University of California, San Diego

Retinoblastoma Protein Partners in Apoptosis and Invasion of Colon Cancer Cells

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

Edison Tse

Committee in charge:

Professor Jean Y.J. Wang, Chair
Professor Cornelius Murre
Professor Michael David

2014

The thesis of Edison Tse, and it is acceptable
in quality and form for publication on microfilm and electronically:

_____

_____

_____

Chair

University of California, San Diego

2014

Table of Contents

List of Figures and Tables

Acknowledgments

The first person I would to thank is Dr. Jean J.Y. Wang. She provided me an opportunity to participate in her lab. With this opportunity, I have been trained with the basic skills for scientific research, but more importantly with the skills to ask scientific questions and critically interpret results. Under Dr. Wang's guidance and the experience in the lab, my passion to pursue science was ignited.

Her technical guidance and training was essential for my progress and success in the lab setting; Dr. Kirsten Ludwig, a very passionate mentor and scientist, has worked tirelessly with me. I would like to thank her. Under her guidance, I learned valuable lessons in experimental design, results interpretation ,and troubleshooting.

Essential to my research, was the work done by Dr. Jinbo Han. I would like to thank her for her contribution and for setting the foundation in which my research project could advance from. Additionally, she has always been supportive and has always extended a helping hand whenever needed.

Proudly, I would like to additionally thank Dr. Priya Sridevi. She has always been kind and supportive of my research. Always willing to share her experience in research, she has provided invaluable insight and technical guidance.

I would like to express my gratitude to all members of the Wang lab. I would like to especially thank Daniel Quan and Louis Nguyen for their scientific insight and discussions. Additionally, I would like to thank Wilson Tang and David Michael whose constant support has made working in a lab a pleasure.

Guiding and supporting me unconditionally were my family and friends, I would to thank you.

ABSTRACT OF THESIS

Retinoblastoma Protein Partners in Apoptosis and Invasion of Colon Cancer Cells

by

Edison Tse

Master of Science in Biology

University of California, San Diego, 2014

Professor Jean Y.J. Wang, Chair

Retinoblastom protein, lacking in any catalytic domain, is a scaffolding protein. It functions through bringing protein complexes together and has been shown to regulate cells processes such as cell cycle progression and programmed cell death, apoptosis. Regulation of RB and its binding partners occurs in a variety of ways. In the E2F transcription factor case, RB is hyperphosphorylated to induce intra-domain

interaction thus releasing E2F. Other avenues to regulating RB includes the process of TNF induced apoptosis which proceeds with a specific caspase cleavage of the last 42 amino acids of RB. By applying mass spectrometry and a number of in vitro binding assays, we have identified several proteins that interact with the C-terminal region of RB. Our results demonstrate that survivin and β-catenin interact with the C-terminal portion of RB. Nonmuscle Myosin Heavy Chain II A has been shown to specifically interact with the terminal 42 amino acids of RB. Our results demonstrate multiple binding partners of RB C-terminal region; a region that is relevant to apoptosis regulation.

I.
Introduction

Colon Cancer and RB relevance

Colorectal cancer is a leading cause of cancer-related death in the United States. In 2010, 131,607 people in the United States were diagnosed with colorectal cancer and 52,045 people died from it [1]. Comprehensive genomic characterization of colorectal (CRC) patient samples by the Cancer Genome Atlas (TCGA) network has found consistent up-regulation of the RB1 gene [2]. It is believed that RB promotes colon cancer survival [3]. The retinoblastoma protein (RB) is a 928 amino acid scaffolding protein; it possesses no catalytic or enzymatic domain [4]. RB consists of 3 major domains: the N-terminal domain, the central "Pocket" domain, and the C-terminal domain [4]. The N-terminal and Central "Pocket" domains are composed of two highly structured helical domains; in between the structured domains are linker sequences that lack definite structure [4]. RB is most famously known as a tumor suppressor through its ability to bind and sequester E2F transcription factors [4, 5]. E2F and its heterodimer DP bind to RB through the Central "Pocket" and C-terminal domain; phosphorylation of RB promotes inter-domain interaction causing the release of its binding partners [4, 6]. Our focus concerns mainly the C-terminal domain of RB. The C-terminal domain is "intrinsically disordered"; however, 30 residues adopt a structured helical conformation [4, 6]. Our study focuses on the interactions between proteins and the C-terminal tail of RB.

RB Caspase-Dependent Cleavage and RB-MI

A caspase cleavage site is located 42 amino acids upstream of the C-terminal end of the RB protein. In this lab, the cleavage sequence of (DEAD/GS) in wild-type RB was mutated into (DEAA/ES) and the mutant RB was termed RB-MI [7]. This mutant RB is resistant to caspase cleavage. After endotoxic shock, apoptosis is inhibited in the intestines but not the spleen of homozygous RB-MI mice [7]. The inhibition of apoptosis by RB-MI is signal dependent; RB-MI inhibits Tumor Necrosis Factor Receptor I (TNFRI) induced apoptosis but not TNFRII induced apoptosis [7]. In a p53-null background, RB-MI mice have a substantially increased incidence of developing colon cancer with no increase seen in lymphoma development [8].

Nuclear β-catenin and Survivin

Intestinal epithelial cells of RB-MI mice are resistant to apoptosis induced by endotoxic shock and TNF unlike the intestinal epithelial cells of RB-WT mice [7]. In this lab, RB-WT and RB-MI mice were placed on the AOM/DSS protocol to induce colon tumors; and it was discovered that tumors developed in RB-WT mice were resistant to TNF induced apoptosis despite expressing wild-type RB and TNFRI [9]. Histological assays of these tumors revealed that nuclear expression of β-catenin is associated with resistance to TNF induced apoptosis [9]. Similarly, cancer cell lines with nuclear β-catenin are resistant to TNF-induced apoptosis [9]. In over 90% of human cancers, the canonical Wnt pathway is constitutively activated through mutational activation of β-catenin or inactivation of DKK, APC, Axin2 [2]. The Wnt pathway proceeds with Wnt protein binding to Frizzeled/LRP receptors leading to β-

catenin stabilization which, in the nucleus, promotes the transcription Wnt target genes [10]. One of the Wnt target genes is survivin [11]. Survivin is a 165 amino acid protein belonging to the inhibitor of apoptosis protein (IAP) family [12]. Survivin plays a role in promoting mitosis through its interaction with Auroa B-kinase, INCENP, and Borealin in the formation of the Chromosomal Passenger Complex (CPC) [12]. As a possible cancer target, survivin is over-expressed in a variety of cancers but is undetectable in differentiated normal tissues [12]. The crystal structure of survivin has been elucidated and has been shown to exist in two forms: a monomeric and dimeric form [13]. Experiments done with siRNA knockdown of β-catenin and survivin have shown reduction in RB protein levels with no significant effect on RB mRNA level [9]. This has led us to determine whether nuclear β-catenin and survivin interacts with RB to stabilize it.

