. 610153), SATB1_L745 (Cell Signaling Tech, Cat No. 3650), SATB2 (Abcam, Cat No. Ab92447), GAPDH (Abcam, Cat No. G041), FLAG (Sigma #F3165), β-Actin (Sigma #A2228).

Annexin V FITC & PI staining kit for flow cytometry (Abcam #AB14085 Annexin V-FITC apoptosis staining/detection kit) was used for validating the apoptosis in the spheroids on statin treatment. The protocol provided in the kit was followed. Briefly, the spheroids after statin treatment of 24 hr, were harvested and stained for annexin V to identify apoptotic cells as well as PI (propidium iodide) to identify dead cells. The staining was accomplished by adding the 1X annexin-binding buffer to the cells. The stained cells, (along with unstained cells as control) were washed with 1X PBS, subjected to flow cytometry and the number of events were recorded. We did not see any alteration in the annexin V staining recorded for the untreated and statin treated cells. Therefore, suggesting that there was no extra apoptosis on statin treatment.

Quantitative RT-PCR

Total RNA was extracted from cells grown under 2D and 3D culture conditions and vehicle control and statin treated sets using Trizol reagent (Taraka RNAiso Plus, Code: 9109). Extracted RNA was subjected for PCR based gene expression analysis. For qPCR-based gene expression analysis, cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems # 4368814). The cDNA was utilized for relative gene expression analysis using gene specific primers, (Chapter 1, Table 1) with 18srRNA as endogenous control.

In vivo study of tumor in mice

HCT116 cells and SW480-FLAG overexpression SATB1 cells, SW480-FLAG control cells (1 X 106) were injected subcutaneously in 8 NOD-SCID mice (male or female respectively, 6-8 weeks old). They were equally divided into two sets, where one set was designated vehicle control and

the other set was given statin treatment. Statin was dissolved in 0.5% methyl-cellulose and administered orally on a daily basis for 45 days, post-7 days of injection of cells. The dose provided was 40 mg/kg/day and no side effects were observed, except a slight reduction in body weight. The mice were sacrificed after 45 days of the assay and the tumor was isolated and weighed. All the murine experiments were approved by the Institutional Animal Ethics Committee.

Clinical trial (IECHR/VB/2018/014)

The phase II/III clinical trial was conducted at Tata Memorial Hospital (TMH), Parel, Mumbai, India under ethical approval by Institutional Ethics Committee for Human Research, after a written informed consent from the patients. The paired samples of the rectal tumor and adjacent normal tissues were obtained from the rectal cancer patients participating in the study. The major aim of the study is to validate whether statins show a better pathological complete response (pCR) in combination with Neo-adjuvant chemo-radiotherapy (NACTRT). The secondary aims include the overall comparison and survival of the patients over a period of 5 years, in the two arms of the study, one with statin added to NACTRT (arm A) and the other with only NACTRT (arm B). Rosuvastatin (20 mg/day) was provided to the patients in arm A, with NACTRT for 6 weeks, followed by only Rosuvastatin (20 mg/day) for another 6-10 weeks, until the surgery. The patients were followed up for approximately 1 year for relapses and survival.

The inclusion criteria for the randomized trial for receiving rosuvastatin were carefully structured. The patients should be between the age group of 18 to 70, and should be willing to consent to the study. They should be confirmed for adenocarcinoma of the rectum and should be fit for receiving neoadjuvant chemotherapy. The disease should not be metastatic and there should be no history of Crohn’s disease or ulcerative colitis. Patients should not be on any medication reactive to statin or prior aspirin consumption. Female patients should not be nursing or pregnant. All the patients were recorded for any comorbidities.

We have recorded 60 patients so far, abiding by all the criteria. The percentage of patients showing better prognosis in the follow-up routines were manually calculated for these 60 patients. 30 patients belonged to arm A with statin in their regime and 30 in arm B with only the NACTRT. We could also conduct immunohistological study in 10 patients from arm A, with paired biopsy samples from pre-statin treated condition and post-statin treated condition.

The tissue sample collection was procured with standard endoscopic and surgical procedures. Immunohistochemistry was performed for the tissue biopsies embedded in paraffin blocks and probed for SATB1 (C-6 SATB1 Santa Cruz #sc-376096) and SATB2 (Abcam #ab92446 anti-

SATB2) antibodies. H score was calculated with intensity levels, weak=1, moderate=2, strong=3 and % of cells positive for signal, 0-33%=1, 34-66%=2 and 64-100%=3. The score for intensity was multiplied with score for percentage of cells to give the final H score. Tissue samples for protein expression assays were collected and snap-frozen for shipment in dry-ice. The tissues were immediately homogenized in RIPA buffer and lysates were run for 9 pair of normal adjacent and tumor samples.

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Chapter 4

To elucidate the effect of Statins on the stability of β-catenin in colorectal cancer

4.1 Introduction

The data presented in the previous chapters (Chapters 2 and 3) alluded to specificity in the mode of action of statins in colorectal cancer (CRC), both *in cellulo* and *in vivo*. Initial results of the human CRC clinical trial further strengthened our hypothesis towards establishing statins as an anti-neoplastic drug, being repurposed for CRC therapy. We also proposed a mechanistic regulation by statins specific to the pivotal Wnt/ β-catenin signaling pathway. In the first two aims we discussed the effect of statins on the tumorigenic properties of CRC and narrowed down to the two major molecular players of the canonical Wnt pathway, SATB1 and β-catenin. In the second aim we further elaborated the effect of statins on SATB1 and SATB2 due to their dynamic expression profile in CRC, also underscoring the importance of SATB proteins as tumor markers. Here, as part of the third aim, (Chapter 1, section 1.5) we delved further into the mechanism by which statins affect β-catenin and augment the specificity of mode of action of statins.

It has been reported and widely accepted that β-catenin along with APC mutation is present in 75% cases of colorectal cancer (1-3), making this player a critical oncogenic factor when mutated to become constitutively active. In the normal conditions, Wnt/ β-catenin signaling is required for maintaining the stemness of the colonic stem cells at the base of the crypt (4-6). However, oncogenic mutation in β-catenin is one of the factors responsible for aberrant upregulation of the canonical Wnt pathway in upper epithelial cells, initiating the adenomatous polyp formation (7-9). Mechanistically, when β-catenin is not able to get degraded and there is excess of this protein present in the cytoplasm, it enters the nucleus and binds to transcription factors like TCF7L2 (10) and SATB1 (11), further upregulating the expression of Wnt target genes. The β-catenin protein can escape degradation by either loss of phosphorylation by GSK3β which indicates β-TrCP ubiquitin ligase mediated ubiquitination and proteasomal degradation (12-16), by saturation of the degradation complex (17-19) or by stabilization of the protein (20, 21). However, some reports suggest mechanisms by which β-catenin protein has been shown to be degraded independent of the GSK3β mediated phosphorylation (22). There are ubiquitin ligases which do not require an S33 or S37 phosphorylation to recognize β-catenin as target for degradation (23-25).

Furthermore, there have been reports suggesting a lysosomal accumulation and ultimately degradation of β-catenin instead of a proteasomal degradation pathway (17, 26-28). These findings have suggested the existence of a very tight regulation of β-catenin which is context-dependent and highly complex.

One of the primary mechanisms by which the stringent regulation of β-catenin involves post-translational modifications (PTMs) occurring on various residues of the protein. β-catenin undergoes multiple PTMs on lysine, serine, threonine, and tyrosine residues, which dictate the protein's fate under different signaling contexts. Among these, lysine residues exhibit particularly dynamic behavior, with various modifications on K19, K48, and K133 influencing degradation, methylation, or acetylation of β-catenin depending on the desired outcome. For instance, under Wnt activation, PCAF competes with ubiquitin ligase βTrCP for K19 and K49 residues to acetylate and stabilize β-catenin, preparing it for nuclear translocation (29). Recent studies have shed light on the effects of lysine methylation on β-catenin, with one suggesting protein stabilization via K133 methylation mediated by SMYD2 (30), while another demonstrated β-catenin degradation through K180 methylation by SET7/9 (30). Interestingly, novel modifications have emerged that either stabilize or degrade β-catenin; for example, a recent study revealed a Neddylation modification crucial for degradation of β-catenin (31). Neddylation is a ubiquitin-like modification and was proposed to play a vital role in β-catenin turnover under canonical Wnt signaling (32).

In our study, the effect of statins on Wnt/ β-catenin signaling, via such a crucial upstream player, advocated for a specific mode of action. Interestingly, we were able to report a probable mechanism that might function in a dual manner, first, by affecting the degradation and second, by affecting the stability of the protein. In the process of inferring the results obtained, we were also able to uncover a novel modification on β-catenin. Our first observations led us to explore the possibility of degradation of the β-catenin protein, as the transcript levels were unaltered on statin treatment. The degradation of β-catenin was confirmed by the ubiquitin ladder accumulation upon statin treatment after inhibiting the two degradation pathways, proteasomal and lysosomal. We further observed a shift towards a lysosomal degradation rather than a proteasomal degradation, as our immunofluorescence analysis showed increased LAMP1 accumulation on statin treatment. Interestingly, we also observed a phosphorylation independent degradation of β-catenin mediated by statin. In our subsequent results, we inferred that GSK3β mediated phosphorylation might not be essential for priming β-catenin to degradation as observed in the canonical conditions.

The incongruity of the statin mediated degradation of β-catenin further led us to explore the interacting partners of β-catenin upon statin treatment by conducting a pull down followed by a mass spectrometry analysis. Apart from the known interactors, we were intrigued to observe multiple novel partners as well. One of them particularly caught our attention because of its direct link to statin mode of action. We observed farnesyl pyrophosphate as an interacting partner in the β-catenin pull down MS-MS. Statins are known to inhibit mevalonate pathway and its downstream cascade, including the addition of isoprenylation modification to target proteins. The statin induced removal of this modification is known to cause destabilization of the protein and subsequent loss of function. Therefore, by using *in silico* analysis, we validated the presence of a farnesylation motif 'CAAX' on β-catenin amino acid sequence, with a significant prediction of the mark on the Cysteine 419. We further tried to explore the physiological significance of the Cys 419 residue and its role in statin mediated effect on β-catenin.

Our biochemical assays revealed that C419 was important for nuclear localization of β-catenin and might disrupt the stability of the protein. We validated the importance of C419 by mutating the residue and tagging the protein with RFP. We observed a loss of farnesylation modification on the mutated residue further strengthening our inference regarding the novel modification on β-catenin. However, the C419A modified β-catenin protein did not undergo spontaneous degradation, like in the case of statin treatment. Therefore, we further investigated other stabilization factors of β-catenin that statin might affect. It is known that PCAF acetylates β-catenin and stabilizes the protein (20, 29, 33). Therefore, we observed whether PCAF was affected on statin treatment. Intriguingly, PCAF levels were significantly reduced and there was loss of interaction of β-catenin and PCAF upon statin treatment. Collectively, our results suggested that statin treatment not only seemed to affect the farnesylation modification, but also reduce PCAF, the other stabilization mechanism of β-catenin. This dual mode of action on β-catenin protein elucidates this Wnt player as a direct target of statins and puts forward a compelling case for the usage of statins in anti-neoplastic therapeutics.

4.2 Results

4.2(i) Statin treatment causes degradation of β-catenin independent of GSK3β phosphorylation

We validated the effect of statin on the major canonical Wnt pathway player, β-catenin in multiple CRC lines, namely DLD1 and highly aggressive COLO205. The downregulation of β-catenin at the protein level was reproducible in both the cell lines, as observed earlier (Figure 1A). However,

we wanted to further investigate whether this downregulation was because of degradation of the protein, as we observed a smear and a product of degradation at lower molecular weight. We took two approaches to deduce the same. First, we validated the presence of a ubiquitin ladder which would be a result of the β-catenin protein degradation, and second, we observed the status of GSK3β mediated phosphorylation that primes the β-catenin protein to ubiquitination. We performed an immunoprecipitation of β-catenin and probed the immunoblot for ubiquitin ladder above the molecular weight of β-catenin, after inhibiting the two degradation pathways, proteasomal as well as lysosomal (Figure 1B). MG132 was used for inhibiting the proteasomal degradation pathway and Chloroquine was used to inhibit the lysosomal degradation pathway. Indeed, we observed a ubiquitin ladder over β-catenin in the MG132 and Chloroquine treated set as expected. However, we observed a higher enriched ubiquitin ladder (red box) on statin treatment, along with the MG132 and Chloroquine treatment, which suggested that β-catenin might be getting primed for degradation on statin treatment (Figure 1B). The effect was specific to statin, as in the absence of inhibition of the degradation pathways, the ubiquitin ladder was absent in the only statin treated set, suggesting that the protein got degraded.

To further validate the statin mediated degradation of β-catenin we wanted to observe whether it was dependent on the canonical degradation complex of Wnt pathway. It is known that GSKβ phosphorylation primes the proteasomal degradation of β-catenin by phosphorylating it on Serine 33, Serine 37 and Threonine 41, out of which Serine 33 and Serine 37 are recognized by βTrCP ubiquitin ligase (34) . However, there have been reports suggesting the phosphorylation independent ways by which β-catenin can be degraded (24). To delineate the dependence of β-catenin on the phosphorylation event, we first looked at the protein levels of Phosphorylated β-catenin, total and Phosphorylated GSK3β (Serine 9) on statin treatment, (Figure 1C). The serine 9 specific phosphorylation of GSK3β is the inactive form which is unable to perform its kinase activity (35, 36). Therefore, we observed the Serine 9 specific inactive GSK3β levels on statin treatment to check for accumulation of the phosphorylation mark on β-catenin and further determine the degradation priming. We observed a reduction in phospho- β-catenin levels and a subsequent increase in the inactive form of S9 specific phospho-GSK3β, whereas, the total GSK3β is slightly reduced (Figure 1C). The ratio of the Phospho-GSK3β and the total GSK3β is increased on statin treatment due to an increase in the inactive form at protein level (Figure 1D). This might suggest the accumulation of the inactive form of phosphorylated GSK3β on statin treatment. However, the degradation of β-catenin, irrespective of the higher levels of the inactive form of GSK3β, interestingly pointed us towards an evidence of GSK3β-mediated phosphorylation independent mechanism on statin treatment.

To further investigate the significance of degradation without phosphorylation, we knocked down GSK3β in CRC line HCT15 and treated with statin (Figure 1F, Appendix Section 3). We observed that irrespective of the GSK3β reduction by siRNA, β-catenin was degraded on statin treatment. We also saw the levels of phosphorylated β-catenin to be reduced on statin treatment and a slight upregulation of the S9 phosphorylated GSK3β, suggesting that statin mediated degradation of β-catenin might not be affected by GSK3β.

We further wanted to observe whether the effect of statin on the β-catenin levels as well as the phosphorylation status could be rescued with either lysosomal degradation inhibition using chloroquine or proteasomal degradation inhibition by MG132 (Figure 1G, H). We recorded a rescue in the protein levels of β-catenin, Phospho- β-catenin and total GSK3β on chloroquine plus statin treatment whereas, no rescue in protein level was observed in MG132 plus statin treatment, suggesting that β-catenin degradation observed might be specific to lysosomal degradation.

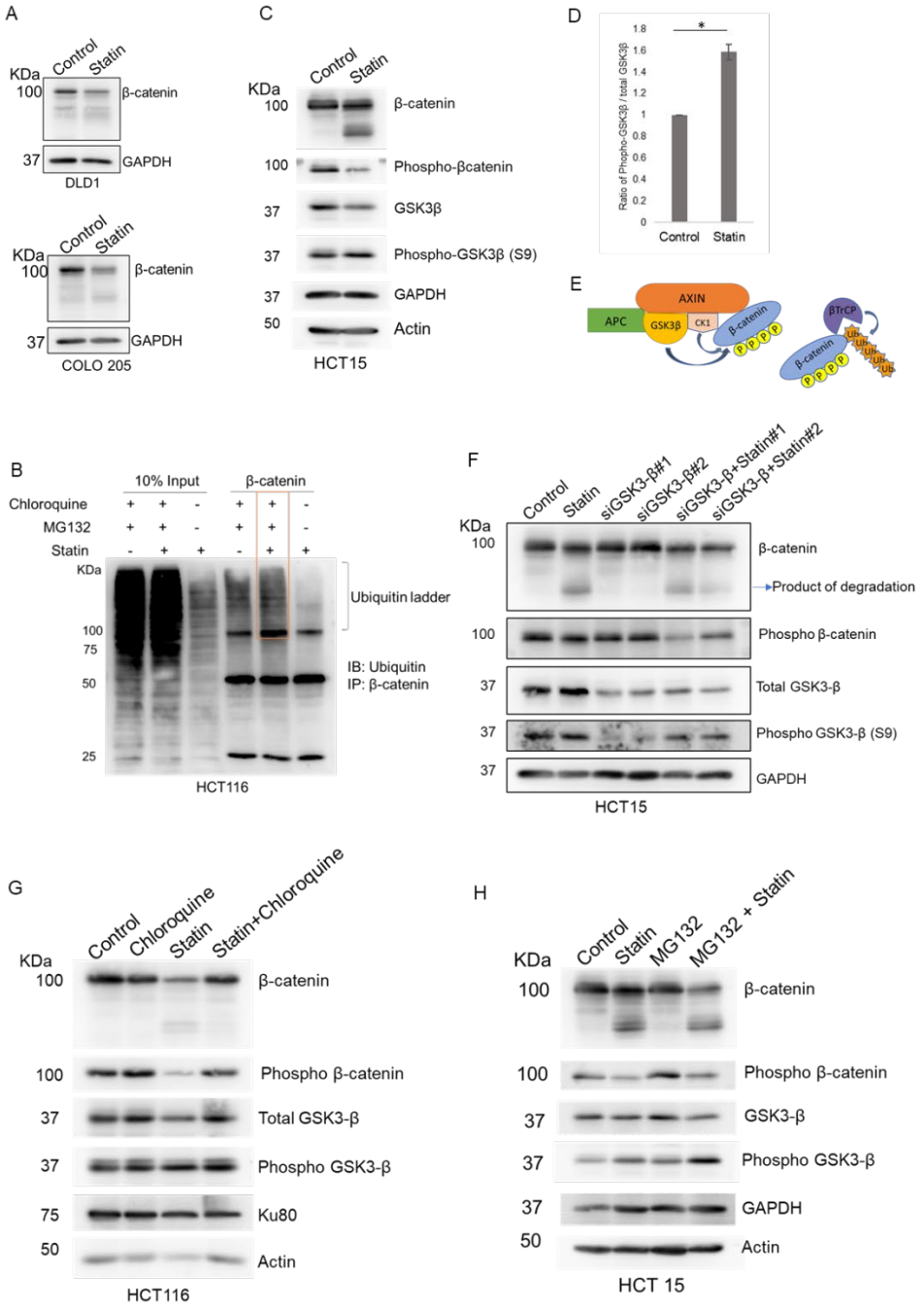

Figure 1: Statin mediated degradation of β-catenin seems to be independent of phosphorylation by GSK3β (A) Immunoblots to observe the degradation of β-catenin on statin treatment in DLD1 (upper immunoblot) and COLO205 cell lines (lower immunoblot) respectively. (B) Ubiquitin ladder accumulation on β-catenin in Chloroquine, MG132 and Statin treatments in immunoblot probed for ubiquitination on immunoprecipitation of β-catenin. The red box highlights the Ub ladder in the combination treatment of chloroquine, MG132 and statin (C) Statin mediated degradation of β-catenin seems to be independent of phosphorylation by GSK3β. Immunoblot to observe, the phospho- β-catenin, total GSK3β and the inactive form of phospho-GSK3β protein levels on statin treatment (D) Densitometry graph for intensity ratio of Phospho-GSK3β / total

GSK3β. On statin treatment, the phospho-GSK3β /total GSK3β is higher (E) Schematic to show the canonical degradation of β-catenin mediated by CK1 that phosphorylates β-catenin at S45 that further primes GSK3β to phosphorylate the following residues of β-catenin, S33, S37 and Thr41. The phosphorylated sites of S33 and S37 are recognized by βTrCP ubiquitin ligase to degrade the protein via proteasomal degradation. (F) Immunoblot to observe the effect of siRNA mediated downregulation of GSK3β along with statin treatment, on the inactive GSK3β, β-catenin and phospho- β-catenin. (G) Immunoblots for the effect on β-catenin, Phospho- β-catenin, GSK3 β and Phospho-GSK3 β in Chloroquine treatment and (H) MG132 respectively. The protein levels were observed to determine whether rescue of degradation takes place on chloroquine or MG132 treatment along with statin. We observed a rescue in the protein levels on chloroquine treatment and not MG132. Biological replicate n=3. *$p<0.05$ according to Student's t-test analysis.

