ROLE OF BUTYRATE IN POST-TRANSCRIPTIONAL REGULATION OF INFLAMMATORY GENES BY REGULATING RNA BINDING PROTEINS

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ABSTRACT

ROLE OF BUTYRATE IN POST-TRANSCRIPTIONAL REGULATION OF INFLAMMATORY GENES BY REGULATING RNA BINDING PROTEINS

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Short chain fatty acids (SCFAs) are generated in the gut by commensal bacteria and are known to reduce inflammation by inhibiting the expression of inflammatory mediators. There are indications that SCFAs can also affect gene expression post transcriptionally. We have hypothesized that butyric acid, a SCFA, can mediate the post-transcriptional regulation of inflammatory mediators such as Cyclooxygenase-2 (COX-2) by affecting the global expression or cytoplasmic translocation of RNA binding proteins (RBPs). We treated colorectal cancer cell lines with sodium butyrate (NaBt) and observed that butyrate reduced the general protein expression of various RBPs and inhibited the cytoplasmic translocation of the stabilizing RBP, HuR. This was supported by a reduction in the NanoLuc reporter activity of several different AU-rich element (ARE) sequences when treated with butyrate and this reduction was maintained even when HuR was overexpressed. We showed that the observed reduced activity of HuR was a consequence of a decrease in p38 and MK2 phosphorylation and increased phosphorylation of Chk2 due to butyrate treatment. Our results suggest that butyrate can reduce the expression of inflammatory genes not only transcriptionally but also post-transcriptionally by affecting the subcellular localization or expression of mRNA stabilizing proteins.

Keywords: NaBt, HuR, ARE, post-transcriptional regulation, inflammation

ÖZ

RNA'YA BAĞLANAN PROTEİNLERİ DÜZENLEYEREK İNFLAMATUVAR GENLERİN POST-TRANSKRİPSİYONEL DÜZENLEMESİNDE BUTİRATIN ROLÜ

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Kısa zincirli yağ asitlerinin (KZYAlar) bağırsakta kommensal bakteriler tarafından üretildiği ve siklooksijenaz-2 (COX-2) dahil olmak üzere sitokinleri ve inflamatuvar aracıları transkripsiyonel olarak inhibe ederek inflamasyonu azalttığı gösterilmiştir. Hipotezimiz, bir KZYA olan bütiratın, RBPlerin ifadelerini global olarak veya sitoplazmik translokasyonlarını etkileyerek transkripsiyon sonrası düzenlenmelerinde rol oynayabileceğidir. Kolorektal kanser hücre hatlarını sodyum bütiratla (NaBt) muamele ettik ve bütiratın, çeşitli RBPlerin genel protein ifadesini azalttığını ve stabilize edici RBP, HuR'nin sitoplazmik translokasyonunu inhibe ettiğini gözledik. Bu, butirat ile muamele edildiğinde birkaç farklı AU-zengin element (ARE) sekansının NanoLuc raportör aktivitesindeki azalma ile desteklendi ve bu azalma HuR fazla ifade edildiğinde bile sürdürüldü. HuR'nin gözlenen azalan aktivitesinin, butirat muamelesinden kaynaklı p38 ve MK2 fosforilasyonundaki azalmanın ve Chk2nin fosforilasyonunda artışın bir sonucu olduğunu gösterdik. Sonuçlarımız, bütiratın, inflamatuvar genlerin ifadesini sadece transkripsiyonel olarak değil, transkripsiyon sonrası mRNA stabilize edici proteinleri inhibe ederek azaltabileceğini göstermektedir.

Anahtar Kelimeler: NaBt, HuR, ARE, post-transkripsiyonel düzenleme, inflamasyon

To ODTÜ, where it feels like home

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ABBREVIATIONS

3'UTR: 3' untranslated region

ARE: Adenylate/Uridylate rich elements

COX-2: Cyclooxygenase 2

CRC: Colorectal Cancer

CYT: Cytoplasmic

HDACi: Inhibition of histone deacetylases

IL: Interleukin

MAPKs: Mitogen-activated protein kinase

MK-2. MAPK-activated protein kinase 2

NaBt: Sodium butyrate

NF-κB: Nuclear factor κB

NSAIDs: Nonsteroidal anti-inflammatory drugs

NUC: Nuclear

RBPs: RNA Binding proteins

RNA IP: RNA immunoprecipitation

TNFα: Tumor necrosis factor α

WCE: Whole cell extract

CHAPTER 1

INTRODUCTION

1.1. Colorectal Cancer

Colorectal cancer (CRC) was the second most common cancer type both in prevalence and mortality worldwide in 2018 (Globocan, 2018). The risk factors for CRC include lifestyle factors such as being overweight or obese, lack of physical activity, a diet high in red meat, smoking and heavy alcohol use. Other risk factors include advanced age, personal history of adenomas or inflammatory bowel disease, family history of CRC, having an inherited syndrome, racial and ethnic background and having type 2 diabetes (American Cancer Society, 2018). Chronic inflammation, seen in inflammatory bowel diseases including ulcerative colitis and Crohn's disease, is considered to be a predisposing factor for CRC (Itzkowitz & Yio, 2004; Ullman, Odze, & Farraye, 2009) and patients with IBD are at higher risk of developing CRC as much as patients with a hereditary history of CRC compared to the general population (Itzkowitz & Yio, 2004; Ullman et al., 2009). Epigenetic changes such as conversion of 5'CpG to 5'TpG or mutations that result in the activation of oncogenes can lead to CRC as well (Wood et al., 2007) Additionally, the gut microbiome is considered to play a major role in the development and progression of CRC and also in the treatment of inflammatory disease (Drewes, Housseau, & Sears, 2016) .