MYH9 Interaction with RB-C-Terminal 42 amino acids

MYH9, also known as non-muscle myosin heavy chain-A, belongs to the myosin II subfamily and has been implicated in cell mobility and adhesion [14, 15]. Non-muscle myosin IIA is composed of two heavy chains encoded by the MYH9 gene; it possesses 3 major domains: the head domain contains the actin-binding domain and ATPase domain, the coiled-coil rod domain, and the non-helical tail domain [16]. The C-terminal 42 amino acids of RB, which is cleaved off during apoptosis, is shown to interact with MYH9 (unpublished data). In colon cancer cells sensitive to TNF-induced apoptosis, this offers insight into the role of RB cleavage and MYH9 interaction disruption has on apoptosis.

II.

Results

Discovery of interaction between Retinoblastoma Protein (RB), β-catenin, and Survivin

To further investigate the mouse model of inflammation associated colon cancer, Dr. Jinbo Han, a former post-doctoral investigator from our lab, observed nuclear expression of β-catenin is associated with resistance to TNF-induced apoptosis. She additionally observed this in colon cancer cell lines; HCT116 cells, lacking nuclear β-catenin, were sensitive to TNF-induced apoptosis, while LIM1899 cells, possessing nuclear β-catenin, were resistant to TNF-induced apoptosis [9]. To further understand the role of nuclear β-catenin, she performed a siRNA knockdown of β-catenin in LIM1899 cells. This resulted in a significant reduction in RB protein, but had no significant impact on the RB mRNA [9]. This suggests β-catenin plays a role in RB protein stability. Survivin is a target gene of the Wnt/β-catenin pathway [2]. SiRNA knockdown of survivin in LIM1899 cells also revealed a small but significant reduction in RB protein levels with no significant reduction in RB mRNA levels. She performed a co-immunoprecipation assay with survivin and pulled down β-catenin and RB [unpublished]. Further study was done to investigate whether β-catenin and survivin associated with RB protein to stabilize it.

Interaction between Survivin and RB is observed in In Vitro GST-Pulldown assay with GST-RB-C fusion protein

To confirm the interaction between survivin and RB protein, a RB C-terminal tail was fused with Glutathione-S-Transferase (GST) protein. This recombinant protein was bacterially expressed and affinity purified. We then carried out an in vitro GST-pulldown assay using whole-cell extracts from HCT116 cells incubated with

GST-RB-C bound Sepharose beads. Cell lysates were employed into the pull-down assay in 1000µg, 500µg, 250µg, 100µg, and 50µg titrations. Whole-cell extracts from HCT116 cells were also incubated correspondingly with GST-only bound Sepharose beads to account for non-specific binding to the GST portion of the fusion protein (figure 1a, lanes 2-6). Whole cell extract inputs were loaded to serve as comparison marker (Fig. 1b Lanes 1 & 12). Survivin pull-down was observed in the GST-only samples at the 1000µg titration but absent in the lower titrations (figure 1b lanes 2-6). This is most likely trapping, due to the fact that the signal rapidly drops off with decreasing titrations. Survivin pull-down was observed in all the titration in the GST-RB-C samples, which a saturation characteristic in the 500µg, 250µg, 100µg, and 50µg titrations (figure 1b, lanes 7-11). GST-specific antibody was used for Western blot analysis to confirm the presence of GST-only and the GST-RB-C fusion protein. As shown in figure 1b, despite the saturation characteristics of survivin in the GST-RB-C samples, there are not stoichiometric amounts of survivin being pulled down by the GST-RB-C protein.

Increased interaction between Survivin and RB is not clearly observed in In Vitro GST-Pulldown assay with GST-RB-C fusion protein with RB-knockdown cells

A possible explanation to the non-stoichiometric pull-down of survivin by GST-RB-C is that endogenous RB is sequestering a pool of survivin. To increase the interaction of survivin and GST-RB-C in the in vitro GST-pulldown assay, HCT116 shRNA-RB whole cell extracts were used. HCT116 shRNA-RB cells have been stably transfected with a shRNA that targets the UTR of RB mRNA for degradation. HCT116 shRNA-RB cells have significantly reduced levels of RB protein but

maintain similar levels of survivin when compared to wild-type HCT116 cells (data not shown). We then carried out an in vitro GST-pulldown assay using whole-cell extracts from HCT116 shRNA-RB cells incubated with GST-RB-C bound Sepharose beads. Cell lysates were employed into the pull-down assay in 1000µg, 500µg, 250µg, 100µg, and 50µg titrations. Whole-cell extracts from these cells were also incubated correspondingly with GST-only bound Sepharose beads to account for non-specific binding to the GST portion of the fusion protein (figure 2a, lanes 2-6). Whole cell extract inputs were loaded to serve as comparison marker (Fig. 2b Lanes 1 & 12) Survivin pull-down was again observed only in the GST-only samples at the 1000µg titration but absent in the lower titrations (figure 2b lanes 2-6). Survivin pull-down was observed in all the titration in the GST-RB-C samples, which a saturation characteristic in the 500µg, 250µg, 100µg, and 50µg titrations (figure 2b, lanes 7-11). GST-specific antibody was used for Western blot analysis to confirm the presence of GST-only and the GST-RB-C fusion protein. As shown in figure 2b, despite the saturation characteristics of survivin in the GST-RB-C samples, there are not stoichiometric amounts of survivin pulled down by the GST-RB-C protein. Comparing the pull-down of surivin to the input of whole cell extract, there was no significant increase in pull-down efficiency between the HCT116 shRNA-RB extracts and the HCT116 extracts.

Interaction between dimeric Survivin and RB is not clearly observed in In Vitro GST-Pulldown assay with GST-RB-C fusion protein

Survivin has been shown to exist in multiple forms: a monomeric and dimeric form [3]. Due to the elution and solubilization steps of the GST pull-down and SDS-PAGE running, multimers are dissociated and run as monomers on SDS-PAGE. To determine whether the dimeric form of survivin binds RB protein, disuccinimidyl suberate (DSS), a non-cleavable and membrane permeable cross linker, was utilized to covalently crosslink survivin in cultured HCT116 shRNA-RB cells. As shown in Figure 3a, the DSS crosslinking reactions increased the amount of dimeric survivin (approx. 34kD) and the pool of monomeric survivin (17kD) decreased substantially.