4.2(ii) Statin mediated β-catenin degradation might take place via lysosomal pathway

After observing a rescue in the levels of β-catenin on lysosomal degradation inhibition we validated the localization of β-catenin on chloroquine treatment along with statin. We probed for the lysosomal marker LAMP1 along with β-catenin (Figure 2). The intensity for LAMP1 significantly increased in chloroquine plus statin treatment set (lower panel) as compared to the only chloroquine panel (upper panel).

We further quantified the intensity of the β-catenin as well as the LAMP1 signal in the four conditions of untreated, only chloroquine, only statin and chloroquine plus statin sets (Figure 3A, B). As seen previously, the β-catenin levels corroborated with the immunoblots, as we observed a significant reduction on statin treatment and a rescue in the levels on chloroquine plus statin condition. LAMP1 intensity is observed to be significantly upregulated in chloroquine plus statin treated set as compared to only chloroquine treatment. This trend was observed in three independent biological replicates, therefore, was recorded as a significant result (Figure 3C). Therefore, the above immunofluorescence assay suggested that statin might cause β-catenin degradation via lysosomal pathway as indicated by the LAMP1 intensity, which was highest on statin plus chloroquine treatment.

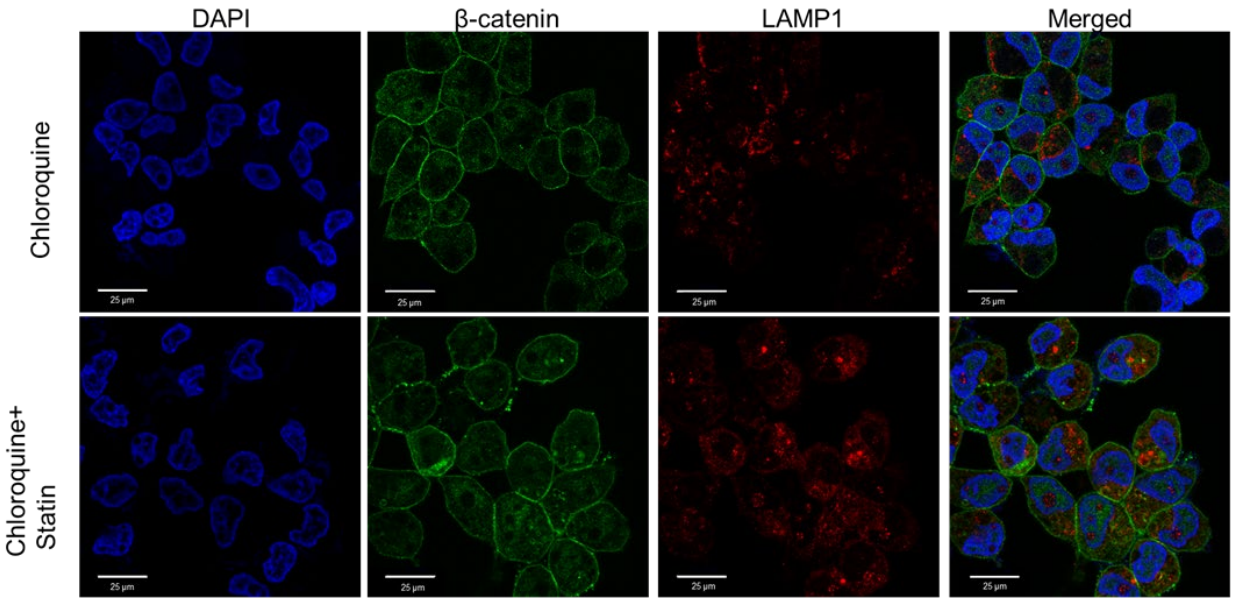

Figure 2: Lysosomal degradation of β-catenin on statin treatment as indicated by increased intensity of LAMP1 lysosomal marker. Immunofluorescence assay for determining the localization of β-catenin and LAMP1 on chloroquine (upper panel) and chloroquine plus statin (lower panel) conditions, indicating a probable preference to lysosomal degradation of β-catenin. The scale bar is 25 µm. The intensity as well as number of LAMP1 foci seem to increase in chloroquine plus statin treated cells.

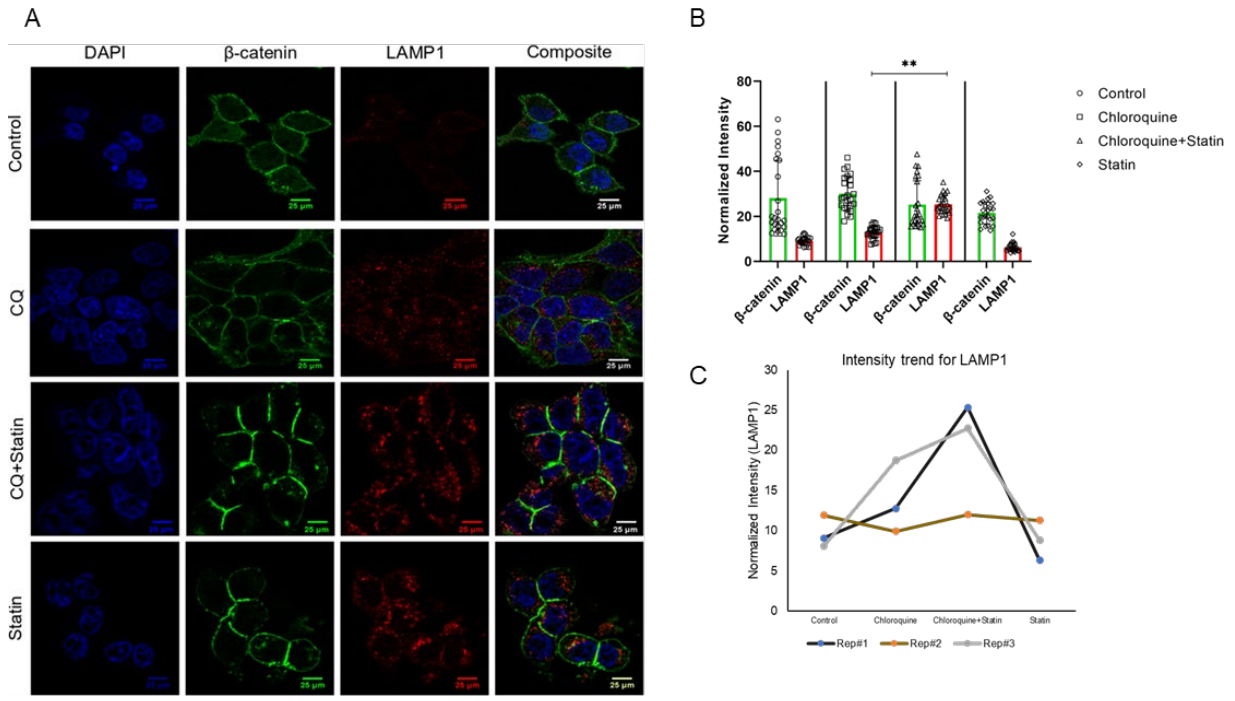

Figure 3: Quantification of the LAMP1 lysosomal marker intensity in HCT15 cells treated with statin and chloroquine, singularly and in combination, respectively. (A)

Immunofluorescence assay (IFA) for localization of β-catenin and LAMP1 indicating lysosomal degradation in untreated control (upper most panel), only chloroquine (second to upper most panel), chloroquine plus statin (second last panel), and only statin treated (lower most panel). LAMP1 intensity can be observed to be increased in chloroquine plus statin condition. Scale bar 25 μm. (B) Intensity graph for β-catenin and LAMP1. There was a significant increase in LAMP1 intensity on chloroquine plus statin set as quantified by the intensity measurement using ImageJ software. (C)Intensity trend for three biological replicates, showed the similar trend of LAMP1 and β-catenin intensities. Biological replicates n=3, **p<0.005 significance value as per the Student's t-test analysis.

4.2(iii) MS-MS analysis of proteins immunoprecipitated with β-catenin reveal multiple novel interactors and a possible prenylation mark

After some intriguing inferences observed regarding a phosphorylation independent and lysosomal degradation specific mechanism mediated by statin on the β-catenin protein, we decided to further explore the interacting partners of β-catenin on statin and chloroquine plus statin conditions. We performed the immunoprecipitation of β-catenin, followed by mass spectrometry of the pulled-down interactors in three different conditions in CRC line HCT116 (Figure 4A). An interactome was constructed for each condition, namely, only chloroquine treated, only statin treated and chloroquine plus statin treated. The huge repertoire of data retrieved from each condition was separately complied and a list of interacting proteins was prepared. We checked for overlapping and exclusive proteins in combinations of the three conditions. However, we focused on the intersecting number of proteins in conditions of chloroquine plus statin and only statin (Figure 4B).

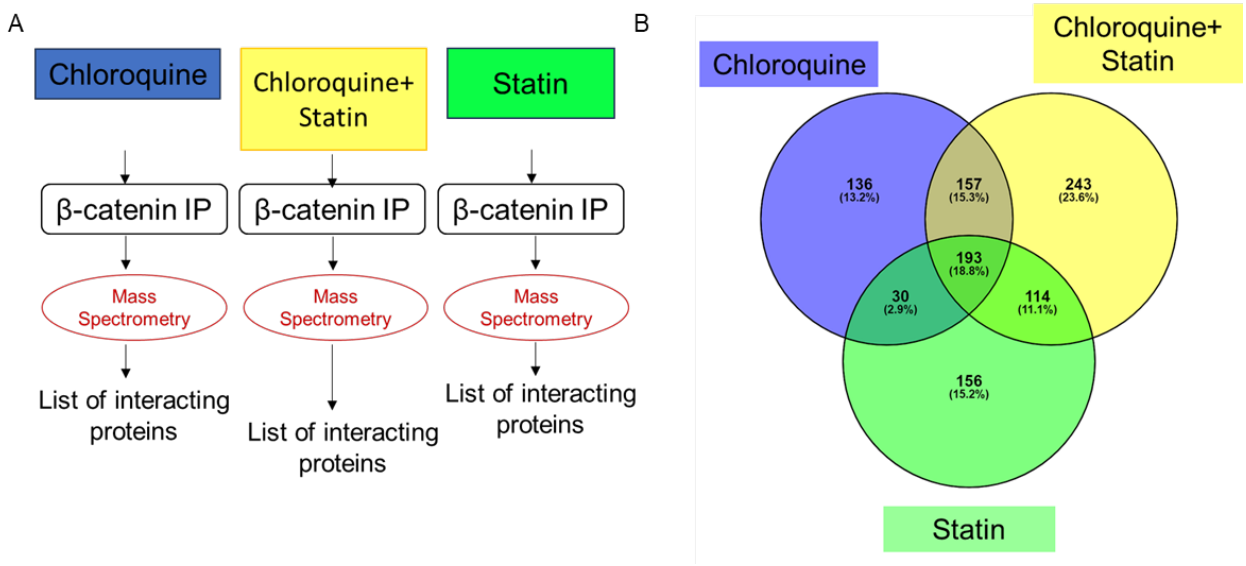

Figure 4: The analysis for elucidating the interactome of β-catenin on statin treatment (A) Schematic for experimental set up in HCT116 cell lines. The three conditions, only Chloroquine, chloroquine plus statin and only statin were used to treat the cells respectively. Immunoprecipitation (IP) of β-catenin was performed for each condition, followed by mass spectrometry of individual samples, which generated a list of interacting proteins in these three conditions. These lists of candidate interacting proteins was used to generate a Venn diagram (B) which provided an overview of the overlapping and mutually exclusive proteins in each condition. We focused on the chloroquine plus statin and only statin lists of interacting proteins for further analysis.

The MS-MS analysis revealed multiple novel interacting proteins along with the known interactors of β-catenin. Among many fascinating interaction factors, we recorded an interesting E3 ubiquitin ligase TRIM21 as one of the β-catenin interacting proteins in chloroquine and statin treatment set specifically. TRIM21 is a lesser-known interactor of β-catenin (25), however, there is one report which suggests the TRIM21 lead to degradation of β-catenin independent of phosphorylation (37). We also observed LAMP1 as one of the interactors concurring with our immunofluorescence assay for co-localization.

However, we noticed a very interesting protein in the list of overlapping proteins for two conditions, chloroquine plus statin and only statin condition, Farnesyl pyrophosphate synthase. Farnesyl pyrophosphate is known to be the precursor for farnesylation modification on proteins that is mediated by the enzyme farnesyl pyrophosphate synthase (38, 39). The mevalonate pathway has three major products, cholesterol, isoprenylation modification on target proteins, and peptide synthesis. The post-translational modification (PTM) of isoprenyl on target residue is essential for

cell signaling (40-43). The well-known targets of this PTM are Ras, Rho small GTPase proteins that are involved in multiple cascades to relay information from the cell membranes to the interior of the cell. The PTM requires a CAAX motif on target protein, Cysteine residue being the one harboring the isoprenyl modification. The “X” stands for any amino acid that determines the prenyl transferase activity, whereas, two aliphatic amino acid residues “AA” are required after the cysteine residue for the PTM. Farnesyl transferase helps in transferring the farnesyl group from farnesyl pyrophosphate into the target protein Cys residue. Therefore, the presence of farnesyl pyrophosphate synthase as one of the interactors of β-catenin was very surprising, as it is not known to have a farnesyl PTM.

To further validate the interaction, we performed an in-silico motif search on the amino acid sequence of β-catenin. We wanted to check if the motif CAAX, required for farnesylation of peptide residue is present on β-catenin, where C is Cysteine that is isoprenylated, followed by two alanine and X as any amino acid. We used the online portal MemeSuite for the analysis and indeed observed one significant prediction at Cysteine position 419 residue, which could be a probable site for farnesylation post translational modification (Figure 5A).

The significance of farnesylation on target peptides is known in stability of the protein. It has been reported that farnesylation and gerynylation are the two forms of isoprenylation that help in providing stability to the target protein (44). Statin, inhibiting the mevalonate pathway, has been observed to inhibit the downstream processes as well. A few reports have suggested that statins remove prenylation or reduce the PTM levels in proteins as a result of inhibition on its upstream mevalonate pathway (45-47). This results in destabilization of the target proteins further resulting into their degradation. Therefore, we wanted to observe if statin mediated β-catenin degradation is a result of destabilization of protein via reduction in farnesylation levels on its C419 residue. This was significant, as we do not see a change in the transcript level of β-catenin on statin treatment, but we do report reduction at the protein level via degradation.

Therefore, to confirm the presence of a farnesyl PTM on C419 of β-catenin, we re-analyzed our IP β-catenin MS-MS data repertoire further (Figure 5B). We searched for the shift in the peptide mass/charge (m/z) ratio for trypsin digested β-catenin fragments containing the C419 residue. We tried to match the mass/charge ratio of the peptide, with and without the addition of farnesyl motif on C419, in all three conditions respectively. First observation was to check for addition of the farnesyl mass to the peptide fragment mass and corresponding charge alteration in chloroquine or chloroquine plus statin conditions (Figure 5C). The second observation was to check for the

absence of farnesyl PTM mass on the peptide in only statin condition, which would validate the removal of the farnesylation (Figure 5C).

The trypsin digested β-catenin peptide containing C419 residue was 38 amino acid long, which would have been very difficult to capture in the Triple TOF MS-MS system (Figure 5B). Addition of an extra mass of farnesyl group to the peptide might have added extra mass to the peptide to make it further difficult to get ionized and captured in the MS-MS. Therefore, with regard to the first observation, we were unable to find a mass/charge value corresponding to MS-MS peak of a peptide with farnesyl mass added, in chloroquine and chloroquine plus statin sets (Figure 5C, D).

However, when we searched for an unmodified peptide mass/charge in only statin condition, without the added farnesyl group, we were able to map the peak with the exact m/z for this peptide, i.e., the 38 amino acid long peptide harboring the Cys 419 but without a farnesyl motif in only statin condition (Figure 5C, D). The significance of this peptide MS-MS peak was, that on statin treatment, the absence of farnesylation, as reported earlier with other protein targets, might hold true for β-catenin as well. The presence of an unmodified peptide in statin set might suggest absence of isoprenylation. However, to capture the modified peptide with farnesyl PTM in chloroquine conditions might require alterations in the MS-MS method for better read-out of the mass/charge ratio. Therefore, with the above indirect evidence of the “absence” of farnesyl modification on C419 residue of β-catenin, in statin treatment, our next objective was to check for the stability of the β-catenin protein.

So far, we suggested a significant role of Cysteine 419 residue of β-catenin which might get farnesylated in control conditions, but the PTM is probably removed as an effect of statin, further destabilizing the protein β-catenin. However, further work would be required to study this novel PTM and its role in β-catenin regulation.

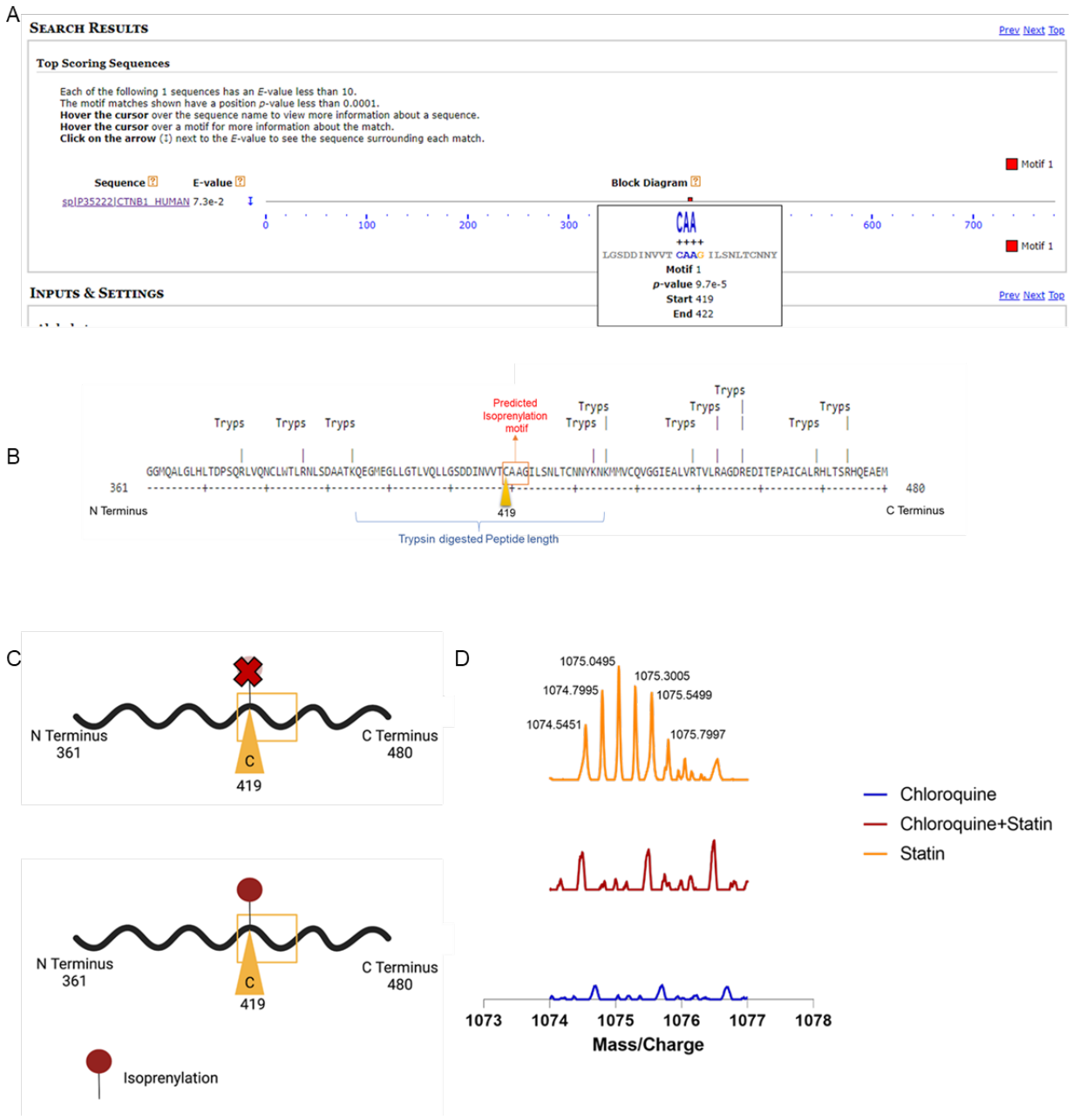

Figure 5: The *in silico* and the peptide mass/charge analysis in MS-MS indicate the presence of a farnesyl PTM on Cysteine residue at position 419 on β-catenin (A) The *insilico* search for Isoprenylation motif 'CAAX' was performed in Meme suite (Motif alignment and search tool, version 5.5.4). The screenshot of the analysis result showed a predicted Isoprenylation motif on β-catenin amino acid sequence, with its significance value. (B) The schematic of trypsin-digested peptides, incorporating the predicted isoprenylation site (utilizing the peptide digestion prediction tool ExPASy, developed by the Swiss Institute of Bioinformatics), validated the presence of the CAAX motif with a likely modified Cys 419 residue. Additionally, it provided insight into the peptide length following trypsin digestion, aiding us in manually determining the mass-to-charge ratio of the peptide, both with and without the modification. (C) Schematic depicting the unmodified peptide mass shift in statin-treated set as compared to the modified peptide present

in the chloroquine and chloroquine plus statin conditions. (D) The MS-MS peak corresponding to the schematic peptide for each condition. We noted a distinct peak with mass-to-charge values indicated above each signature peak, representing the unmodified peptide exclusively in the statin-treated condition. This observation bolsters our hypothesis regarding the presence of this novel isoprenyl modification and its subsequent elimination from the β-catenin peptide upon statin treatment.

4.2(iv) Site directed mutagenesis of Cysteine 419 of β-catenin shows cytoplasmic localization exclusively

To validate the above observation of a farnesylation motif present on β-catenin we performed a pull down with β-catenin and probed for farnesylation mark with a pan-farnesyl antibody. We observed the presence of a farnesyl modification over β-catenin molecular weight in untreated control condition, as well as chloroquine treated conditions. Furthermore, we also observed a significant reduction in farnesylation intensity on statin treatment (Figure 6A). The densitometric analysis of the intensity showed a significant reduction in farnesylation on statin treatment as well (Figure 6B).

We had initially discussed about the stability of the protein being compromised on removal of isoprenylation. Therefore, we wanted to elucidate whether the stability of the β-catenin protein is also affected on statin mediated removal of farnesylation. Furthermore, we wanted to delineate the significance of the C419 residue in the function of β-catenin regulation. Therefore, we performed a site directed mutagenesis for the C419 into alanine and tagged the modified β-catenin protein with RFP. We transfected the construct transiently in HCT116 cell line and interestingly observed a loss in nuclear localization upon performing an immunofluorescence assay (Figure 6C). The C419A modified β-catenin was observed to be exclusively localized in cytoplasm. The nuclear localization of the C419A modified β-catenin was significantly reduced by less than 50% when quantified exclusively for the RFP tagged protein (Figure 6D, E). The total number of nuclei positive for RFP signal as well as the nuclear intensity for RFP, both were significantly reduced for the C419A modified β-catenin as compared to the wildtype protein.

We further performed an immunofluorescence assay with ChiR treatment in HEK293T cell line (Figure 7). The significance of using the HEK293T cell line was that the endogenous levels of β-catenin as well as Wnt target gene expression is low. Therefore, the endogenous β-catenin protein would not interfere with the transiently transfected RFP tagged protein. The chemical ChiR

is a GSK3 β kinase inhibitor which is used to activate Wnt signaling by causing loss of phosphorylation on β-catenin and thereby result in its nuclear localization. The β-catenin protein, free of the degradation complex, enters the nucleus and targets the downstream Wnt responsive genes for upregulation. The rational for this observation was to check whether the modified β-catenin protein is able to enter the nucleus on Wnt activation in an otherwise low Wnt expressing cell line. Therefore, we transfected HEK293T cells with the RFP tagged wildtype (WT) and C419A modified β-catenin constructs respectively, and gave ChiR treatment for Wnt activation. We observed endogenous levels of β-catenin in all conditions, and as seen in Figure 7A upper two panels, the endogenous protein (green) got translocated to the nucleus upon ChiR mediated Wnt activation. Surprisingly, we observed that the C419A β-catenin did not enter the nucleus even on Wnt activation by ChiR (Figure 7A). We quantified the nuclear RFP signal intensity for validating the localization of the WT and C419A β-catenin upon ChiR treatment (Figure 7B). The intensity graph showed a reduced nuclear localization for the C419A β-catenin protein. Furthermore, no significant change in nuclear localization was observed for the C419A β-catenin with or without ChiR treatment (Figure 7B).

Therefore, the Cysteine 419 residue, which we predicted to harbor a farnesylation PTM, seemed to be essential for nuclear localization of the β-catenin protein. Absence of this mark in the C419A modified protein altered its localization from being nuclear to cytoplasmic, even on activation of Wnt pathway.

To further observe whether there was a farnesylation mark on WT and loss of this modification in C419A β-catenin, we pulled down the RFP tagged β-catenin specifically (Figure 7C). The RFP antibody was used to pull down the WT as well as the C419A β-catenin protein and probed with the pan-farnesylation antibody previously used. We observed the reduction of farnesylation on C419A β-catenin specifically as compared to the WT protein, validating a significant reduction of PTM in the modified β-catenin protein (Figure 7C). When conducting pull-down assays with RFP specific WT β-catenin, a robust farnesylation signal is evident, as depicted in lane 1. However, the intensity of this modification is notably reduced in the pull-down of C419A modified β-catenin, as observed in lane 2. In lane 2, a smear is still apparent, indicating multiple bands. This is attributed to the pan-farnesyl antibody utilized, which recognizes farnesylation marks on various proteins that may have co-purified with the RFP-tagged β-catenin. *In silico* analysis did not identify any other residues displaying a predicted farnesylation mark on β-catenin. Although the RFP antibody yielded multiple bands, our focus was on the band running slightly above 100 kDa,

corresponding to the protein ladder. The input lanes of the lower blot exhibit a ladder-like pattern, attributable to the pan-farnesyl antibody's recognition of all farnesylated proteins in the lysate.

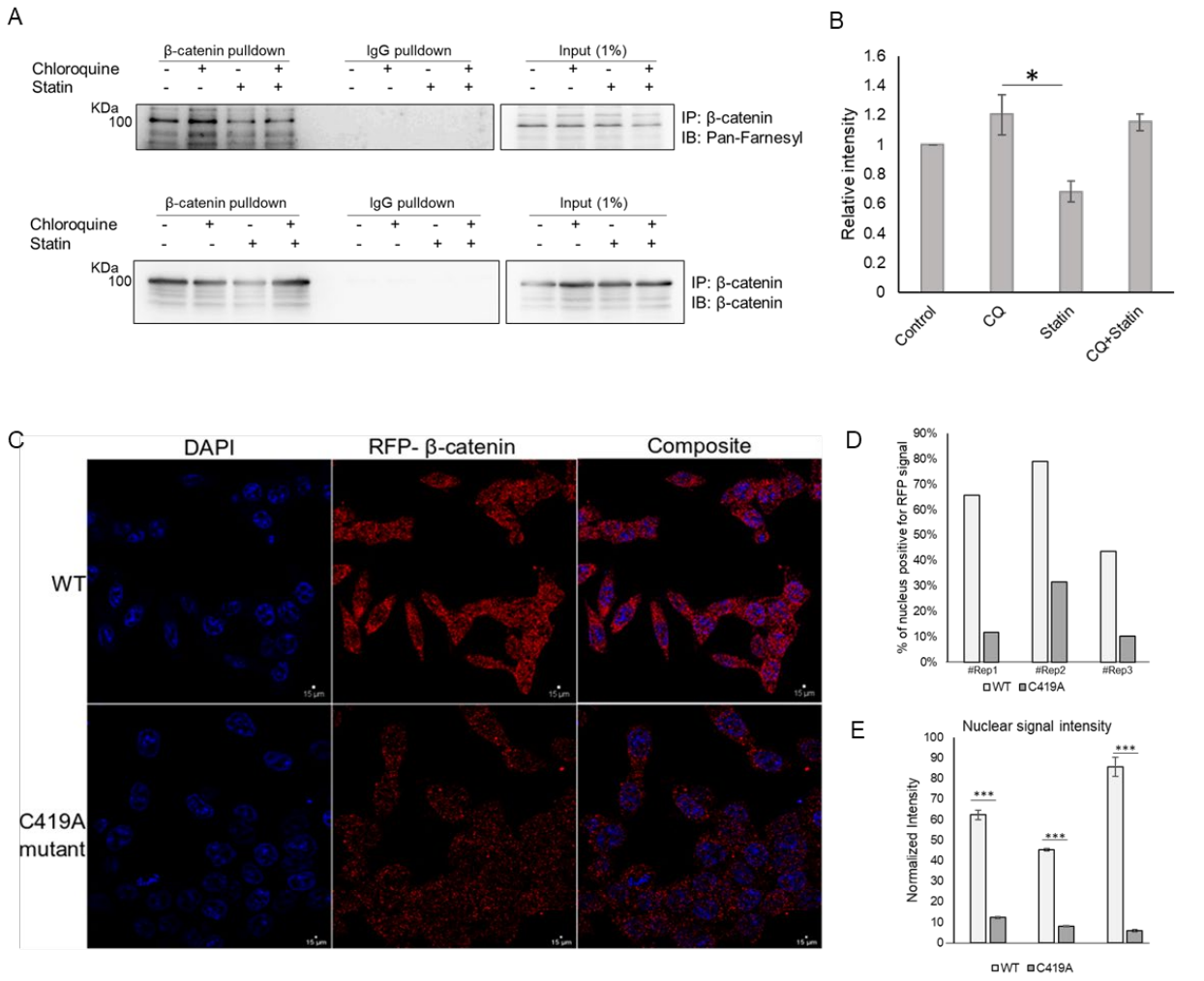

Figure 6: Validation of the novel farnesyl modification on β-catenin protein in chloroquine and chloroquine plus statin conditions, with a loss of farnesylation mark on β-catenin in only statin treated set. (A) Immunoprecipitation (IP) for validation of presence of isoprenylation by using pan-farnesyl antibody for probing, after performing a pull down with β-catenin antibody (upper blot) and corresponding β-catenin pull down, probed with β-catenin antibody as a positive control of IP (lower blot), (B) supported by the densitometry graph to further validate the reduction in intensity of the farnesylation at β-catenin molecular weight. Notable reduction in relative intensity was observed in only statin as compared to chloroquine plus statin lanes, whereas the other conditions did not exhibit a significant alteration. (C) Immunofluorescence assay to observe the RFP localization in HCT116 cells transfected with the WT β-catenin and C419A modified β-catenin (both tagged with RFP). We observed a loss of nuclear localization of the RFP tagged β-catenin protein, in the cells transfected with the C419A β-catenin construct. (Scale bar 15 μm) (D)Intensity graph for RFP signal in the nucleus and (E) the percentage of nuclei positive for the RFP signal showed a significant and drastic reduction in nuclear localization of the C419A β-

catenin as compared to the WT protein. Total no. of cells considered were 300, each replicate 100 cells. Biological replicate n=3, *p<0.05, **p<0.005, ***p<0.0005 significance value as per the Student's t-test analysis.

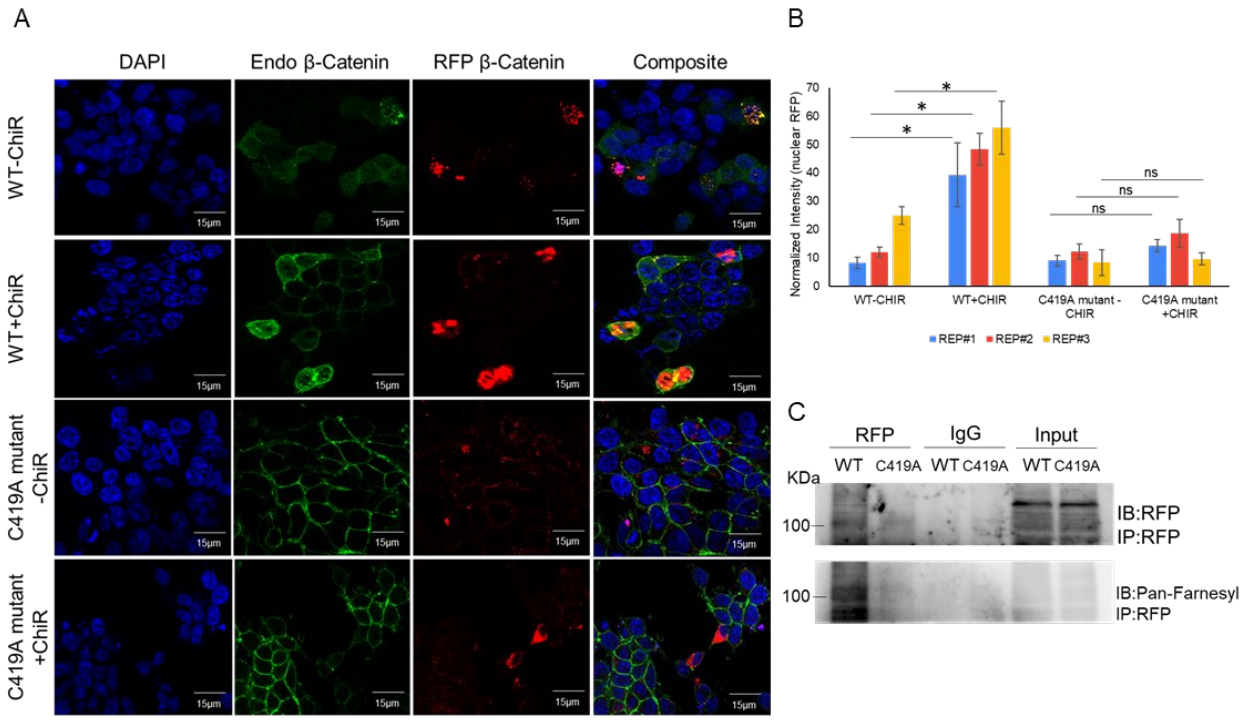

Figure 7: The C419A modified β-catenin does not show nuclear localization even after ChiR treatment in HEK293T cells. (A) The immunofluorescence assay for observing the transiently transfected RFP tagged WT and C419A β-catenin localization in ChiR treated HEK293T cells, along with localization of the endogenous β-catenin protein. The WT minus ChiR showed lower expression of endogenous β-catenin(green), and, RFP tagged WT protein was present in both cytoplasm as well as nucleus. However, on ChiR treatment, both the endogenous and the WT- RFP proteins were localized primarily in the nucleus, indicating activation of Wnt signaling. In the C419A β-catenin transfected HEK293T cells, the localization of both endogenous and the RFP tagged protein was cytoplasmic in the absence of ChiR as expected. In the presence of ChiR, the endogenous protein seemed to be equally present in cytoplasm as well as nucleus, however, the C419A RFP tagged β-catenin protein was exclusively present in the cytoplasm and not the nucleus. (B) The intensity graph for the nuclear RFP signal indicated a significant upregulation for the WT protein but no change in the C419A β-catenin protein, indicating no nuclear localization for the modified β-catenin (C) Immunoprecipitation was performed with RFP antibody to specifically pull down the tagged WT or C419A β-catenin modified protein, and probed with pan-farnesyl antibody to observe the status of farnesylation in the WT and C419A β-catenin. We did observe a significant reduction in the intensity of the PTM in the C419A β-catenin protein as compared to the WT protein. Biological replicate n=3, ns = non-significant, *p<0.05 significance value as per the Student's t-test analysis.