We then carried out an in vitro GST-pulldown assay using whole-cell extracts from the DSS-crosslinked HCT116 shRNA-RB cells incubated with GST-RB-C bound Sepharose beads. Cell lyates were employed into the pull-down assay in 1000µg, 500µg, 250µg, 100µg, and 50µg titrations. Whole-cell extracts from DSS-crosslinked HCT116 shRNA-RB cells were also incubated correspondingly with GST-only bound Sepharose beads to account for non-specific binding to the GST portion of the fusion protein (figure 3c, lanes 2-6). Whole cell extract inputs were loaded to serve as comparison marker (Fig. 3c Lanes 1 & 12). There was no detectable signal form the areas corresponding to both the monomeric survivin (17kD) and the dimeric survivin (34kD). The input suggests that both dimeric and monomeric survivin was present in the whole cell extract. Interestingly there was a in the 70kD region that was pulled down in the GST-RB-C samples and was completely undetectable in the GST-only samples and is reactive to the survivin –specific antibody.

Discovery of interaction between the RB-C-terminal 42 amino acids and MYH9 protein

RB protein possesses a caspase cleavage site at the C-terminus that generates a 42 amino acid tail fragment. To determine if any proteins interact specifically with this tail fragment, Dr. Jinbo Han generated the C-terminal 42 amino acid tail attached to a Strep and 10X His tag and pulled down associated proteins from Hela whole cell extracts. The pull down was separated via SDS-PAGE and silver stained (Table 1a.). In collaboration with Dr. Chen at the University of Arizona, specific bands were isolated and identified by LC-MS/MS (liquid chromatography-mass spectrometry). The results of the mass spectrometry pull down experiment generated a list of possible interacting proteins (Table 1b.). The most significant protein pulled down was MYH9.

To validate these results, Dr. Kirsten Ludwig, a former post-doctoral researcher at our lab, performed a reciprocal co-immunoprecipitation assay with both RB and MYH9 as bait in HCT116 shRNA-RB cells with RB and RB mutants transiently transfected in (data not shown). The results of the reciprocal co-immonpreciptation validated that MYH9 interacted specifically with the C-terminal 42 amino acids (C42) of RB. Wild-type RB was able to pull down MYH9 while RB-ΔI, a RB mutant with the last 42 amino acids deleted, was unable to pull MYH9 down; the reciprocal co-immunoprecipitation reciprocated this result (data not shown). She also performed immunofluorescence co-localization assay to determine whether the proteins co-localized together in the cell. In mitotic cells RB and MYH9 can be seen

co-localizing, however in non-mitotic cells, MYH9 is localized to the periphery of the cell while RB is mainly localized in the nucleus (data not shown).

Caspase-dependant cleavage of MYH9 in Retinoblastoma knockdown cells

Experiments were performed to determine the functional relevance of this MYH9 and the RB interaction. RB protein has a caspase cleavage site 42 amino acids upstream of the C-terminal end, and this cleavage can be induced in HCT116 cells with treatment with Tumor Necrosis Factor α (TNF) and cyclohexamide (CHX) for four hours. A drug treatment assay was performed using wild-type HCT116 cells, HCT116 RB-KD cells, and a clonal cell line derived from the HCT116 RB-KD cells. As seen in figure 4, upon treatment with TNF/CHX, there is reduced RB signal as well as an appearance of cleaved RB signal (Fig. 4b, Lanes 3, 7, 11). Across the cell lines, MYH9 signal was consistent with no treatment, TNF-only treatment, and CHX-only treatment. Interestingly, TNF/CHX treatment led to a significant decrease in MYH9 signal in HCT116 RB-KD cell lines but there was no decrease seen in the wild-type HCT116 cell line (figure 4b, lanes 3,7,11). Western blot analysis probing for GAPDH and activated caspases were performed as loading controls and confirmation of TNF-induced apoptosis.

To determine whether this MYH9 signal loss was caspase-dependent, an additional treatment with ZVAD-fmk was added. ZVAD-fmk is a cell permeable, irreversible pan-caspase inhibitor. The drug treatment assay was performed using wild-type HCT116 cells, HCT116 RB-KD cells, and a clonal cell line derived from the HCT116 RB-KD cells. As seen in figure 4, upon treatment with TNF/CHX, there is

reduced RB signal as well as an appearance of cleaved RB signal (Fig. 4c, Lanes 15, 19, 23). Across the cell lines, MYH9 signal was consistent in the no treatment and TNF-only treatment samples. With TNF/CHX treatment, there was a significant decrease in MYH9 signal in HCT116 RB-KD cell lines but there was no decrease seen in the wild-type HCT116 cell line (Fig.4c, lanes 15, 19, 23). In the TNF/CHX + ZVAD-fmk treatment groups, there was restoration of the MYH9 signal (Fig. 4c, Lanes 20 & 24). Western blot analysis probing for GAPDH and activated caspases were performed as loading controls and confirmation of TNF-induced apoptosis. These results correspond well with another study that showed that MYH9 possesses a caspase-cleavage site and is cleaved during apoptosis in Jurkat T cells [4].

Comparison between RB mutants and MYH9

To determine if the MYH9 signal loss is due to RB knockdown leading to apoptosis sensitization or if RB bound to MYH9 protects it, a transient transfection assay was performed. Several RB mutants were transiently transfected into HCT116 shRNA-RB cells. RB-WT expresses the wild-type version of the RB protein. RB-MI expresses a RB protein with a 2 amino acid mutation in the caspase-cleavage site of RB making the protein resistant to cleavage. RB-ΔI expresses a RB protein in which the C-terminal 42 amino acids are truncated. RB-KF expresses a RB protein in which a $K^{896}F^{897}$ are deleted and substituted with Ile^{896}; this mutated RB has been to shown to be unable to bind MYH9 and is implicated in bladder cancer [5]. FKBP-caspase-8 was also transiently transfected into all the conditions. FKBP-caspase-8 is a modified caspase-8 in which the DED domains have been replaced with inducible dimerization

domains which are activated upon AP20187 treatment [6]. This system was utilized to circumvent the sensitization to TNF induced apoptosis resulting from the RB knockdown. To determine the reason for MYH9 loss, this transient transfection assay was performed. Upon treatment with AP21087, there was subsequent increased signal of the activated caspases and RB signal reduction. MYH9 signal remained consistent across all sample groups. Western blot analysis on GAPDH was used as loading control.

The previous experiment did not generate the initial observation of reduced MYH9 signal. A possibility for this is attributed to the lower than 50% transfection efficiency rate; for Western blot analysis, greater than 50% is required when looking for reduction in endogenous proteins. A different assay was utilized to circumvent this issue. Utilizing immunofluorescence (IF), single cells could be characterized on their basis of RB transfection, nucleus condition, and MYH9 staining. The MYH9 specific antibody utilized was targeted to the tail end of the MYH9 beyond the caspase cleavage site; in cells, MYH9 staining is lost in cell in which MYH9 undergoes cleavage (fig. 6a). The IF assay was performed with HCT116 RB-KD cells transiently transfected with RB-mutant expression plasmids. These were treated with TNF/CHX. Results of the IF revealed that all cells that displayed pyknotic nuclei, a feature of apoptosis, showed lack of reactivity to the MYH9 antibody and were minimally stained (Fig. 6a) [7]. Highly RB-stained cells were assessed for nucleus condition and were designated as either normal nuclei or pyknotic nuclei. Treatment with TNF/CHX resulted in significant increase in proportion of RB stained pyknotic nuclei. Across all

the transiently transfected RB mutants, there were no significant difference between the proportion of pyknotic nuclei in the TNF/CHX treatment groups (Fig. 6b).