4.2(v) The C419A modified β-catenin stable expression cell lines show a reduced tumorigenesis *in vivo*

Further we wanted to extend our analysis *in vivo* and deduce the significance of the C419A modified β-catenin physiologically. We wished to monitor the physiological relevance of the C419 residue as the modified β-catenin was unable to enter the nucleus in the *in cellulo* model system. To this effect we induced subcutaneous tumor by injecting the NOD-SCID mice with stably transfected WT and C419A β-catenin expressing cell lines, HCT116 and SW480 respectively (Figure 8A, B). We observed a significant reduction in tumor burden in the mice injected with the C419A β-catenin expressing cell lines. The tumor tissues were harvested at the end of 35 days of cells injection and the samples were analyzed for whole tissue lysate proteome as well as transcriptome analysis, with the rationale to reveal the set of proteins and genes getting differentially expressed in the WT vs C419A modified β-catenin expressing tumors.

However, we conducted some initial immunoblot assays from the protein lysates of the tumors obtained from the SW480 cell line stably expressing the WT and C419A β-catenin (Figure 8C). We first observed the levels of RFP to validate the expression of the tagged protein in the tumor tissues. We further checked for the alteration in the protein levels of β-catenin itself as well as proliferation marker PCNA. However, we did not observe any change in the protein levels of either. We analyzed the whole tissue lysate proteome by running the tumor samples in an MS-MS protocol and obtained differentially expressed set of proteins (Figure 8D). We observed a total of 109 proteins exclusively expressed in the C419A modified β-catenin tumor tissue. We performed a Gene ontology analysis of these 109 proteins, which revealed a GO term enrichment of pathways related to trafficking and transportation, COP-I mediated trafficking, Golgi to ER retrograde trafficking, Gap-junction movement and assembly, protein localization and translocation, RHO and RAS GTPase related Reactome database hits (Appendix, Section 2: Table 1).

Furthermore, we performed a transcriptome analysis on the tumor tissues, to validate the expression profile of differentially expressed genes in the C419A modified β-catenin containing tissue as compared to the wildtype. Interestingly, we did not observe much significant alteration in the transcript profile of the C419 β-catenin vs the WT tumors. A few genes were significantly downregulated, namely RAD51 paralog D, ZP1, and CD27. However, intriguingly, the 200 most significantly upregulated genes belonged to transportation of proteins, transmembrane signaling, cytoskeletal regulation by Rho GTPase, extracellular protein with motor activity, CCKR signaling, apoptosis signaling and G protein signaling (Appendix, Section 2: Figure 1A, B). These GO terms

depicting the upregulated transcripts corresponded to the terms of the 109 unique peptides expressed in the C419A β-catenin modified tumor samples (Figure 9A).

Additionally, we also observed the significant upregulation of the Wnt antagonist DKK1 in the C419A modified tumor tissues. We plotted the average normalized counts of DKK1 in both the tumor samples, and indeed observed higher expression in the mutation harboring tumor tissue (Figure 9B). Collectively, the above proteomics and transcriptomics analysis of the tumors isolated showed a slight difference in phenotype and genotype of the WT and the C419A harboring mutant β-catenin, with a correlation between the transcription and translation. The mutation in the β-catenin protein expressed in the SW480 and HCT116 cell lines, caused a reduction in tumor burden, which might be linked to the differentially expressed genes. The upregulation of the protein transportation, translation, GTPase activity, Rho mediated signaling, at both the transcript and protein level indicated a functional alteration mediated by the C419A mutant β-catenin. The *in cellulo* observations of a cytoplasmic localization of the C419A β-catenin also corroborated the importance of the Cysteine 419 residue, that might be carrying a post translational modification.

Our inferences have led us to the hypothesis of a potential lipid PTM of isoprenylation on β-catenin protein, which might be responsible for its stabilization, along with other modifications. The Cys 419 residue might be a significant motif for the addition of the PTM, mutation of which caused the β-catenin protein to lose nuclear localization. Furthermore, the mutant β-catenin harboring cell lines showed a reduction in tumor burden when injected subcutaneously in murine experiments. The tumor samples extracted for the proteomic and transcriptomic profile showed an alteration in the protein trafficking and small GTPase signaling as the effect of the C419A mutation in β-catenin. Also, the expression of Wnt antagonist DKK1 gene was observed to be upregulated in the mutant tumor samples. DKK1 acts as a negative regulator of Wnt by binding to LRP6 receptor, and its expression might be controlled directly by the canonical Wnt pathway as part of a negative feedback (48) or by independent mechanisms (49). However, further exploration is required in this direction to validate the phenotypic effect of the C419A mutant β-catenin, the exact function of this residue and the confirmation of the proposed PTM.

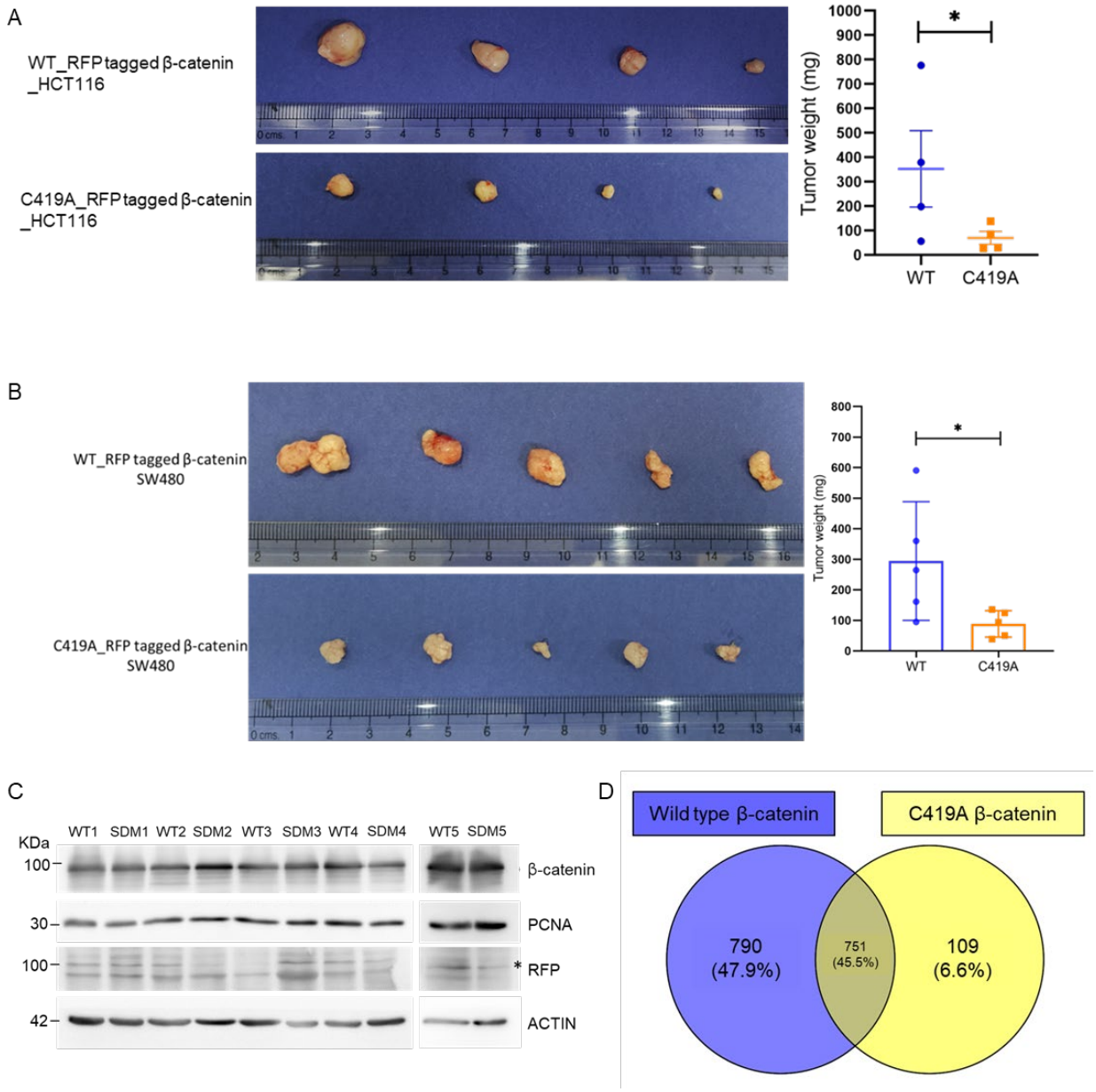

Figure 8: The cell line induced tumors harboring the C419A modified β-catenin show reduced burden in the *in vivo* study, central for deducing the physiological relevance of the C419A residue in modified β-catenin. The cell lines HCT116 (A) and SW480 (B) stably expressing the WT and the C419A β-catenin were injected in NOD-SCID mice respectively. The tumors developed at the end of 30 days of injection and were measured for their weight post-harvesting the tumor tissue. The corresponding graphs showed a significantly reduced tumor burden for the C419A modified β-catenin, in the case of both the cell lines respectively (C) Immunoblots, for the protein level alteration in WT and C419A β-catenin induced tumor tissues were run, to validate the expression of RFP tagged protein in the samples harvested. We also observed no alteration in the protein levels of β-catenin and the proliferation marker PCNA in the tissues. (D) The whole lysate proteome analysis was performed to check for the set of altered or differentially expressed proteins in WT vs C419A modified β-catenin harboring tumors. We

obtained 109 proteins specifically expressed in the C419A tumors. The GO term analysis (Appendix, Section 2, Table 1) for these 109 proteins showed pathways related to trafficking and protein transportation as well as translocation. *p<0.05 significance value as per the Student's t-test analysis.

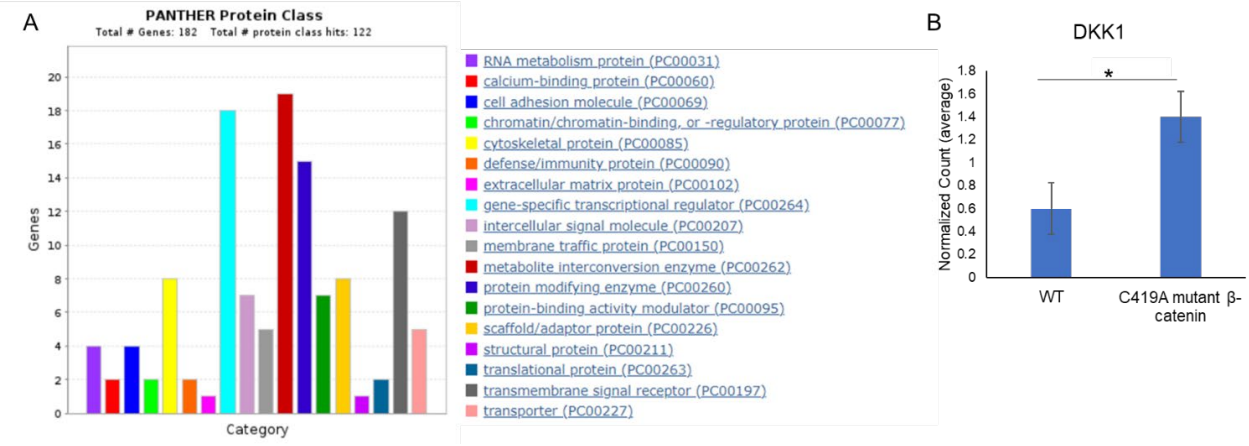

Figure 9: Quant-seq analysis of differentially expressed genes upregulated in the C419A β-catenin tumor samples as compared to the control samples. (A) The differentially expressed genes were subjected to Gene Ontology (GO) term analysis, specifically focusing on the Protein class category. Out of the analyzed genes, 200 were found to be upregulated, with the highest representation of terms related to transportation, cytoskeletal organization, cell adhesion, and membrane trafficking. The analysis was conducted using the online portal PANTHER 17.0, employing the Gene List analysis tool. The resulting bar graph illustrates the terms, with each category color-coded along the x-axis, and the number of genes associated with each term depicted on the y-axis. (B) The normalized counts (average) of DKK1 were plotted separately to highlight the significant upregulation of this Wnt antagonist in the tumors harboring the C419A modified β-catenin. Students' t test for biological replicates of four (n = 4), with *p<0.05.

4.2(vi) Stability of β-catenin is compromised by Statin treatment via loss of PCAF

Further, we wanted to explore the effect on the C419A modified β-catenin in the context of the statin treatment. It is known that isoprenylation lies downstream of the mevalonate pathway that statins inhibit, as an effect of which, the isoprenylation of target proteins is also reduced. Since we were able to simulate an isoprenylation motif and predict a farnesyl modification on the Cys residue 419, we wanted to observe whether statin treatment would cause a reduction in the PTM in both WT and the C419A β-catenin. Also, we wanted to delineate whether the degradation of β-catenin protein observed on statin treatment would be affected because of the modification.

The C419A β-catenin represented the absence of isoprenylation PTM which showed alteration in β-catenin localization and also had a less tumorigenic propensity. Since statin is known to cause reduction of isoprenylation, this loss of PTM alone should have recapitulated the statin mediated reduction of isoprenylation PTM theoretically. However, surprisingly, the C419A β-catenin did not undergo spontaneous degradation like the effect of statin on WT β-catenin. The only phenotypic effect of C419A β-catenin was cytoplasmic localization and not degradation, which might contribute to the first step towards destabilization of the protein.

However, this suggested that the statin mediated degradation of β-catenin might require another factor to lead to the protein to degradation, post destabilization. To validate the above hypothesis, we first treated the WT and C419A β-catenin transfected cells with statin and observed the protein levels (Figure 10A). The endogenous as well as RFP tagged WT and C419A β-catenin was degraded on statin treatment. The protein level of WT and C419A β-catenin were not altered in absence of statin. Therefore, we suggested that, apart from the farnesylation PTM on C419, some other stabilization factors involved in maintaining the stability of β-catenin, might get affected on statin treatment.

It is known that β-catenin undergoes different PTMs, which either cause its degradation or stabilization (Figure 10B). The marks that cause degradation are phosphorylation of S41/45, S33, S37, neddylation at K312 (50), K355 and ubiquitination at Lys19, Lys49 (13). However, the stability of β-catenin protein is majorly maintained by PCAF mediated acetylation (29, 51). Based on the inferences so far, we proposed that isoprenylation PTM was also responsible for the stability of the β-catenin protein. Therefore, statin treatment might affect both, the isoprenylation as well as PCAF mediated acetylation, to destabilize and thereby degrade the β-catenin protein simultaneously.

To validate the above proposition, we observed the protein level alteration of PCAF on statin treatment in CRC lines. The PCAF protein was observed to be significantly reduced on statin treatment (Figure 10C). We further over-expressed PCAF to overcome the low protein amount in HCT116 cell line, and treated the cells with statin. The over-expressed PCAF was also downregulated due to effect of statin in these cells (Figure 10D). We further validated the loss of interaction between β-catenin and PCAF on statin treatment by performing a pulldown of β-catenin and probing the immunoblot with PCAF antibody. The PCAF interaction was accumulated in chloroquine treated condition but was significantly reduced in statin treated set (Figure 10E). This further validated the role of PCAF in the stability of β-catenin protein and the disruption of this interaction upon statin treatment.

Therefore, statin mediated destabilization and subsequent degradation of the β-catenin protein might be regulated via two probable mechanisms, which involves loss of isoprenylation and loss of PCAF. When both the stabilization factors of β-catenin protein are compromised by statin, it leads to degradation via lysosomal pathway, most likely, independent of the phosphorylation mediated by GSK3β (Figure 10F).

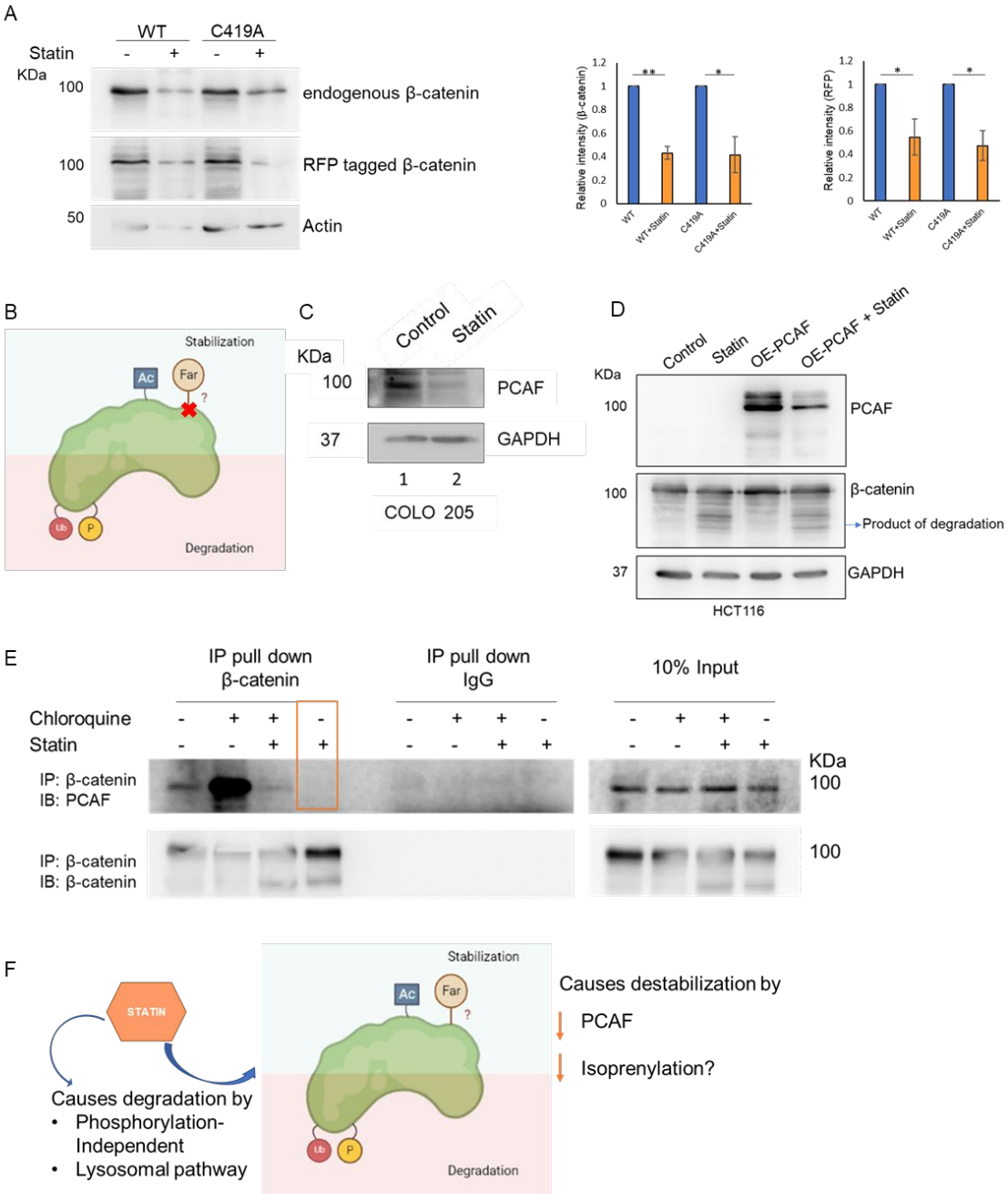

Figure 10: Statin mediated downregulation and loss of interaction of PCAF and β-catenin impacts the β-catenin protein stability. (A) The effect of statin on HCT116 cells expressing endogenous (WT) β-catenin as well as the C419A β-catenin expressing HCT116 cells. We

observed degradation of the modified β-catenin protein as well as the WT protein upon statin treatment, signifying that the statin mediated destabilization of β-catenin might be a result of the involvement of some other factor. The densitometric quantification of the intensity of the immunoblots for both endogenous as well as the RFP tagged β-catenin protein showed a significant reduction upon statin treatment. (B) Schematic to represent different PTMs for stabilization and destabilization of β-catenin. It is known that phosphorylation and subsequent ubiquitination are responsible for β-catenin degradation, whereas, acetylation by PCAF and a probable isoprenylation are responsible for providing the stability to the protein. (C) Immunoblot to monitor the effect of statin on PCAF protein levels. We observed a significant downregulation of PCAF expression. (D) Immunoblot depicting over-expression of PCAF and the effect upon statin treatment. We observed a significant reduction in the protein levels of PCAF upon statin treatment, even after its over-expression. (E) The β-catenin immunoprecipitation was performed in the previous conditions of chloroquine treatment and statin treatment, individually and in combination in HCT15 cells. The interaction of β-catenin and PCAF was recorded in each condition by probing the blot with PCAF antibody. An intact interaction was observed in chloroquine only set, however, there was a significant loss of PCAF and β-catenin interaction in statin treated set (red box). (F) Schematic to summarize the effect of statin on β-catenin in a dual mechanism, first, via loss of isoprenylation as well as PCAF, and second, by degradation via lysosomal pathway independent of phosphorylation by GSK3β. Biological replicate n=3, *$p<0.05$, **$p<0.005$ significance value as per the Student's t-test analysis.