1a.

Cell Lysate: HCT116 Cells			
Sample #	**Pulldown Protein**	**Input (µg)**	**[Prot] (µg/µL)**
1	Input	20	N/A
2	GST	1000	4.0
3	GST	500	2.0
4	GST	250	1.0
5	GST	100	0.4
6	GST	50	0.2
7	GST-RB-C	1000	4.0
8	GST-RB-C	500	2.0
9	GST-RB-C	250	1.0
10	GST-RB-C	100	0.4
11	GST-RB-C	50	0.2
12	Input	20	N/A

1b.

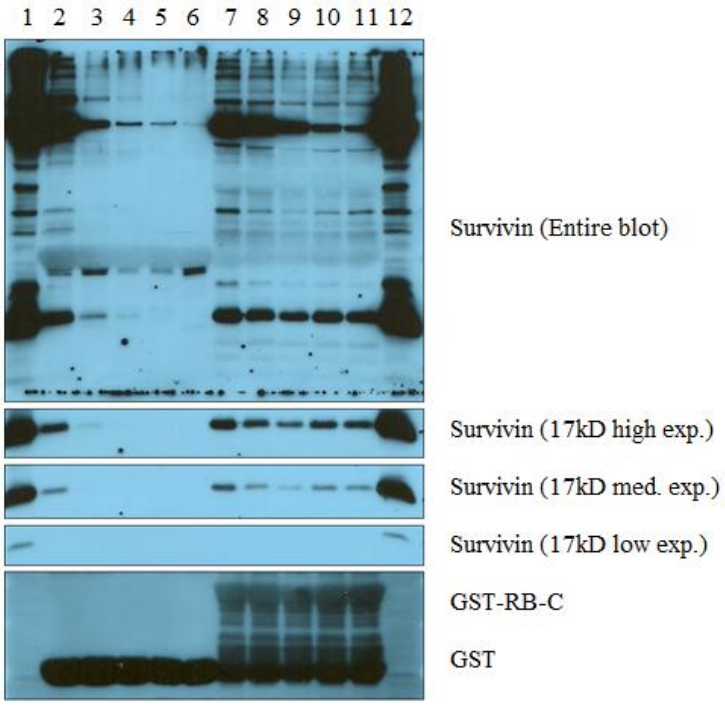


Figure 1. In vitro GST-pulldown assay shows GST-RB-C interaction with Survivin with HCT116 lysate. (a) Schematic table for the GST-RB-C pull-down assay. (b) Autoradiograph of blot probed with Survivin-specific antibody and GST-specific antibody. Lanes 1 & 12 are Inputs with 20µg of whole cell extracts.

2a.

Cell Lysate: HCT116 shRNA-RB Cells			
Sample #	**Pulldown Protein**	**Input (µg)**	**[Prot] (µg/µL)**
1	Input	20	N/A
2	GST	1000	4.0
3	GST	500	2.0
4	GST	250	1.0
5	GST	100	0.4
6	GST	50	0.2
7	GST-RB-C	1000	4.0
8	GST-RB-C	500	2.0
9	GST-RB-C	250	1.0
10	GST-RB-C	100	0.4
11	GST-RB-C	50	0.2
12	Input	20	N/A

2b.

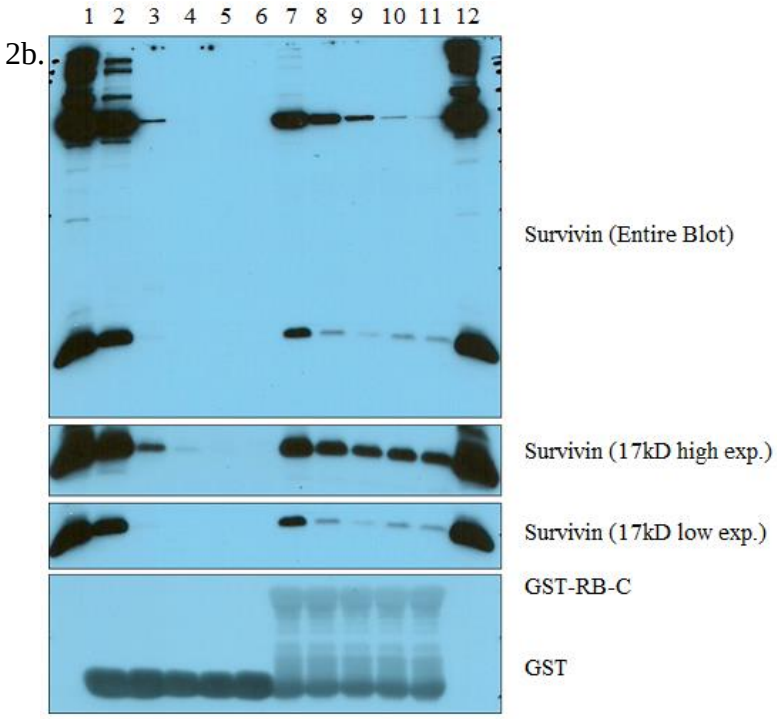
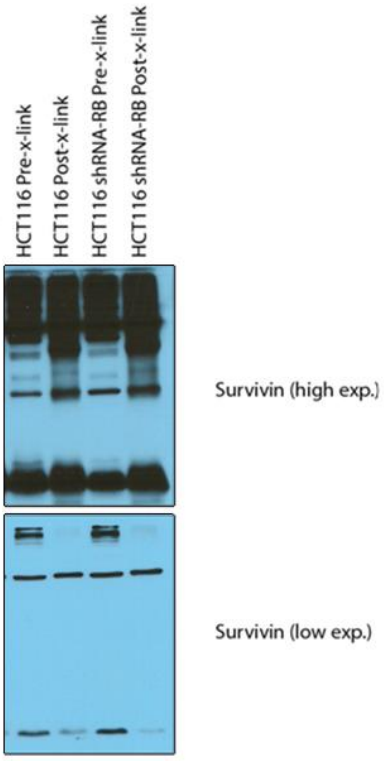


Figure 2. In vitro GST-pulldown assay shows GST-RB-C interaction with Survivin with HCT116 shRNA-RB cell lysate. (a) Schematic table for the GST-RB-C pulldown assay. (b) Autoradiograph of blot probed with Survivin-specific antibody and GST-specific antibody. Lanes 1 & 12 are Inputs with 20µg of whole cell lysates.

3a.



3b.

Cell Lysate: DSS-Crosslinked HCT116 shRNA-RB Cells			
Sample #	Pulldown Protein	Input (µg)	[Prot] (µg/µL)
1	Input	20	N/A
2	GST	1000	4.0
3	GST	500	2.0
4	GST	250	1.0
5	GST	100	0.4
6	GST	50	0.2
7	GST-RB-C	1000	4.0
8	GST-RB-C	500	2.0
9	GST-RB-C	250	1.0
10	GST-RB-C	100	0.4
11	GST-RB-C	50	0.2
12	Input	20	N/A

3c.

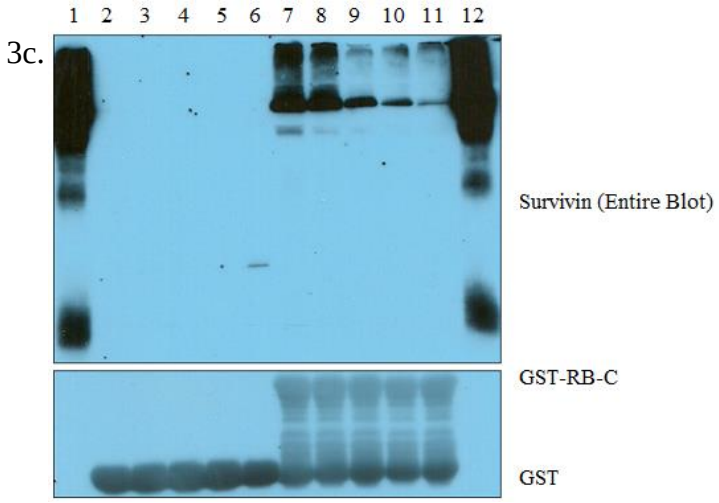
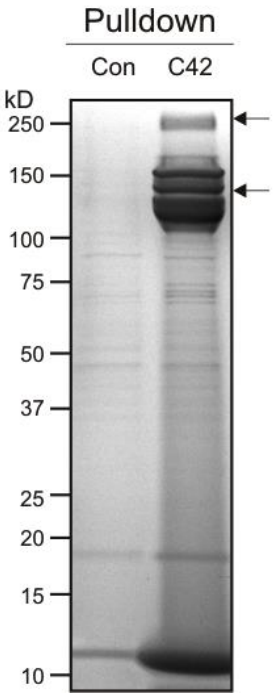


Figure 3. In vitro GST-pulldown assay shows lack of GST-RB-C with dimeric Survivin with DSS cross-linked HCT116 shRNA-RB cell lysates. (a) Autoradiograph of blot probed with Survivin-specific antibody verifying increased dimeric Survivin following DSS crosslinking of HCT116-shRNA-RB cells. (b) Schematic table for the GST-RB-C pulldown assay. (c) Autoradiograph of blot probed with Survivin-specific antibody and GST-specific antibody. Lanes 1 & 12 are Inputs with 20µg of whole cell lysates.

Table 1. MS/MS determined binding partners of Retinoblastoma protein C-terminal 42 amino acid tail. (a) Silver stain of Retinoblastoma protein C-terminal 42 amino acid tail pulldown assay. Indicated bands were underwent MS/MS analysis. (b) MS/MS analysis results identified proteins in indicated bands. Figure contributed by Dr. Han in collaboration with Dr. Chen at University of Arizona

Tbl. 1a.



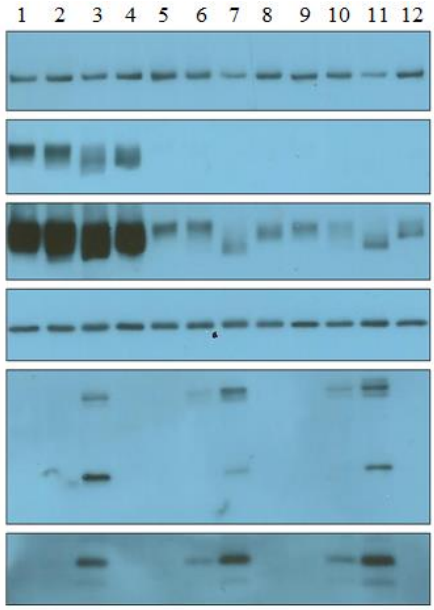
Tbl. 1b.

250 kD band	MW	Unique Peptides
Gene_Symbol=ACACA Acetyl-CoA carboxylase 1	265.5	1
Gene_Symbol=MYH9 Myosin-9	226.5	34
Gene_Symbol=FASN Fatty acid synthase	273.4	3
Gene_Symbol=POLQ DNA polymerase theta	197.6	1
Gene_Symbol=FLNB Isoform 1 of Filamin-B	278.2	1
Gene_Symbol=FLNA filamin A, alpha	280	1
Gene_Symbol=MYH10 Isoform 1 of Myosin-10	228.9	2
110-150 kD bands	**MW**	**Unique Peptides**
Gene_Symbol=MYO1C Myosin-Ic	118	2
Gene_Symbol=ACACA Acetyl-CoA carboxylase 1	265.5	2
Gene_Symbol=MYH9 Myosin-9	226.5	24
Gene_Symbol=FASN Fatty acid synthase	273.4	1
Gene_Symbol=COMP Cartilage oligomeric matrix protein precursor	82.8	1
Gene_Symbol=TTBK2 Tau-tubulin kinase	184.6	1
Gene_Symbol=COPA Coatomer subunit alpha	138.3	3
Gene_Symbol=PC Pyruvate carboxylase, mitochondrial precursor	129.6	4
Gene_Symbol=RB1 Retinoblastoma-associated protein	106.7	1
Gene_Symbol=MYO1B Isoform 1 of Myosin-Ib	131.9	1

4a.

#	Cell Type	Treatment	#	Cell Type	Treatment
1	HCT116	Vehicle	**13**	HCT116	Vehicle
2	HCT116	TNF	**14**	HCT116	TNF
3	HCT116	TNF/CHX	**15**	HCT116	TNF/CHX
4	HCT116	CHX	**16**	HCT116	TNF/CHX + ZVAD
5	HCT116 RB-KD	Vehicle	**17**	HCT116 RB-KD	Vehicle
6	HCT116 RB-KD	TNF	**18**	HCT116 RB-KD	TNF
7	HCT116 RB-KD	TNF/CHX	**19**	HCT116 RB-KD	TNF/CHX
8	HCT116 RB-KD	CHX	**20**	HCT116 RB-KD	TNF/CHX + ZVAD
9	HCT116 RB-KD Clone	Vehicle	**21**	HCT116 RB-KD Clone	Vehicle
10	HCT116 RB-KD Clone	TNF	**22**	HCT116 RB-KD Clone	TNF
11	HCT116 RB-KD Clone	TNF/CHX	**23**	HCT116 RB-KD Clone	TNF/CHX
12	HCT116 RB-KD Clone	CHX	**24**	HCT116 RB-KD Clone	TNF/CHX + ZVAD

4b.