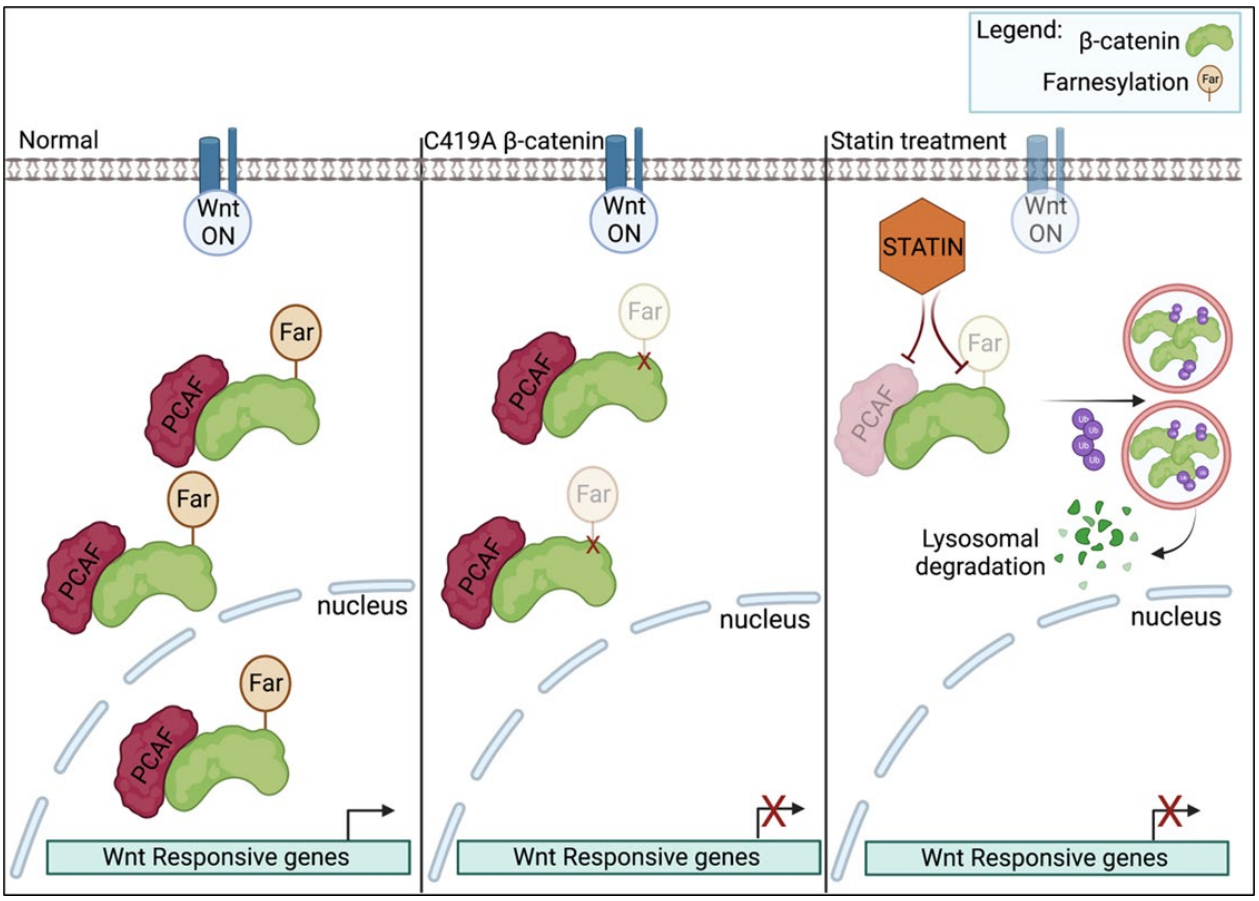

Figure 11: Statin mediated degradation of β-catenin involves the dual mode of action of first, destabilization via PTMs, followed by lysosomal degradation. Graphical abstract to summarize the the mechanistic regulation imposed by statin on β-catenin protein. In the normal Wnt ON conditions (activation), the β-catenin protein escapes the degradation complex and enters the nucleus, probably stabilized by the action of PCAF and isoprenylation. In the second case of the C419A modified β-catenin protein, in Wnt On condition, the protein is not able to enter the nucleus, because of which it might have a reduced tumorigenic effect, as observed in our in vivo analysis. However, in the third case of statin treatment, both the PCAF as well as isoprenylation is affectively reduced, thereby affecting the stability of the β-catenin protein, which might lead to its degradation via a lysosomal pathway.

4.3 Discussion

Our results have augmented our understanding of the intricacies of a regulatory mechanism as tightly wound as that of β-catenin and the effect of a drug like statin on the physiological as well as molecular framework. We have generated high throughput results which have given us a wholistic approach to observe the effects of the mechanism under investigation.

The significance of studying the effect of statin on β-catenin was not only limited to Wnt pathway and its anti-tumor effects. The β-catenin protein has also been extensively studied in correlation to mevalonate pathway (52) and the regulation of cholesterol biosynthesis (53). It has been reported that cholesterol directly affects the canonical Wnt signaling by specifically facilitating the Dvl (Dishevelled) recruitment to LRP 5/6 and Frizzled receptor and activating the downstream Wnt cascade (54, 55). The interplay between cholesterol biosynthesis pathway and Wnt/ β-catenin signaling is physiologically relevant for different processes like wound healing, inflammation (56). However, the aberrant expression is also a function of cholesterol and cholesterol esters in different Wnt-dependent tumors. In pancreatic carcinoma, the cholesterol is known to activate Fzl receptor and thereby upregulating Wnt signaling (57). Therefore, there have been attempts to exploit the interplay between these two major pathways and target the players for anti-neoplastic effects (58).

In our study as well, we have tried to delineate the mechanism that links the cholesterol pathway and a major tumorigenic signaling cascade. We have discussed about the possibility of using this physiological connection in developing novel CRC therapeutics that bring the focus on statins as a repurposed drug. We studied Wnt/ β-catenin signaling as a target of statin treatment in CRC and further elucidated the effect on the major players of this pathway. In this chapter our aim was to elaborate on the statin mediated mechanistic regulation of β-catenin.

We observed that statin treatment in CRC lines targeted β-catenin to degradation thereby directly affecting the Wnt/ β-catenin signaling. The degradation seemed to be GSK3β-mediated phosphorylation-independent and specific to lysosomal pathway. The β-catenin interactome analysis revealed a set of novel interactors, one of them being TRIM21. The TRIM21 E3 Ubiquitin ligase is a lesser-known isoform of TRIM ligases and only one report suggested the interaction of TRIM21 with β-catenin in a phosphorylation-independent manner (37).

We also observed farnesyl pyrophosphate as one of the interactors, which is known to be the precursor for farnesylation on target peptides. Therefore, we searched for a probable farnesyl motif *in silico* on the β-catenin sequence as it is not known to be harboring a farnesylation PTM.

The significance of farnesylation lies in the reports that have emphasized on the protein stabilization effect of the isoprenylation PTM. The lipid modification sits on the Cysteine residue of the targets with a consensus motif 'CAAX'. This cysteine residue undergoes either farnesylation or geranylation depending on the amino acid present on the position denoted by 'X' in the motif sequence (59). For instance, when 'X' is glycine, farnesylation is more likely, whereas geranylgeranylation requires a leucine or phenylalanine at the 'X' position, in addition to two cysteine residues in the motif (CCXX or CCAX) (60). Since β-catenin features glycine at the 'X' position, it is likely to undergo farnesylation, which is why we selected a pan-farnesyl antibody to detect the PTM. However, to conclusively confirm farnesylation, further assays employing methods with increased sensitivity and enrichment of farnesylation detection mechanisms would be necessary.

It has been well established that the isoprenylation modification is important for providing protein stability and loss of this PTM lead the proteins to degradation. Statin affects the mevalonate pathway, thereby inhibiting all the downstream processes as well, which also is responsible for the reduction in the isoprenylation modification (61). Therefore, statin mediated removal of this PTM leads to destabilization and subsequent degradation of the proteins (62).

There seemed to be a direct correlation between the removal of farnesylation by statin and degradation of β-catenin as we did find a CAAX sequence with the Cys 419 residue showing a high significance value for the probability of harboring a farnesyl PTM. To further investigate, we generated a site directed mutation at C419 into alanine, and tagged the modified β-catenin protein with RFP. We observed that C419A β-catenin indeed showed a reduced farnesyl mark. Moreover, the localization of the C419A modified β-catenin was altered from being nuclear to cytoplasmic. However, this C419A β-catenin did not undergo degradation until treated by statin, suggesting that another factor might be playing a pivotal role for the degradation.

It is well established that PCAF acetylates β-catenin and thereby confers stability to the protein. Therefore, we also monitored the status of PCAF on statin treatment, which was found to be downregulated. Furthermore, the interaction between β-catenin and PCAF was significantly reduced upon statin treatment. Thus, we suggested that both, the loss of probable farnesylation and loss of PCAF interaction destabilizes β-catenin and drives it towards degradation.

This study has opened multiple new avenues that need further work to completely understand the physiological role of β-catenin farnesylation. The C419A β-catenin was localized in the cytoplasm but not degraded, as we validated from immunoblots as well as immunofluorescence assays. The

significance of this cytoplasmic localization on loss of farnesyl PTM needs to be further explored. Therefore, the physiological relevance of the prenylation PTM on β-catenin protein is required to be unfolded. The significance of adopting the phosphorylation-independent lysosomal degradation and coupling it with loss of probable farnesylation and PCAF would also be an interesting aspect to further delve into unearthing the direct mode of action of statins on Wnt/ β-catenin signaling. Therefore, the inferences would provide a boost to the therapeutics studies in CRC and thereby help in formulating a mechanistic approach towards the use of statins.

4.4 Materials and Methods

Cell lines and culture

HCT116 and HCT15 CRC cell lines were obtained from European Collection of Cell Cultures (ECACC) and HT29 and HEK293T cell lines was obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). HCT116 and HEK293T were cultured in DMEM media (Gibco, Waltham, MA, USA) with 10% FBS whereas HCT15 and HT29 were cultured in RPMI media (Gibco, Grand Island, NY, USA) with 10% FBS. Simvastatin (Sigma Cat No. #79902-63-9) was used for all assays at a final concentration of 30 μM in activated form. Briefly, for activation of statin, it was dissolved in 0.1N NaOH 300 μl and 100% ethanol 200 μl and heated at 50 °C for 2 hrs. The pH was adjusted to 7.4 with HCl and the volume was made up to 1 ml with Milli Q water. The final drug solution was filtered and stored in aliquots at -20°C. The activation of simvastatin is important to break the lactone ring that is the competitive inhibitor substrate for its target enzyme HMG CoA reductase. Chloroquine (Sigma Cat no. C6628) with final concentration of 25 μM and MG132 (EMB Millipore cat no. 474790) final concentration of 10 μM were used for respective treatments.

Plasmid constructs

For wildtype (WT) β-catenin construct, full length β-catenin was cloned into DsRed construct using primers 5'GCTAGGGAATTCATGGCTACTCAAGCTGATTTGA3' and 5'GCGGTACCTTACAGGTCAGTATCAAACCAGG3'. For C419A modified β-catenin construct, the primers were designed using Quik Change primer design program provided by Agilent as a free access portal, for mutating 419th amino acid of β-catenin. The WT construct was used as the template for PCR amplification with forward primers 5'-gttagaaagaattccagctgcagcggtgaccacatttatatcatct-3' and reverse primer

5’agatgatataaatgtggtcaccgctgcagctggaattctttctaac3’. The PCR product was DpnI digested followed by its transformation. The positive clones were confirmed by Sanger sequencing.

Immunoblotting and Co-immunoprecipitation

The antibodies used for immunoblotting were β-catenin (BD Bioscience #), GAPDH (Abcam, Cat No.#G041), β-Actin (Sigma, A5441), Phospho- β-catenin (THR41/45) MILLIPORE 04-1067, GSK3β (CST 9315S), Phosphorylated GSK3β (S9) CST#9322S, PCAF (Santa Cruz #SC-13124). Lysate generation was performed in RIPA buffer 20 mM Tris pH7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 1% Sodium deoxycholate, PIC (Protease Inhibitor Cocktail #A32963 ThermoFisher).

For immunoprecipitation, the lysate was prepared in the buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5% Sodium deoxycholate, 1 mM EDTA). The Normal Mouse antibody (MILLIPORE Cat no. 12-371) and Normal Rabbit antibody (MILLIPORE Cat no. 12-370) was used as negative IgG controls for interaction studies respectively. Briefly, the lysates were precleared with Protein G beads (ThermoFisher Scientific Dynabeads Protein G Cat no.#10004D) or anti-mouse IgG beads (ThermoFisher Dynabeads M-280 Sheep anti-Mouse IgG Cat no.#112.02D) respectively, to reduce non-specific binding. The pre-cleared lysates (500 µg total protein) were incubated with the pull-down antibody of β-catenin or RFP respectively, overnight at 4°C. The lysates were then incubated with fresh, equilibrated with buffer, beads for min 4 hrs at 4°C. The elute was then collected and loaded on SDS-PAGE gel for Western blotting analysis.

Immunofluorescence assay

HCT116 cells (1 x 10^4) were seeded onto the coverslips in a 6 well dish and treated with statin and chloroquine singularly and in combination, or transiently transfected either with WT-β-catenin or C419A modified β-catenin, respectively. After 48 hrs of either treatment or transfection, cells were fixed with 4% paraformaldehyde (PFA) for 10 min followed by permeabilization with 0.1% Triton-X-100 for 10 min. The cells were then blocked with 5% BSA for 1 hr. Primary antibody (1:100 dilution) incubation was done overnight at 4°C followed by three 1X PBS washes for 10 min each. The slides were then incubated with secondary antibodies (1:200 dilution) for 1h at room temperature followed by three 1X PBS washes for 10 min each. The cells were then stained with DAPI for 10mins followed by two 1X PBS washes for 10 min each. The coverslips were then mounted onto the slides and imaged using a Leica SP8 confocal microscope. HCT116 cells were treated with either chloroquine, statin or both for 48h. The cells were then fixed and stained for LAMP1 (Abcam #ab25630) and β-catenin and/or RFP (Invitrogen_Tag RFP Polyclonal

Antibody_#R10367) antibody respectively, with the abovementioned protocol. For HEK 293T cells, transfection with either WT-β-catenin or C419A modified β-catenin construct was done followed by 3 μM ChiR (99021) treatment for 6h. Abovementioned protocol was followed for staining with RFP (Invitrogen Tag RFP Polyclonal Antibody_#R10367) and β-catenin (Cell Signaling Technology, Cat No. #8480) antibodies. Secondary antibodies were from ThermoFisher, Goat anti-mouse Alexa Fluor 488 (Cat # A-11001), Goat anti-rabbit Alexa Fluor 568 (Cat # A-11011).

In vivo analysis

Stably transfected WT and C419A modified β-catenin cell lines HCT116 and SW480 (1×10^6) were subcutaneously injected in NOD-SCID mice (6-8 weeks old). The tumors were allowed to develop for 35 days and tissue was harvested thereafter. The tumor burden was measured and tissues were divided for transcriptome and proteome analysis. The tissues for RNA isolation were directly homogenized in Trizol reagent (Taraka RNAiso Plus, Code: 9109) and 500 ng RNA was used for Quant seq library prep. The quantification of RNA was performed in Qubit 4.0 (ThermoFisher) and the quality control was done in Bioanalyzer (2100 Agilent Bioanalyzer Systems). Protein lysate was extracted from the tissues by homogenizing the samples in RIPA buffer plus protease inhibitor cocktail tablet (ThermoFisher #A32963).

Interactome and whole tissue lysate proteomics sample preparation and analysis

The in-gel method for sample preparation was adopted for the proteome analysis as per the published protocol (63). Briefly, the cell lysate was run on 10% SDS-PAGE gel and pieces were cut for further processing, after destaining them completely. 10 mM DTT (DL-Dithiothreitol, Sigma #D0632) and 20 mM IAA (Iodoacetamide, Sigma #I1149) is added successively and gel pieces washed and dried with ACN (100% Acetonitrile). Trypsin digestion was performed overnight at 37°C and peptide extraction was done with TFA. The obtained peptides were purified by the method of desalting through a C18 column disk. The air-dried samples were then processed for the proteomics analysis on Sciex TripleTOF6600 mass spectrometer interfaced with an Eksigent nano-LC 425 system using established protocols previously reported (64, 65). All raw data was analyzed using the ProteinPilot software from Sciex using search parameters previously described (65). The GO term for the differential peptides were plotted using g:Profiler. For the MS-MS for interactome analysis, the immunoprecipitation of β-catenin was performed and the elute was loaded on the 10% SDS-PAGE gel, to be further subjected to the in-gel method of proteomics study sample preparation. For the whole tissue lysate proteome analysis, the tumor

tissues harvested from the mice subcutaneously, were thoroughly homogenized in the RIPA buffer and the protein lysate was collected. Hundred µg of total protein was resolved on a 10% SDS-PAGE gel, followed by the in-gel method of proteomics sample preparation.

Quant sequencing and analysis

Total RNA (500 ng) was used for library preparation using Lexogen Quant Seq 3' mRNA-seq Library Prep kit (Vienna BioCenter, Vienna, Austria) using the protocol provided in the kit. The final libraries were purified using the Lexogen Purification Module with magnetic beads (Cat no. M02296-4-0130) and were quantified using the Qubit 1X HS DNA system (ThermoFisher Scientific). The analysis of the libraries was performed using Limma-voom method with eBayes at robust setting. The MD highlighted genes significant at FDR of 0.05 and exhibited Log 2-fold change of at least 0. The software and the method were in accordance with the article published (66-68). The edgeR was used for statistical methods implemented, as referred to in the articles published (69-72).

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Chapter 5

5.1 Summary, conclusion and future directions

Statins are family of drugs that are known to deplete cholesterol in cells by enabling its uptake from the blood stream where it is present in excess in the form of harmful circulating low density lipoprotein (LDL). This mechanism of action establishes statins as the most potent drug for the treatment of hypercholesterolemia as higher LDL levels have been known to lead to coronary heart disease, atherosclerosis, and dyslipidemic complications. There are other drugs that target the cholesterol biosynthesis pathway or mevalonate pathway, however, the efficiency of lowering the serum cholesterol by statins is unmatched. The reason for this potency is their mode of action by directly targeting the rate limiting step of the cholesterol biosynthesis pathway. The enzyme HMG CoA reductase that catalyzes the crucial step, converting HMG CoA into mevalonate, is competitively inhibited by statins. The lactone ring structure of statins competes with HMG CoA for the active site of the target reductase enzyme, thereby, inhibiting the activity (1). The mevalonate product is unable to form and thereby the entire downstream pathway is inhibited, including cholesterol production, as mevalonate is the precursor for multiple important lipids, post translational modifications and protein synthesis as well.

As discussed in the Chapter 1 - section 1.5, the paradigm for repurposing of drugs fits well with statins as they are known to affect the cholesterol biosynthesis pathway that is shown to be dysregulated in CRC tumorigenesis (2, 3). Reports have suggested upregulation of cholesterol biosynthesis pathway in CRC (4, 5), making Statins a potential candidate for inclusion in cancer therapeutics (6, 7). Some studies and a few meta-analysis results have shown the anti-tumor effect of statins in colorectal cancer (8), pancreatic cancer (9), breast cancer (10). There are reports which suggest that statins target the tumor cells via mutated p53 to undergo apoptosis (11-14), and some suggest via downregulation of Hippo pathway (15). However, the mechanism for delineating the effect of statins in CRC via targeting a signaling cascade is not clearly established.

Statins have undergone evolution in terms of the R group attached to the lactone ring of its molecular structure which acts as the competitive inhibitor of the substrate of HMG CoA reductase. The chemically synthesized Statins with modified R groups, now present in the pharmacological market, are better at drug delivery and have reduced side-effects (16). However,

Statins are still presumed to cause pleotropic effects. Therefore, delineating the specific mechanism of the statin mediated effects in CRC seems to be of utmost importance.

In this study, we took the first step towards validating the characterization of statins as a repurposed drug by taking a multi-pronged approach. We generated high-throughput multi-omics data that focused on the whole transcriptome, proteome and targeted lipidome of statin treated colorectal cancer (CRC) cell lines. The reason for working on colorectal cancer and elucidating the effect of statins in this type of tumor specifically lies in multiple factors, one of them being the hepatic portal link between the liver and the large intestine. Liver is known to be the site for maximum cholesterol biosynthesis as well as the most common site for metastasis of CRC (17). Moreover, the intestines have been another important site for cholesterol homeostasis due to dietary absorption regulation (18) as well as the influx governed by the microbiota metabolism (19). Therefore it provides both a physiological as well as mechanistic relevance for studying the statin effect in CRC. Furthermore, to remove any bias in our study related to the use of a hydrophobic or a hydrophilic type of statin, we used both simvastatin and rosuvastatin belonging to each type respectively, and obtained the same results. Therefore, we performed *in cellulo* and *in vivo* murine experiments using both types of statins. However, the human study was conducted exclusively using rosuvastatin as it is the most commonly prescribed therapeutic statin along with atorvastatin.

Our first observation was to validate the mode of action of statins in our model system, CRC. The lipidomics analysis on the CRC cell line treated with statin showed a significant reduction in the levels of cholesterol and its derivatives. Furthermore, we consistently monitored cholesterol as the read-out for the activity of statins in our model system for the next set of experiments. We also considered other lipid categories that are known to affect the tumorigenic signaling, and either observed no alteration or slight downregulation, except arachidonic acid. Our transcriptome data further validated the mevalonate pathway inhibition by showing an upregulation in the cholesterol responsive genes as a function of the feedback mechanism elicited on statin treatment. Interestingly, the same data showed us some novel effectors that belonged to oncogenic pathways. We observed that our transcriptome and proteome data collectively yielded Wnt/β-catenin signaling as one of the targets that was consistently downregulated upon statin treatment.

This observation is of major significance as it not only depicted Wnt/β-catenin signaling as a target, but also showed a pivotal oncogenic pathway being affected by a drug. There have been previous reports that have emphasized on Wnt pathway as target to inhibit tumorigenesis (20). There also have been studies correlating the cholesterol biosynthesis and aberrant Wnt signaling

to promote cancer progression (21, 22). However, a direct mechanism targeting the canonical Wnt/ β-catenin pathway via cholesterol metabolism has not been reported yet (23).

The role of Wnt/β-catenin signaling has been well established in maintaining normal intestinal homeostasis (24, 25) as well as in the initiation of adenoma formation when aberrantly expressed (26, 27). The statin mediated effect on the canonical Wnt cascade essentially meant an inhibitory effect in the early stages of tumor progression. Thereby suggesting a possible preventive effect of statins in the case of early diagnosis of CRC, as initiation of the carcinoma is known to be the most difficult time point for diagnosis. Furthermore, we observed that the major upstream players of Wnt signaling that are also considered to be the markers for tumorigenesis were downregulated at the protein level. Therefore, our study also contributed towards establishing the specificity of the mode of action of statins in CRC.

We delineated the mechanistic regulation of the two essential players of canonical Wnt pathway, namely β-catenin and SATB proteins, in detail and reported intriguing observations. We reported that physically depleting cholesterol was not sufficient to replicate the effect of statin, thereby suggesting a signaling modulation that affected the Wnt pathway either directly or indirectly. We discussed both the possibilities by exploring the statin mediated effect on each Wnt player respectively. We observed a probable indirect effect of statin treatment on SATB proteins via EMT-MET dynamics. It is known that SATB1 and SATB2 are homologs and have an opposite role in CRC tumorigenesis, where SATB1 is over-expressed and drives tumor progression and SATB2 has a converse function. We observed that statin treatment reversed the SATB protein expression in CRC, by significantly downregulating SATB1. Interestingly, we also observed a reversal in the EMT-MET phenotype which correlated with the SATB proteins expression. The markers for mesenchymal phenotype were downregulated on statin treatment, corresponding to the SATB1 expression and the epithelial phenotype were significantly expressed, which corresponded to the expression of SATB2. It appears to corroborate with the previous reports published as well as the *in cellulo* and *in vivo* analysis, regarding the tumorigenicity and SATB protein expression. However, further investigation would be required to delineate whether SATB proteins govern the EMT-MET phenotype or vice versa. Further, it would be interesting to observe whether statin disrupts the dynamic balance between SATB proteins by affecting the EMT-MET phenotype directly.

The direct effect of statin was observed on β-catenin as we deduced a mechanistic regulation affecting the stability of the protein. We observed the degradation of β-catenin protein mediated by statin, which seems to be eliciting a non-canonical degradation pathway. Wnt-dependent

degradation of β-catenin in “OFF” conditions, is carried out by the degradation complex including GSK3β mediated phosphorylation and subsequent ubiquitination by β-TrCP ubiquitin ligase, leading the protein for proteasomal degradation (28, 29). However, upon statin treatment, we observed a GSK3β-independent lysosomal degradation of β-catenin which might be via another ubiquitin ligase. The rich repertoire of interacting partners in an interactome study for β-catenin, revealed multiple novel players, including a novel ubiquitin ligase. However, the most intriguing of the interactors was the precursor for farnesylation post-translational modification.

It is known that proteins, especially small GTPase membrane bound proteins including RAS, RAF, RHO, get farnesylated for stability and show better transmission of signal (30, 31). Therefore, we hypothesized that β-catenin might be getting farnesylated for better stabilization and subsequent function in the nucleus, and statin treatment might result into removal of this modification. Previous reports have suggested that statin inhibits all the downstream processes of the mevalonate pathway, including the isoprenylation of target proteins (31, 32). Therefore, we suggested with our *in silico* and biochemical analysis that indeed a farnesyl modification might help β-catenin in providing stability to the protein. On the other hand, statin treatment might affect this stability by removal of the putative farnesyl modification. This inference provided us with a mechanism of statin mediated effect directly on a major player of the Wnt pathway.

Our further results depicted that degradation of β-catenin might require another stabilization factor to get affected as only removal of farnesylation was not enough. We had generated a modified β-catenin protein with mutated site of farnesyl recognition motif, at the predicted Cysteine 419 residue. This modified protein not only showed a reduction in farnesylation but also showed a unique phenotype of nuclear localization loss. However, the protein did not undergo spontaneous degradation suggesting another factor might be at play to lead the protein to degradation on statin treatment. PCAF is known to acetylate β-catenin and impart stability to the protein (33, 34), and we showed that statin downregulates PCAF significantly. Therefore, with the two factors which provide stability, the PCAF mediated acetylation and farnesylation post-translational modifications being significantly reduced, the β-catenin protein was destined to degradation upon statin treatment. With our inferences we indicated a dual mechanism by which statins affected β-catenin and directly targeted Wnt pathway for inhibition.

There are a few aspects of the study which can be further explored. In our biochemical analysis of the loss of function mutation at the farnesylated residue of β-catenin - Cys 419, we observed an altered localization of the protein. The β-catenin with the mutated Cys 419 could not enter the nucleus even on Wnt activation. Therefore, we suggested that farnesylation of β-catenin might be

important for its localization into the nucleus. However, the physiological significance of this putative farnesylation on β-catenin is not known. One function might be to mediate the stabilization of the protein which is known. However, whether this PTM further helps in interaction or transcriptional factor ability of β-catenin is something that would be interesting to explore as it has not been reported as yet. The β-catenin protein has a very tight regulation via numerous PTMs throughout the sequence of the amino acids. However, a lipid modification of the 15 carbon farnesylation would mean a direct link between cholesterol biosynthesis pathway and Wnt pathway.

Therefore, with the above inferences, we delineated the specific mode of action of statin, targeting a pivotal oncogenic pathway, the canonical Wnt signaling, via its two major players. The significance of the statin mediated effect on these upstream players lies at the critical stage the cascade is affected. The mechanism of inhibition ensured a downregulation right at the initiation of the tumorigenic signaling. The regulation of the SATB proteins and β-catenin, whether via EMT-MET balance or by destabilization leading to degradation respectively, indicated towards a direct mode of action. Furthermore, our preliminary human study where we reported a total of 60 patients, 30 of which were given statin orally along with the neo-adjuvant therapy, has affirmed our inferences. We were not only able to project SATB proteins as reliable markers for adenomatous tumor but also propose them as potential therapeutic targets. Our data collected for the CRC patients also shed light on the importance of including serum cholesterol levels as a prognostic test for better correlation and diagnosis of these adenomatous polyps. Currently, the CRC patients are not monitored for the blood cholesterol which should be included in the prognostic protocol. The cholesterol level readout was a standard analysis in our assays, which turned out to be reliable as well as helped us in validating statin mode of action. With the inclusion of this parameter for patients on statin treatment regime we would be able to manage the after-effects (if any) as well as the therapeutics effects equally efficiently.

Therefore, with this study we provided a clinical as well as mechanistic relevance to support the inclusion of statins in the current therapeutic regime for CRC treatment. Hence, suggesting statins as a suitable candidate for not only using it as re-purposed drug in the case of CRC therapeutic protocols, but also paving the way ahead for a treatment regime affecting the early stages of tumorigenesis.

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List of Publications

1. **Sneha Tripathi**, Ekta Gupta, and Sanjeev Galande. (2024) Statins as anti-tumor agents: a paradigm for repurposed drugs. *Cancer Reports* (Accepted for publication)

2. **Sneha Tripathi**, Ekta Gupta, Satyajeet Khare, Rutika Naik, Rafeeq Mir, Jasmine Kaur Dhall, Saarthi Desai, Swati Humane, Subhash Yadav, Munita Bal, Avanish Saklani, Prachi Patil, Siddhesh Kamat, and Sanjeev Galande. (2024) Statin attenuates Wnt/β-catenin signaling by targeting the SATB family of proteins in Colorectal cancer. (Manuscript submitted)

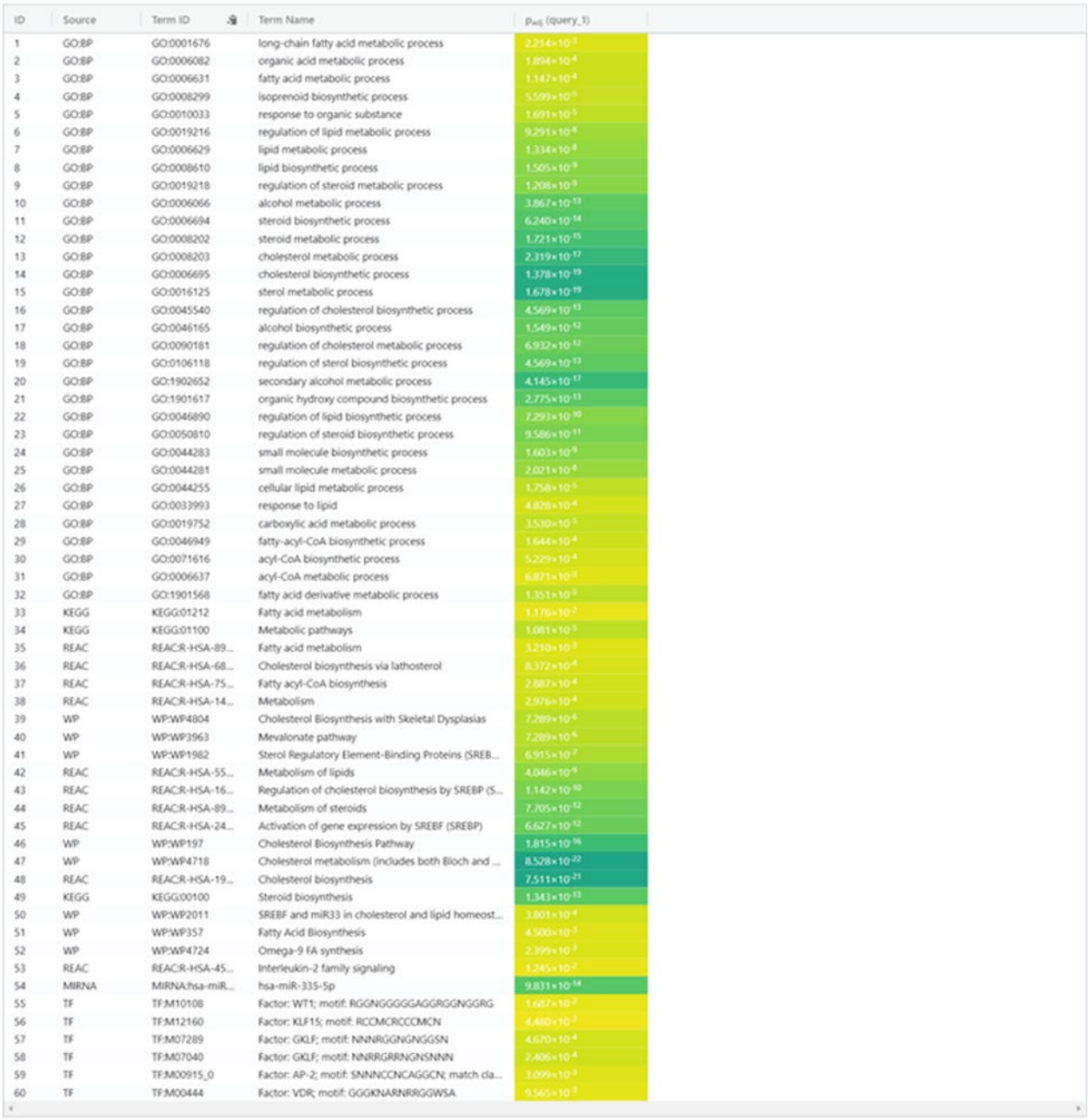
3. **Sneha Tripathi**, Ekta Gupta, Rafeeq Mir, Siddhesh Kamat, Sanjeev Galande. (2024) β-catenin gets destabilized by Statin treatment in a mevalonate pathway-dependent manner.
(Manuscript under preparation)

4. Anupam A. Sawant, **Sneha Tripathi**, Sanjeev Galande, and Sudha Rajamani. (2024) A Prebiotic Genetic Nucleotide as an Early Darwinian Ancestor for Pre-RNA Evolution. *ACS Omega* Article ASAP
DOI: 10.1021/acsomega.3c09949

Appendix

Section 1:

Chapter 2



ID	Source	Term ID	Term Name	p_{adj} (query_1)
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2	GO:BP	GO:0006082	organic acid metabolic process	1.894×10^{-4}
3	GO:BP	GO:0006631	fatty acid metabolic process	1.147×10^{-4}
4	GO:BP	GO:0008299	isoprenoid biosynthetic process	5.599×10^{-5}
5	GO:BP	GO:0010033	response to organic substance	1.691×10^{-5}
6	GO:BP	GO:0019216	regulation of lipid metabolic process	9.291×10^{-8}
7	GO:BP	GO:0006629	lipid metabolic process	1.334×10^{-8}
8	GO:BP	GO:0008610	lipid biosynthetic process	1.505×10^{-9}
9	GO:BP	GO:0019218	regulation of steroid metabolic process	1.208×10^{-9}
10	GO:BP	GO:0006066	alcohol metabolic process	3.867×10^{-13}
11	GO:BP	GO:0006694	steroid biosynthetic process	6.240×10^{-14}
12	GO:BP	GO:0008202	steroid metabolic process	1.721×10^{-15}
13	GO:BP	GO:0008203	cholesterol metabolic process	2.319×10^{-17}
14	GO:BP	GO:0006695	cholesterol biosynthetic process	1.378×10^{-19}
15	GO:BP	GO:0016125	sterol metabolic process	1.678×10^{-19}
16	GO:BP	GO:0045540	regulation of cholesterol biosynthetic process	4.569×10^{-13}
17	GO:BP	GO:0046165	alcohol biosynthetic process	1.549×10^{-12}
18	GO:BP	GO:0090181	regulation of cholesterol metabolic process	6.932×10^{-12}
19	GO:BP	GO:0106118	regulation of sterol biosynthetic process	4.569×10^{-13}
20	GO:BP	GO:1902652	secondary alcohol metabolic process	4.145×10^{-17}
21	GO:BP	GO:1901617	organic hydroxy compound biosynthetic process	2.775×10^{-13}
22	GO:BP	GO:0046890	regulation of lipid biosynthetic process	7.293×10^{-10}
23	GO:BP	GO:0050810	regulation of steroid biosynthetic process	9.586×10^{-11}
24	GO:BP	GO:0044283	small molecule biosynthetic process	1.603×10^{-9}
25	GO:BP	GO:0044281	small molecule metabolic process	2.021×10^{-8}
26	GO:BP	GO:0044255	cellular lipid metabolic process	1.758×10^{-5}
27	GO:BP	GO:0033993	response to lipid	4.828×10^{-4}
28	GO:BP	GO:0019752	carboxylic acid metabolic process	3.530×10^{-5}
29	GO:BP	GO:0046949	fatty-acyl-CoA biosynthetic process	1.644×10^{-4}
30	GO:BP	GO:0071616	acyl-CoA biosynthetic process	5.229×10^{-4}
31	GO:BP	GO:0006637	acyl-CoA metabolic process	6.871×10^{-3}
32	GO:BP	GO:1901568	fatty acid derivative metabolic process	1.351×10^{-5}
33	KEGG	KEGG:01212	Fatty acid metabolism	1.176×10^{-2}
34	KEGG	KEGG:01100	Metabolic pathways	1.081×10^{-5}
35	REAC	REAC:R-HSA-89...	Fatty acid metabolism	3.210×10^{-3}
36	REAC	REAC:R-HSA-68...	Cholesterol biosynthesis via lathosterol	8.372×10^{-4}
37	REAC	REAC:R-HSA-75...	Fatty acyl-CoA biosynthesis	2.887×10^{-4}
38	REAC	REAC:R-HSA-14...	Metabolism	2.976×10^{-4}
39	WP	WP:WP4804	Cholesterol Biosynthesis with Skeletal Dysplasias	7.289×10^{-6}
40	WP	WP:WP3963	Mevalonate pathway	7.289×10^{-6}
41	WP	WP:WP1982	Sterol Regulatory Element-Binding Proteins (SREB...	6.915×10^{-7}
42	REAC	REAC:R-HSA-55...	Metabolism of lipids	4.046×10^{-9}
43	REAC	REAC:R-HSA-16...	Regulation of cholesterol biosynthesis by SREBP (S...	1.142×10^{-10}
44	REAC	REAC:R-HSA-89...	Metabolism of steroids	7.705×10^{-12}
45	REAC	REAC:R-HSA-24...	Activation of gene expression by SREBF (SREBP)	6.627×10^{-12}
46	WP	WP:WP197	Cholesterol Biosynthesis Pathway	1.815×10^{-16}
47	WP	WP:WP4718	Cholesterol metabolism (includes both Bloch and ...	8.528×10^{-22}
48	REAC	REAC:R-HSA-19...	Cholesterol biosynthesis	7.511×10^{-23}
49	KEGG	KEGG:00100	Steroid biosynthesis	1.343×10^{-13}
50	WP	WP:WP2011	SREBF and miR33 in cholesterol and lipid homeost...	3.801×10^{-4}
51	WP	WP:WP357	Fatty Acid Biosynthesis	4.500×10^{-3}
52	WP	WP:WP4724	Omega-9 FA synthesis	2.399×10^{-3}
53	REAC	REAC:R-HSA-45...	Interleukin-2 family signaling	1.245×10^{-2}
54	MIRNA	MIRNA:hsa-miR...	hsa-miR-335-5p	9.831×10^{-14}
55	TF	TF:M10108	Factor: WT1; motif: RGGNGGGGGAGGRGGNGGRG	1.687×10^{-2}
56	TF	TF:M12160	Factor: KLF15; motif: RCCMCRCCCMCN	4.480×10^{-2}
57	TF	TF:M07289	Factor: GKLF; motif: NNNRGGNGNGGSN	4.670×10^{-4}
58	TF	TF:M07040	Factor: GKLF; motif: NNRRGRRNGNSNNN	2.406×10^{-4}
59	TF	TF:M00915_0	Factor: AP-2; motif: SNNNCCNCAGGCN; match cla...	3.099×10^{-3}
60	TF	TF:M00444	Factor: VDR; motif: GGGKNARNRRGGWSA	9.565×10^{-3}

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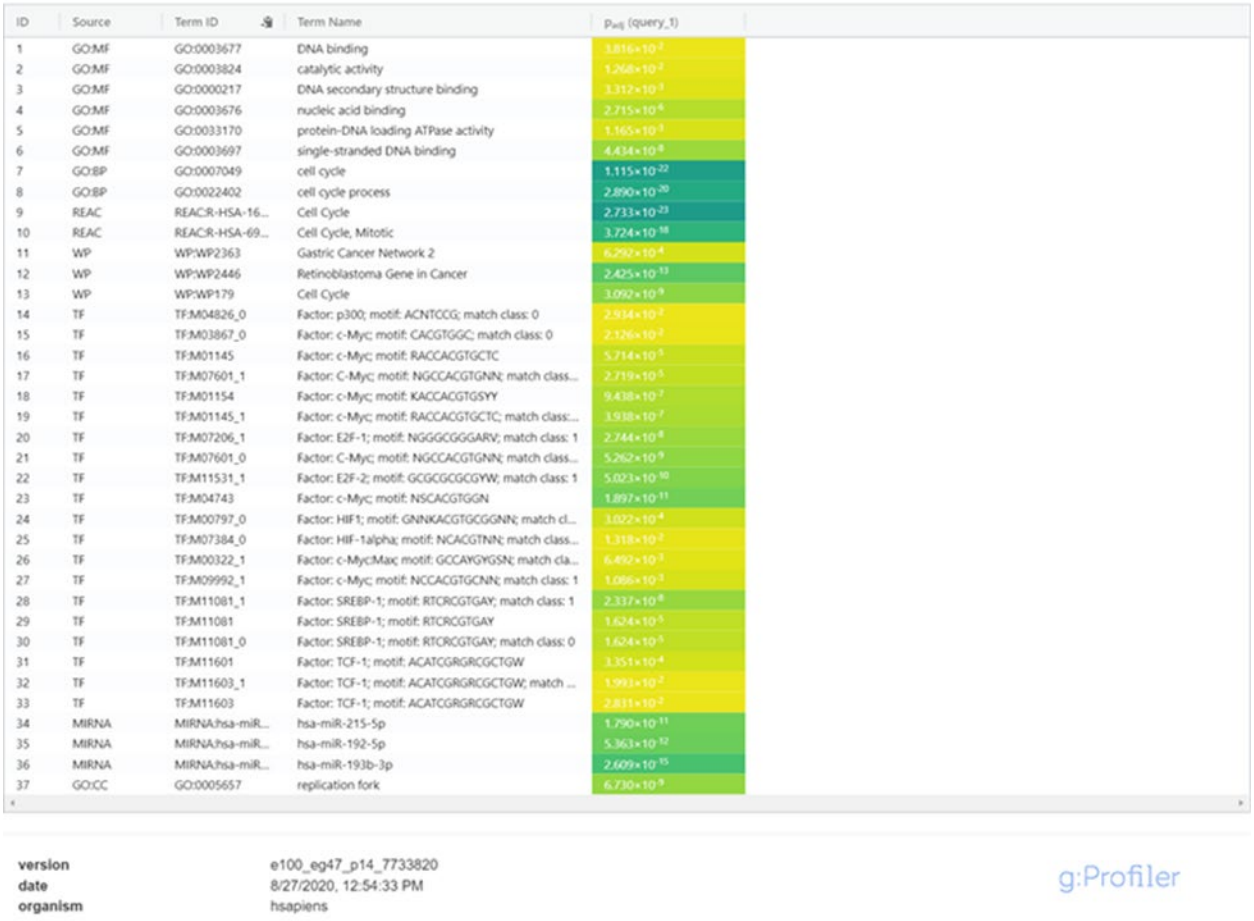
g:Profiler

Table 1: GO terms list for the Manhattan plot (Figure 2A) provided from g:Profiler software for the significantly upregulated genes on statin treatment in transcriptome analysis. The

color coding is dependent on the significance value (p value) assigned to the terms in the software based on the gene list belonging to the term and the statistical significance of their match, blue signifying highly significant to orange signifying least significant. Therefore, all the GO terms listed are highly significant.



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0003677	DNA binding	3.816×10^{-2}
2	GO:MF	GO:0003824	catalytic activity	1.268×10^{-2}
3	GO:MF	GO:0000217	DNA secondary structure binding	3.312×10^{-3}
4	GO:MF	GO:0003676	nucleic acid binding	2.715×10^{-6}
5	GO:MF	GO:0033170	protein-DNA loading ATPase activity	1.165×10^{-3}
6	GO:MF	GO:0003697	single-stranded DNA binding	4.434×10^{-8}
7	GO:BP	GO:0007049	cell cycle	1.115×10^{-22}
8	GO:BP	GO:0022402	cell cycle process	2.890×10^{-20}
9	REAC	REAC:R-HSA-16...	Cell Cycle	2.733×10^{-23}
10	REAC	REAC:R-HSA-69...	Cell Cycle, Mitotic	3.724×10^{-18}
11	WP	WP:WP2363	Gastric Cancer Network 2	6.292×10^{-4}
12	WP	WP:WP2446	Retinoblastoma Gene in Cancer	2.425×10^{-13}
13	WP	WP:WP179	Cell Cycle	3.092×10^{-9}
14	TF	TF:M04826_0	Factor: p300; motif: ACNTCCG; match class: 0	2.934×10^{-2}
15	TF	TF:M03867_0	Factor: c-Myc; motif: CACGTGGC; match class: 0	2.126×10^{-2}
16	TF	TF:M01145	Factor: c-Myc; motif: RACCACGTGCTC	5.714×10^{-5}
17	TF	TF:M07601_1	Factor: C-Myc; motif: NGCCACGTGNN; match class...	2.719×10^{-5}
18	TF	TF:M01154	Factor: c-Myc; motif: KACCACGTGSYY	9.438×10^{-7}
19	TF	TF:M01145_1	Factor: c-Myc; motif: RACCACGTGCTC; match class:...	3.938×10^{-7}
20	TF	TF:M07206_1	Factor: E2F-1; motif: NGGGCGGGARV; match class: 1	2.744×10^{-8}
21	TF	TF:M07601_0	Factor: C-Myc; motif: NGCCACGTGNN; match class...	5.262×10^{-9}
22	TF	TF:M11531_1	Factor: E2F-2; motif: GCGCGCGCGYW; match class: 1	5.023×10^{-10}
23	TF	TF:M04743	Factor: c-Myc; motif: NSCACGTGGN	1.897×10^{-11}
24	TF	TF:M00797_0	Factor: HIF1; motif: GNNKACGTGCGGNN; match cl...	3.022×10^{-4}
25	TF	TF:M07384_0	Factor: HIF-1alpha; motif: NCACGTNN; match class...	1.318×10^{-2}
26	TF	TF:M00322_1	Factor: c-Myc:Max; motif: GCCAYGYGSN; match cla...	6.492×10^{-3}
27	TF	TF:M09992_1	Factor: c-Myc; motif: NCCACGTGCNN; match class: 1	1.086×10^{-3}
28	TF	TF:M11081_1	Factor: SREBP-1; motif: RTCRCGTGAY; match class: 1	2.337×10^{-8}
29	TF	TF:M11081	Factor: SREBP-1; motif: RTCRCGTGAY	1.624×10^{-5}
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32	TF	TF:M11603_1	Factor: TCF-1; motif: ACATCGRGRCGCTGW; match ...	1.993×10^{-2}
33	TF	TF:M11603	Factor: TCF-1; motif: ACATCGRGRCGCTGW	2.831×10^{-2}
34	MIRNA	MIRNA:hsa-miR...	hsa-miR-215-5p	1.790×10^{-11}
35	MIRNA	MIRNA:hsa-miR...	hsa-miR-192-5p	5.363×10^{-12}
36	MIRNA	MIRNA:hsa-miR...	hsa-miR-193b-3p	2.609×10^{-15}
37	GO:CC	GO:0005657	replication fork	6.730×10^{-9}

version	e100_eg47_p14_7733820
date	8/27/2020, 12:54:33 PM
organism	hsapiens

g:Profiler

Table 2: GO terms list for the Manhattan plot (Figure 2B) provided from g:Profiler software for the significantly downregulated genes on statin treatment in transcriptome analysis. The color coding is dependent on the significance value (p value) assigned to the terms in the software based on the gene list belonging to the term and the statistical significance of their match, blue signifying highly significant to orange signifying least significant. Therefore, all the GO terms listed are highly significant.

ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0045296	cadherin binding	9.638×10^{-12}
2	GO:MF	GO:0050839	cell adhesion molecule binding	1.710×10^{-8}
3	GO:MF	GO:0097159	organic cyclic compound binding	2.638×10^{-4}
4	GO:MF	GO:1901363	heterocyclic compound binding	3.704×10^{-4}
5	GO:MF	GO:0031625	ubiquitin protein ligase binding	2.489×10^{-2}
6	GO:BP	GO:1901575	organic substance catabolic process	1.332×10^{-10}
7	GO:BP	GO:0043161	proteasome-mediated ubiquitin-dependent protei...	1.756×10^{-5}
8	GO:BP	GO:0010498	proteasomal protein catabolic process	1.065×10^{-4}
9	GO:BP	GO:0051603	proteolysis involved in cellular protein catabolic pr...	4.716×10^{-4}
10	GO:BP	GO:0006511	ubiquitin-dependent protein catabolic process	5.869×10^{-3}
11	GO:BP	GO:0017015	regulation of transforming growth factor beta rece...	6.255×10^{-3}
12	GO:BP	GO:1903844	regulation of cellular response to transforming gr...	7.426×10^{-3}
13	GO:BP	GO:0070498	interleukin-1-mediated signaling pathway	1.620×10^{-2}
14	GO:BP	GO:0016055	Wnt signaling pathway	3.712×10^{-2}
15	GO:BP	GO:0198738	cell-cell signaling by wnt	3.870×10^{-2}
16	REAC	REAC:R-HSA-21...	Downregulation of TGF-beta receptor signaling	6.832×10^{-7}
17	REAC	REAC:R-HSA-89...	Regulation of PTEN localization	3.356×10^{-6}
18	REAC	REAC:R-HSA-21...	TGF-beta receptor signaling activates SMADs	3.834×10^{-6}
19	REAC	REAC:R-HSA-46...	Degradation of DVL	2.260×10^{-5}
20	REAC	REAC:R-HSA-17...	Signaling by TGF-beta Receptor Complex	1.304×10^{-4}
21	REAC	REAC:R-HSA-21...	Regulation of activated PAK-2p34 by proteasome ...	1.609×10^{-4}
22	REAC	REAC:R-HSA-12...	Downregulation of ERBB4 signaling	2.027×10^{-4}
23	REAC	REAC:R-HSA-34...	Autodegradation of the E3 ubiquitin ligase COP1	2.121×10^{-4}
24	REAC	REAC:R-HSA-75...	Ubiquitin-dependent degradation of Cyclin D	2.121×10^{-4}
25	REAC	REAC:R-HSA-69...	Ubiquitin Mediated Degradation of Phosphorylate...	2.121×10^{-4}
26	REAC	REAC:R-HSA-89...	Regulation of RUNX3 expression and activity	2.764×10^{-4}
27	REAC	REAC:R-HSA-46...	Degradation of AXIN	3.143×10^{-4}
28	REAC	REAC:R-HSA-17...	SCF-beta-TrCP mediated degradation of Emi1	3.143×10^{-4}
29	REAC	REAC:R-HSA-88...	PTK6 Regulates RTKs and Their Effectors AKT1 and...	3.623×10^{-4}
30	REAC	REAC:R-HSA-90...	Signaling by NOTCH4	3.643×10^{-4}
31	REAC	REAC:R-HSA-56...	NIK-->noncanonical NF-kB signaling	5.121×10^{-4}
32	REAC	REAC:R-HSA-56...	Hedgehog 'on' state	5.281×10^{-4}
33	REAC	REAC:R-HSA-18...	SCF(Skp2)-mediated degradation of p27/p21	5.752×10^{-4}
34	REAC	REAC:R-HSA-56...	GLI3 is processed to GLI3R by the proteasome	5.752×10^{-4}
35	REAC	REAC:R-HSA-56...	Degradation of GLI2 by the proteasome	5.752×10^{-4}
36	REAC	REAC:R-HSA-56...	Degradation of GLI1 by the proteasome	5.752×10^{-4}
37	REAC	REAC:R-HSA-93...	IRAK2 mediated activation of TAK1 complex	5.993×10^{-4}
38	REAC	REAC:R-HSA-56...	Dectin-1 mediated noncanonical NF-kB signaling	5.752×10^{-4}
39	REAC	REAC:R-HSA-90...	TICAM1,TRAF6-dependent induction of TAK1 com...	9.349×10^{-4}
40	REAC	REAC:R-HSA-53...	Hedgehog ligand biogenesis	9.971×10^{-4}
41	REAC	REAC:R-HSA-17...	APC/C:Cdc20 mediated degradation of Securin	1.356×10^{-3}
42	REAC	REAC:R-HSA-13...	Downregulation of ERBB2:ERBB3 signaling	1.392×10^{-3}
43	REAC	REAC:R-HSA-90...	TICAM1-dependent activation of IRF3/IRF7	1.392×10^{-3}
44	REAC	REAC:R-HSA-90...	Signaling by TGFB family members	1.815×10^{-3}
45	REAC	REAC:R-HSA-17...	Cdc20:Phospho-APC/C mediated degradation of C...	2.192×10^{-3}
46	REAC	REAC:R-HSA-17...	APC/C:Cdh1 mediated degradation of Cdc20 and ...	2.402×10^{-3}
47	REAC	REAC:R-HSA-18...	EGFR downregulation	3.235×10^{-3}
48	REAC	REAC:R-HSA-97...	IRAK1 recruits IKK complex upon TLR7/8 or 9 stim...	2.774×10^{-3}
49	WP	WP:WP183	Proteasome Degradation	4.742×10^{-3}
50	TF	TF:M04515_1	Factor: E2F-1; motif: WWTGGCGCCAAA; match cla...	1.641×10^{-5}
51	TF	TF:M04826_1	Factor: p300; motif: ACNTCCG; match class: 1	1.561×10^{-4}
52	TF	TF:M11530	Factor: E2F-2; motif: NWTTTGGCGCCAWWNN	1.912×10^{-4}
53	TF	TF:M10438	Factor: ZF5; motif: GGSGCGCGS	5.816×10^{-4}
54	TF	TF:M00932_1	Factor: Sp1; motif: NNGGGGCGGGGNN; match cla...	8.032×10^{-4}
55	TF	TF:M12160	Factor: KLF15; motif: RCCMCRCCCMCN	1.247×10^{-3}
56	TF	TF:M03925	Factor: YY2; motif: NCCGCCATNTY	3.093×10^{-3}
57	TF	TF:M03924_1	Factor: YY1; motif: NNCGCCATTNN; match class: 1	4.267×10^{-3}
58	TF	TF:M10530	Factor: sp4; motif: NNNGCYCCGCCCCY	3.445×10^{-2}
59	MIRNA	MIRNA:hsa-miR...	hsa-miR-615-3p	5.288×10^{-11}
60	MIRNA	MIRNA:hsa-miR...	hsa-miR-324-3p	2.115×10^{-2}

version	e103_eg50_p15_68c0e33
date	5/18/2021, 10:26:39 PM
organism	hsapiens

g:Profiler

Table 3: GO terms list for the Manhattan plot (Figure 3A) provided from g:Profiler software for the significantly downregulated proteins on statin treatment in whole lysate proteomics MS-MS analysis. The color coding is dependent on the significance value (p value) assigned to the terms in the software based on the protein list belonging to the term and the statistical significance of their match, blue signifying highly significant to orange signifying least significant. Therefore, all the GO terms listed are highly significant.