4c.

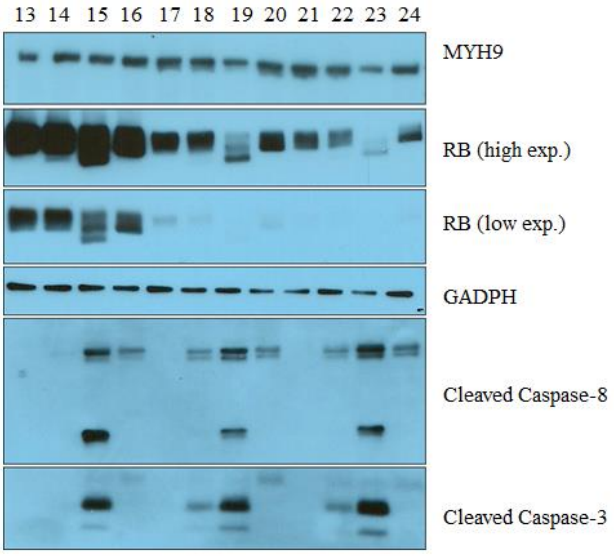


Figure 4. Caspase-dependent MYH-9 cleavage in HCT116 shRNA-RB cells. (a) Schematic table for cell types and treatment conditions. Cells were treated with TNF (10ug/mL), CHX (2.5µg/mL), and/or ZVAD-fmk (10mM) for four hours. (b) Autoradiograph of blot probed with MYH9-specific antibody and RB-specific antibody. Additionally probed with GAPDH antibody for loading control and activated/cleaved caspase antibodies.

5a.

Cell Type: HCT116 RB-KD		
Sample#	Expression Plasmid	AP20187 Treatment
1	Vector + FKBP-C8	-
2	Vector + FKBP-C8	+
3	RB-WT + FKBP-C8	-
4	RB-WT + FKBP-C8	+
5	RB-MI + FKBP-C8	-
6	RB-MI + FKBP-C8	+
7	RB-ΔI + FKBP-C8	-
8	RB-ΔI + FKBP-C8	+
9	RB-KF +FKBP-C8	-
10	RB-KF +FKBP-C8	+

5b.

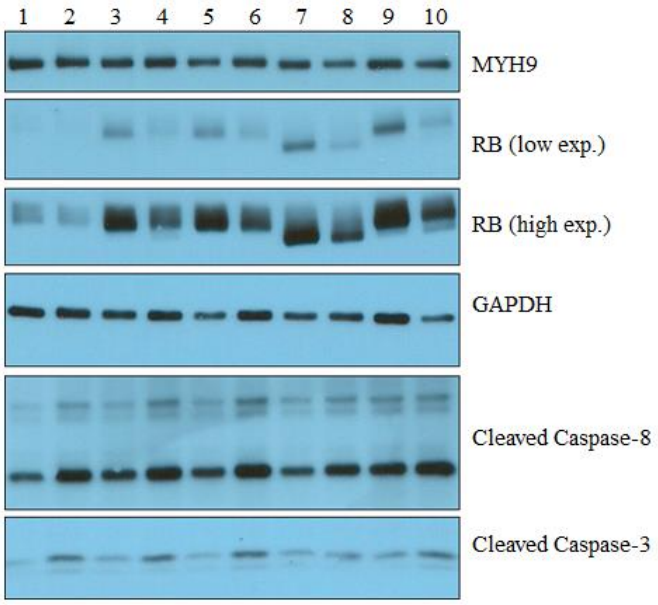
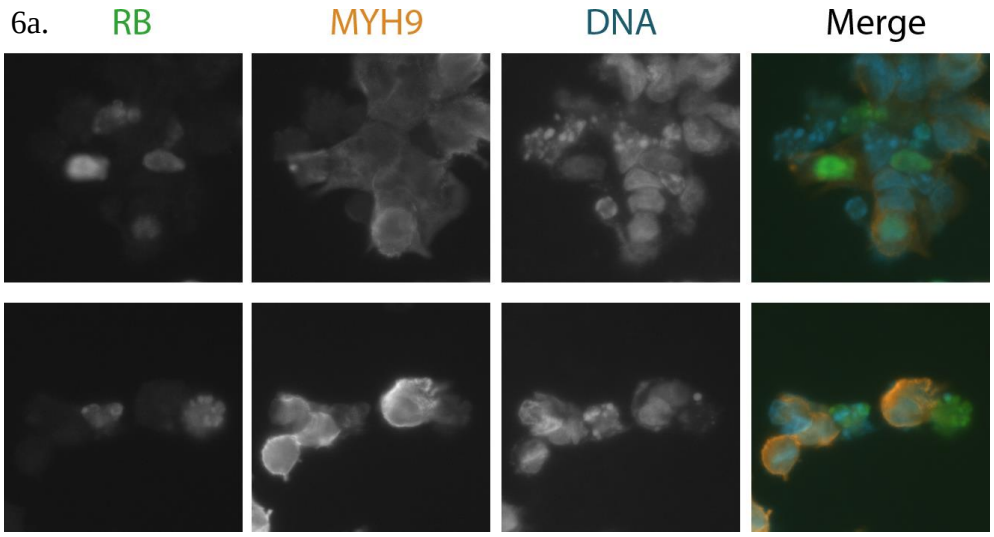


Figure 5. Transient co-transfection RB and FKBP-Caspase-8 into HCT116 shRNA-RB cells shows lack of MYH9 cleavage. (a) Transfection and treatment table for HCT116 RB-KD cells with FKBP-caspase-8. (b) Autoradiograp of blot probed with MHY9 and RB specific antibodies. Additionally probed with GAPDH antibody for loading control and activated/cleaved caspase antibodies.

6a.



6b.