S.No.	Gene name	qPCR Primer sequence
1	SATB1	Forward- AACTCAGGGCTTGCTTTC Reverse- CCTGGTATATTCGGTCTCTTTC
2	SATB2	Forward- AGAGATGAACCAGAGCACATTAG Reverse- GTTGCTGACACATTGGCATAAT
3	β-catenin	Forward- AAGGTGTGGCGACATATGCA Reverse- GTAATCTTGTGGCTTGTCCTCAGA
4	18srRNA	Forward- CGCCGCTAGAGGTGAAATTCT Reverse- CGAACCTCCGACTTTCGTTCT
5	SREBF2	Forward- GCTGCCAAGGAGAGTCTAT Reverse- AGGTTTCACCAAGGACTCTAT
6	HMGR	Forward- ACAAGAATTTAGTGGGCTCTG Reverse- TCCTGTCCACAGGCAAT
7	CDH1	Forward- GGCTGGACCGAGAGAGTTTC Reverse- CCTGACCCTTGTACGTGGTG
8	Vimentin	Forward- GCGAGGAGAGCAGGATTTCT Reverse- TGGGTATCAACCAGAGGGAGT
9	E-cadherin	Forward- GTCCTGGGCAGAGTGAAT Reverse- GGGTTATGAAACCGTAGAGGC

Table 4: The list of qPCR primers used for the analysis

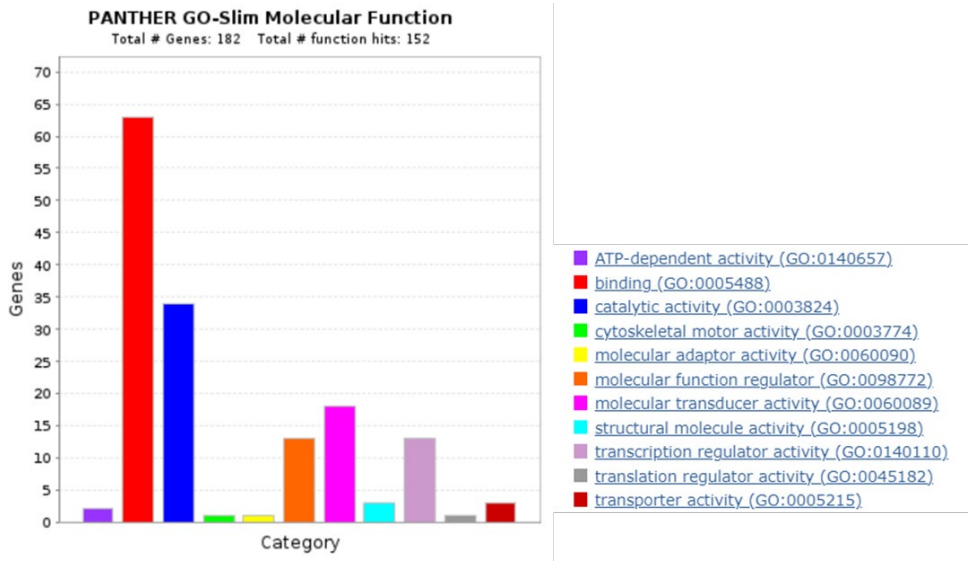
Section 2:

Chapter 4

REAC		stats	
Term name	Term ID	p_{adj}	$-\log_{10}(p_{adj})$ 0 ≤16
Translocation of SLC2A4 (GLUT4) to the plasma membrane	REAC:R-HSA-14...	1.375×10^{-5}	
Post NMDA receptor activation events	REAC:R-HSA-43...	2.117×10^{-5}	
RHO GTPases activate IQGAPs	REAC:R-HSA-56...	3.096×10^{-5}	
Activation of NMDA receptors and postsynaptic events	REAC:R-HSA-44...	7.371×10^{-5}	
Microtubule-dependent trafficking of connexons from Golg...	REAC:R-HSA-19...	8.545×10^{-5}	
Transport of connexons to the plasma membrane	REAC:R-HSA-19...	1.115×10^{-4}	
RAS GTPase cycle mutants	REAC:R-HSA-96...	1.317×10^{-4}	
Post-chaperonin tubulin folding pathway	REAC:R-HSA-38...	2.292×10^{-4}	
Recycling pathway of L1	REAC:R-HSA-43...	3.834×10^{-4}	
Activation of AMPK downstream of NMDARs	REAC:R-HSA-96...	5.183×10^{-4}	
COPI-independent Golgi-to-ER retrograde traffic	REAC:R-HSA-68...	6.975×10^{-4}	
Sealing of the nuclear envelope (NE) by ESCRT-III	REAC:R-HSA-96...	1.039×10^{-3}	
HSP90 chaperone cycle for steroid hormone receptors (SHR...	REAC:R-HSA-33...	1.078×10^{-3}	
Gap junction assembly	REAC:R-HSA-19...	2.506×10^{-3}	
Assembly and cell surface presentation of NMDA receptors	REAC:R-HSA-96...	4.675×10^{-3}	
Aggrephagy	REAC:R-HSA-96...	5.245×10^{-3}	
Carboxyterminal post-translational modifications of tubulin	REAC:R-HSA-89...	5.868×10^{-3}	
The role of GTSE1 in G2/M progression after G2 checkpoint	REAC:R-HSA-88...	7.305×10^{-3}	
L1CAM interactions	REAC:R-HSA-37...	7.669×10^{-3}	
Gap junction trafficking	REAC:R-HSA-19...	8.960×10^{-3}	
Gap junction trafficking and regulation	REAC:R-HSA-15...	9.901×10^{-3}	
Formation of tubulin folding intermediates by CCT/TriC	REAC:R-HSA-38...	1.188×10^{-2}	
Golgi-to-ER retrograde transport	REAC:R-HSA-88...	1.574×10^{-2}	
Kinesins	REAC:R-HSA-98...	2.416×10^{-2}	
COPI-dependent Golgi-to-ER retrograde traffic	REAC:R-HSA-68...	2.634×10^{-2}	
Neurotransmitter receptors and postsynaptic signal transmi...	REAC:R-HSA-11...	2.669×10^{-2}	
COPI-mediated anterograde transport	REAC:R-HSA-68...	3.113×10^{-2}	
Cooperation of Prefoldin and TriC/CCT in actin and tubulin f...	REAC:R-HSA-38...	3.133×10^{-2}	
PTK6 Regulates RHO GTPases, RAS GTPase and MAP kinases	REAC:R-HSA-88...	3.580×10^{-2}	
ER to Golgi Anterograde Transport	REAC:R-HSA-19...	4.158×10^{-2}	
Protein localization	REAC:R-HSA-96...	4.688×10^{-2}	

Table 1: GO term list as per the REAC database, for the 109 proteins which were uniquely expressed in the modified β-catenin expressing tumor tissue (venn diagram in Figure 8D, section 4.2(v)). This list was generated using g:Profiler software. The color coding is dependent on the significance value (p value) assigned to the terms in the software based on the protein list belonging to the term and the statistical significance of their match, blue signifying highly significant to orange signifying least significant. Therefore, all the GO terms enlisted are highly significant.

(A)



(B)

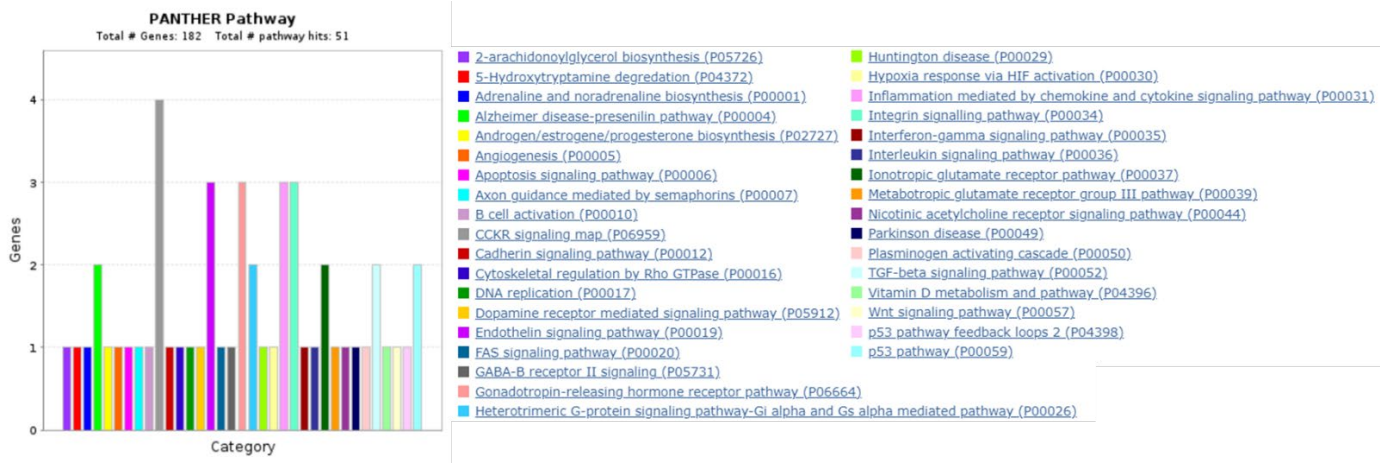


Figure 1: The transcriptomics analysis of the differentially expressed genes upregulated in the C419A β-catenin tumor samples as compared to the WT samples. (A) The GO terms for the Molecular function for the 200 genes upregulated in the C419A β-catenin modified tumor sample. (B) The GO terms for the Pathway function for the 200 genes upregulated in the C419A β-catenin modified tumor sample. The terms were plotted using the online portal PANTHER 17.0, with the Gene List analysis tool. The bar graph represents the terms, colour coded for each category on the x axis, and the number of genes in each term on the y axis.

Chapter 4: The β-catenin sequence validation for generation of C419 site directed mutant

ACCGGGATTGTCTGTTCTACGCCATCACGACACTGCATAATCTCCTGCTCCATCAGGAAGGAGCTAAAATGGCAGTGC
GCCTAGCTGGTGGACTGCAGAAAATGGTTGCTTTGCTCAACAAAACAAACGTGAAATTCTTGGCTATTACAACAGAC
TGCCTTCAGATCTTAGCTTATGGCAATCAAGAGAGCAAGCTCATCATTCTGGCCAGTGGTGGACCCCAAGCCTTAGTA
AACATAATGAGGACCTACACTTATGAGAAGCTTCTGTGGACCACAAGCAGAGTGCTGAAAGTGCTGTCTGTCTGCTC
TAGCAACAAGCCGGCCATTGTAGAAGCTGGTGGGATGCAGGCACTGGGGCTTCATCTGACAGACCCAAGTCAGCGA
CTTGTTCAAAACTGTCTTTGGACTCTCAGAAACCTTTCAGATGCAGCGACTAAGCAGGAAGGGATGGAAGGCCTCCT
TGGGACTCTAGTGCAGCTTCTGGGTTCCGATGATATAAATGTGGTCACCCTGCAGCTGGAATTCTTTCTAACCTCACTT
GCAATAATTACAAAAACAAGATGATGGTGTGCCAAGTGGGTGGCATAGAGGCTCTTGTACGCACCGTCCTTCGTGCT
GGTGACAGGGAAGACATCACTGAGCCTGCCATCTGTGCTCTTCGTCATCTGACCAGCCGGCATCAGGAAGCCGAGA
TGGCCCAGAATGCCGTTCGCCTTCATTATGGACTGCCTGTTGTGGTTAAACTCCTGCACCCACCATCCCACTGGCCTCT
GATAAAGGCAACTGTTGGATTGATTCGAAACCTTGCCCTTTGCCCAGCAAATCATGCGCCTTTGCGGGAACAGGGTG
CTATTCCACGACTAGTTCAGCTGCTTGTACGAGCACATCAGGACACCCAACGGCGCACCTCCATGGGTGGAACGCAG
CAGCAGTTTGTGGAGGGCGTGCGCATGGAGGAGATAGTAGAAGGGTGTACTGGAACTCTCCACATCCTTGCTCGGG
ACGTTCACAACCGGATTGTAATCCGAGGACTCAATACCATTCCATTGTTTGTGCAGTTGCTTAATTCTCCCATTGAAAT
ATCCAAAGAGTAGCTGCAGGGGTTCCTCGTGAACTTGCTCAGGACAAGGAGGCTGCAAAGGCCCTTGAAGCTTAG
GGAACCCCGCCTCCCCTGAAAAATTTCCCCCCCCCCGAAAAGAAGGCGTGCTAAAAAACGAGGGG

Figure 2: The above sequence was obtained after plasmid sequencing validation. The grey region marks the primer which was used to mutate the Cysteine at the 419 position. The blue region marks the sequencing primers used to validate the site directed mutagenesis. The vector backbone used was dsRed which consists of an RFP tag at the C terminus of the protein being expressed. The WT full length β-catenin was first cloned into the dsRed vector and this was further used for making the single residue mutation for generate the C419A construct.

Section 3:

Chapter 2

Result section 2.2(iii) Figure 3B

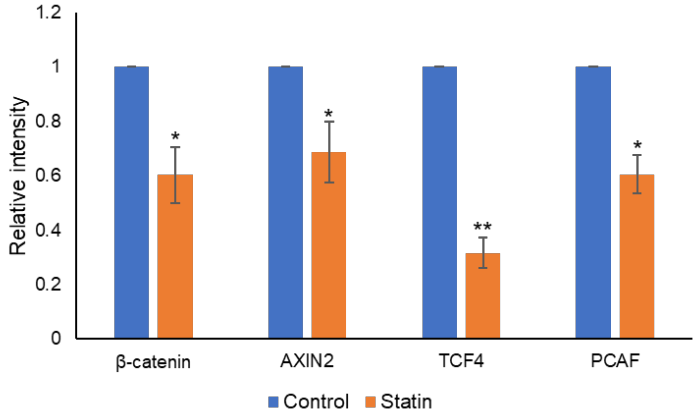


Figure 3B: Densitometric analysis of the relative intensity of protein levels of β-catenin, AXIN2, TCF4 and PCAF in control vs statin treated HCT15 cells. All the proteins show a significant reduction in levels upon statin. Student t test analysis, *p<0.05, **p<0.005, N=3

Result section 2.2(iv) Figure 4B

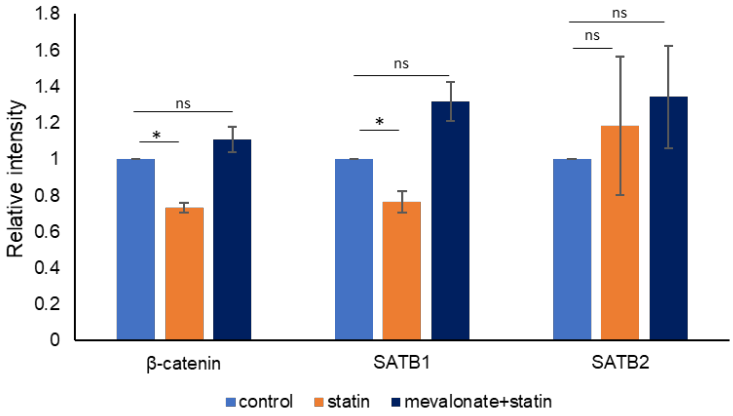


Figure 4B: Densitometric analysis of relative intensity for β-catenin, SATB1 and SATB2 in control vs statin and statin plus mevalonate conditions. The analysis shows a significant reduction in β-catenin and SATB1 levels upon statin treatment, however no significant alteration is observed in SATB2 protein levels. Student t test analysis, *p<0.05, ns= non-significant. N=3

Chapter 3

Result section 3.2(i) Figure 1C

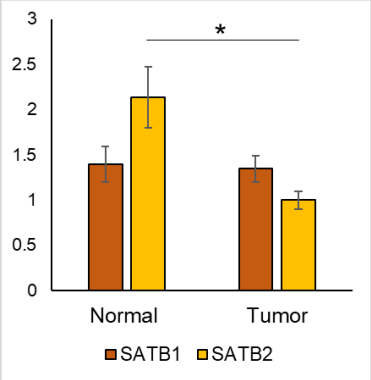


Figure 1C: Densitometry analysis of the 9 set of patient samples with paired adjacent normal and tumor tissue. The densitometric analysis shows a significant reduction in SATB2 proteins in tumor whereas adjacent normal tissue has higher levels of SATB2. SATB1 protein levels are however not significantly altered. Student's t test analysis, *p<0.05, ns= non-significant.

Chapter 4

Result Section 4.2(i), Figure 1F

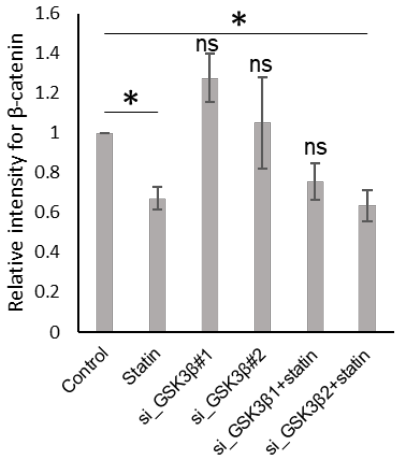


Figure 1F: Densitometry analysis for the relative intensity of β-catenin in conditions statin, siGSK3β#1 and #2, and statin plus siGSK3β#1 and #2. The significant reduction in protein levels is seen in only statin treatment and statin plus siGSK3β#2. Student's t test analysis, *p<0.05, ns= non-significant. N=3