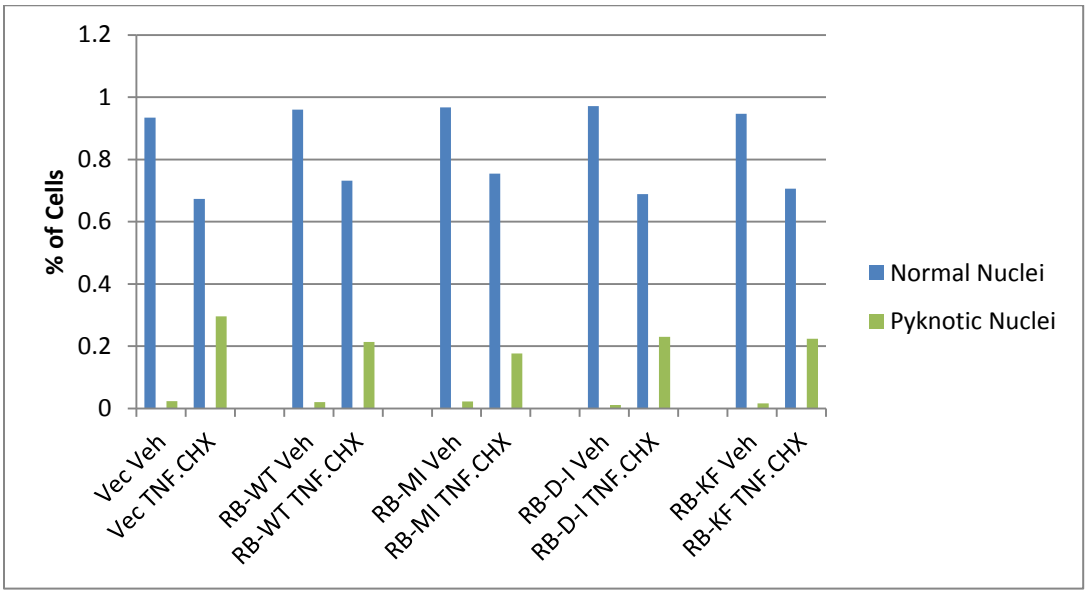


Figure 6. Immunofluorescence assay shows effects of transiently transfected RB and RB-mutants into HCT116 shRNA-RB cells treated with TNF/CHX. (a) Immunoflourescence image of RB-transfected cells with normal or pykontic nuclei. (b) Bar chart showing results of immunofluorescence assay. Cells were assessed based on nuclei condition (normal or pyknotic).

III.

Discussion

Retinoblastoma protein functions as a scaffolding protein; it interacts with multiple proteins and acts as a platform for their interaction. RB protein has well known interactors such as the E2F transcription factors that drive cell cycle [4, 5]. RB protein plays a role in tumor suppression as well as a role in regulating apoptosis. Our focus was on the study of apoptosis in colon cancer cells in relation to the RB C-terminal region. Nuclear β-catenin and survivin has been implicated in stabilizing RB [9]. β-catenin and survivin have been shown to interact with RB through co-immunoprecipitation and in-vitro GST pulldown assays. The mass spectrometry assay with the RB-C42 fragment pulldown assay demonstrated an interaction between MYH9 and the caspase-cleaved tail fragment of RB protein.

Survivin interacts with the C-terminal tail of RB

In the first GST pulldown experiment involving the GST-RB-C fusion protein, survivin was pulled down and displayed saturation characteristics with whole cell extract titrations. In an attempt to increase the efficiency of the GST pulldown assay, whole cell extract from RB-knockdown cells were utilized to eliminate the possibility that endogenous RB was sequestering a pool of survivin protein. The results were similar to the first pulldown, with no significant increase in pulldown efficiency when compared with input signals. Survivin has been shown previously to exist in two major forms: a monomeric form and a dimeric form [3 results]. Cells were incubated with a covalent crosslinker to preserve multimeric protein complexes. The pulldown with these whole cell extracts did not generate any signal in the molecular weight regions of the dimer (34kD) or the monomer (17kD). These results implicate that

survivin interacts with RB but the interaction is weak. There was saturation of the monomeric survivin pulldown in Fig 1 and 2 is not stoichiometric with the amount of GST-RB-C. A possible rationalization of this is that the interaction between the RB-C terminal region and survivin is a weaker part of a larger complex.

Interestingly, for all the pulldown assays, there was a consistent band being pulled down near the 70kD region and was reactive to the survivin-specific antibody. A tetramer of survivin would have an expected molecular weight of 68kD. A further possible route of experiment is to determine whether this consistent high molecular weight band is a tetramer of survivin or if it is possibly a protein complex with survivin incorporated.

MYH9 interacts with the C-terminal 42 amino acids of RB

The mass spectrometry assay revealed MYH9 as a significant interacting partner with the RB-C42 tail. These results were validated with co-immunoprecpitation pull-down assays and co-localization assays with immunofluorescence. HCT116 cells are sensitive to TNF-induced apoptosis; the results show RB cleavage following treatment with TNF/CHX but no significant change with MYH9 levels. In RB-KD HCT116 cells treated with TNF/CHX, RB is also cleaved and there is a significant decrease in MYH9 signal. This reduction in MYH9 levels is shown to be caspase-dependent and is substantiated by previous literature [18]. Transient transfection assays were performed with Western blot analysis and immunofluorescence to further understand the relation between RB and MYH9. The results revealed no major differences across the RB mutants.

There are several possible explanations explaining this phenomenon. A possible explanation is the sensitization to apoptosis is an off-target effect of the RB-shRNA. However, there is unlikely due to previous evidence that showed that RB-null cells also have increased sensitivity to TNF-induced apoptosis. Another possibility is that transient transfection of RB is insufficient to suppress apoptosis. Transient transfection is limited temporally in that the expression vector is not replicated/maintained indefinitely and this may be insufficient for enough RB to effectively suppress apoptosis. Another possibility is that these cells do not possess nuclear β-catenin, and RB may require its presence to suppress apoptosis. In future experiments, we can utilize a constitutively active S37A β-catenin test the hypothesis that apoptosis suppression requires RB and nuclear β-catenin. Another avenue for future research is to introduce stable RB constructs back into the HCT116 shRNA-RB cell line to investigate further in the effects of the RB mutants and MYH9.

IV.

Materials and Methods

Cell Culture and Transfection. HCT116 cells were maintained in DMEM media supplemented with Penicillin/Streptomycin and 10% Fetal Bovine Serum. Cells were culture at 37 degrees Celsius and 5% CO_2. Cells were cultured to a confluency of 80-90% and treated with TNF (10ng/mL) and CHX (2.5µg/mL) for 4 hours. For transient transfection, cells were seeded at 80-90% confluency, and then transiently transfected with expression plasmid, using GeneTran following manufacturer's protocol.

Protein Extraction and Western blotting. Protein extracts were harvested and sonicated in cold RIPA lysis buffer (50mM Tris, 150mM NaCl, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, 1mM PMSF, supplemented with protease and phosphatase inhibitors). Following centrifugation at 14,000 r.p.m at 4 degrees Celsius for 15 minutes, the whole cell lysate supernatant was isolated from remaining cellular debris. Protein lysates were prepared in SDS sample buffer and boiled for 10 minutes. For RB protein probing, lystates were incubated at 37 degrees for 15 minutes. Protein lystates were separated on Tris-glycine gels and transferred onto nitrocellulose membranes. Membranes were block with 5% non-fat milk for one hour. Blots were incubated in primary antibody for four hours to overnight. Blots were washed and then incubated in secondary antibody for 1 hour. Blots were incubated with chemiluminescent reagents for 10 minutes and then exposed onto x-ray film.

Immuno-fluorescence. Cells were cultured on collagen or poly-L-lysine coated coverslips at 80-90% confluency. Cells were fixed using 4% para-formaldehyde in PBS for 15 minutes; followed by a permeabilization step with 0.3% Triton-X in PBS.

Coverslips were incubated with primary antibody for 1 hour at 37 degrees Celsius. Following a wash step with PBS, coverslips were incubated with secondary antibody for 1 hour at 37 degrees Celsius. Following a wash step with PBS, coverslips were incubated with Hoerst DNA stain for 10 minutes. Coverslips were mounted onto microscope slides using PermaFluor Mounting media.

GST-Fusion Protein Bound Beads. BL21 competent cells were transformed with GST-fusion protein expression plasmid. Overnight culture of was generated and when optimal OD600 was achieved IPTG was added to induce protein expression. BL21 cells were freeze thawed and sonicated. Following centrifugation at 10,000r.p.m. for 10 minutes at 4 degrees Celsius, supernatant was run through a column with Glutathione Sepharose beads. The column was then washed twice and maintained in a 20% glycerol PBS solution.

In vitro protein binding assay by GST-fusion protein pulldown. Protein extracts were harvested and sonicated in cold NETN lysis buffer (50mM Tris, 150mM NaCl, 0.5mM EDTA, 0.5% NP-40). Following centrifugation at 14,000 r.p.m at 4 degrees Celsius for 15 minutes, the whole cell lysate supernatant was isolated from remaining cellular debris. Protein lysates were then incubated with the GST-fusion protein bound Sepharose beads at 4 degrees Celsius on a rotator for overnight. The beads were centrifuged down and washed three times with PBS and eluted with SDS sample buffer. Samples were boiled for 10 minutes. The samples were loaded onto Tris-Glycine gels for Western blotting analysis.

References:

1. U.S. Cancer Statistics Working Group. United States Cancer Statistics: 1999–2010 Incidence and Mortality Web-based Report. Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer

2. Network, T.T., (2012). Comprehensive molecular characterization of human colon and rectal cancer. Nature, 487(7407): p. 330-337.

3. Yamamoto, H., et al., (1999). Paradoxical increase in retinoblastoma protein in colorectal carcinomas may protect cells from apoptosis. Clin Cancer Res, 5(7): p. 1805-1815.

4. Dick, F. A., & Rubin, S. M. (2013). Molecular mechanisms underlying RB protein function. Nat Rev Mol Cell Biol, 14(5), 297–306.

5. Morris, E. J., & Dyson, N. J. (2001). Retinoblastoma protein partners. Advances in Cancer Research, 82, 1–54.

6. Rubin, S. M., Gall, A.-L., Zheng, N., & Pavletich, N. P. (2005). Structure of the Rb C-terminal domain bound to E2F1-DP1: a mechanism for phosphorylation-induced E2F release. Cell, 123(6), 1093–106.

7. Chau, B. N., Borges, H. L., Chen, T.-T., Masselli, A., Hunton, I. C., & Wang, J. Y. J. (2002). Signal-dependent protection from apoptosis in mice expressing caspase-resistant Rb. Nat Cell Biol, 4(10), 757–765.

8. Borges, H.L., Bird, J., Wasson, K., Cardiff, R.D., Varki, N., Eckmann, L., Wang, J.Y.J. (2005). Tumor promotion by caspase-resistant retinoblastoma protein. Proc. National Academy of Sciences, 102(42), 15587-115592.

9. Han, J., Soletti, R.C., Sadarangani, A., Sridevi, P., Ramirez, M.E., Eckmann, L., Borges, H.L., Wang, J.Y.J., (2013). Nuclear expression of β-catenin promotes RB stability and resistance to TNF-induced apoptosis in colon cancer cells. Mol Cancer Research, 11(3), 207-218.

10. Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell, 127(3), 469–480.

11. Arend, R. C., Londoño-Joshi, A. I., Samant, R. S., Li, Y., Conner, M., Hidalgo, B., … Buchsbaum, D. J. (2014). Inhibition of Wnt/β-catenin pathway by niclosamide: A therapeutic target for ovarian cancer. Gynecologic Oncology, 134(1), 112–20.

12. Athanasoula, K. C., Gogas, H., Polonifi, K., Vaiopoulos, A. G., Polyzos, A., & Mantzourani, M. (2014). Survivin beyond physiology: orchestration of multistep carcinogenesis and therapeutic potentials. Cancer Letters, 347(2), 175–82.

13. Chantalat, L., Skoufias, D. A., Kleman, J.-P., Jung, B., Dideberg, O., & Margolis, R. L. (2000). Crystal Structure of Human Survivin Reveals a Bow Tie–Shaped Dimer with Two Unusual α-Helical Extensions. Molecular Cell, 6(1), 183–189.

14. Xia, Z.-K., Yuan, Y.-C., Yin, N., Yin, B.-L., Tan, Z.-P., & Hu, Y.-R. (2012). Nonmuscle myosin IIA is associated with poor prognosis of esophageal squamous cancer. Diseases of the Esophagus, 25(5), 427–436.

15. Vicente-Manzanares, M., Ma, X., Adelstein, R. S., & Horwitz, A. R. (2009). Non-muscle myosin II takes centre stage in cell adhesion and migration. Nat Rev Mol Cell Biol, 10(11), 778–790.

16. Kopp, J.B., (2010). Glomerular pathology in autosomal dominant MYH9 spectrum disorders: what are the clues telling us about disease mechanism?. Kidney International 78, 130-133

17. Gedaly, R., Galuppo, R., Daily, M. F., Shah, M., Maynard, E., Chen, C. Spear, B. T. (2014). Targeting the Wnt/β-Catenin Signaling Pathway in Liver Cancer Stem Cells and Hepatocellular Carcinoma Cell Lines with FH535. PLoS ONE, 9(6), e99272.

18. Kato, M., Fukuda, H., Nonaka, T., Imajoh-Ohmi, S., (2005). Cleavage of Nonmuscle Myosin Heavy Chain-A during Apoptosis in Human Jurkat T Cells. J Biochem, 137(2), 157-166.

19. Miyamoto, H., Shuin, T., Torigoe, S., Iwasaki, Y., Kubota, Y., (1995). Retinoblastoma gene mutations in primary human bladder cancer. British Journal of Cancer, 71(4), 831-835.

20. Chang Xiaolu, D. W. Y. (2003). Activation of Procaspases by FK506 Binding Protein-Mediated Oligomerization. Science Signaling, 2003(167), pl1–pl1.

21. Ulukaya, E., Sarimahmut, M., Cevatemre, B., Ari, F., Yerlikaya, A., & Dimas, K. (2014). Additive enhancement of apoptosis by TRAIL and fenretinide in metastatic breast cancer cells in vitro. Biomedicine & Pharmacotherapy = Biomédecine & Pharmacothérapie, 68(4), 477–482.