1.2. Inflammation

Inflammation is a well-coordinated, adaptive response to restore the body's homeostasis mostly due to infection (pathogen-associated molecular patterns (PAMPs) and virulence factors) or injury (toxic compounds, allergens and irritants) but there are other factors that trigger inflammation such as signals from

malfunctioning or dead cells as well as stressed cells, ECM breakdown products or formation of endogenous crystals (Medzhitov, 2008). The initial response of inflammation is the recognition of the threat by tissue-resident macrophages and mast cells of the innate immune system, which then release several inflammatory mediators such as cytokines, chemokines and bioactive lipids. This is followed by the activation of blood vessels which leads to the delivery of plasma and leukocytes to the site of injury or infection and extravasation of neutrophils, which then eliminate the infectious or invading agents (Medzhitov, 2008). After the intervention of the neutrophils, inflammation continues with resolution and repair phases. For the resolution of inflammation, lipid mediators have critical roles. The switch of lipid mediators from pro-inflammatory prostaglandins to pro-resolution lipoxins and resolvins prevents the recruitment of neutrophils while it supports the non-phlogistic recruitment of monocytes that are involved in the tissue-remodelling and removal of dead cells (Medzhitov, 2008). If the inflammation process is not followed by resolution, it may lead to chronic inflammation.

1.2.1. Inflammation and CRC

The initiation, progression and resolution of inflammation are closely regulated; deregulation of inflammation is pathogenic and is observed in many cancer types including CRC (Coussens & Werb, 2002). Long term, low dose use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin is known to be associated with reduced risk of CRC and other cancer types (Friis, Riis, Erichsen, Baron, & Sørensen, 2015). Several cytokines are involved in the promotion phase of CRC such as interleukin-1 (IL-1), IL-6, IL-8 and tumor necrosis factor (TNFα) which are target genes of the pro-inflammatory transcription factor nuclear factor κB (NF-κB) pathway (Karin & Greten, 2005). NF-κB is also a critical regulator for the transition from IBD to CRC and its activation can lead to initiation, promotion and progression of cancer (Schwitalla et al., 2013).

1.2.2. Role of COX-2 in Inflammation and CRC

In addition to the secreted cytokines and chemokines that are involved in the chronic inflammation, pro-inflammatory mediators such as cyclooxygenases (COX) and lipoxygenases (LOX) can promote CRC (Janakiram & Rao, 2014; Tuncer & Banerjee, 2015). COX and LOX are enzymes that catalyze the generation of prostaglandins from polyunsaturated fatty acids such as arachidonic acid (Janakiram & Rao, 2014; Tuncer & Banerjee, 2015). Two different COX enzymes are expressed: the constitutively expressed COX-1 and the inducible form COX-2 (Tuncer & Banerjee, 2015). The expression of COX-2 is generally induced during inflammation and leads to the synthesis of prostaglandin E2 (PGE2) which can promote tumor cell proliferation and angiogenesis and inhibits apoptosis in CRC (Castellone, Teramoto, & Gutkind, 2006). COX-2 is known to be overexpressed in colon cancer (Eberhart et al., 1994) and COX-2 deficiency in mouse models with colon cancer showed a decrease in tumorigenesis (Chulada et al., 2000). NSAIDs like aspirin inhibit the COX-2 enzyme and subsequently suppress colon tumor development (Wright, 2002) and thus are used for decreasing the risk of CRC (Chan et al., 2005). Therefore, suppression of COX-2 is an essential preventive strategy in CRC.

1.3. Butyrate

Butyrate is a short-chain fatty acid that is produced in the intestinal lumen particularly by *Clostridium* clusters IV and XIVa (Louis & Flint, 2009; Van Den Abbeele et al., 2013) by the fermentation of undigested dietary carbohydrates that pass through upper gut (Hamer et al., 2008; Louis & Flint, 2009). Butyrate is important for gut homeostasis such that patients with IBD have reduced levels of butyrate in their fecal samples (Marchesi et al., 2007). Furthermore, butyrate producing bacteria are reduced in the feces of patients with CRC compared to healthy people (Wu et al., 2013). Additionally, butyrate responsive genes are known to be involved in proliferation, differentiation and apoptosis of colonic epithelial cells and are deregulated in CRC

(Daly & Shirazi-Beechey, 2006). Regulation of gene expression in the presence of SCFAs such as butyrate can be via the hyper-acetylation of histones through the inhibition of histone deacetylases (HDACi) (Sealy & Chalkley, 1978). In colon cancer cells, butyrate, by its HDACi activity, was shown to suppress the activation of NF-κB (Inan et al., 2000), which is involved in the regulation of expression of inflammatory cytokines (Baeuerle & Henkel, 1994). Butyrate can also increase the expression of peroxisome proliferator-activated receptor γ (PPAR γ) (Wächtershäuser, Loitsch, & Stein, 2000), which plays a role in the reduction of intestinal inflammation (Dubuquoy et al., 2006) and in tumor suppression in colon cancer (Girnun et al., 2002) suggesting an anti-inflammatory role of butyrate.

1.4. RNA Binding Proteins (RBPs)

RNA Binding proteins (RBPs) are involved in the post-transcriptional regulation of messenger RNAs (mRNAs) of proto-oncogenes, transcription factors and cytokines by binding to Adenylate/Uridylate rich elements (ARE) such as AUUUA or UUAUUUAUU (Chen & Shyu, 1995) found in their 3'untranslated region (3'UTR) (Anderson, 2008). RBPs regulate the stability of mRNAs by binding to ARE regions through their RNA recognition motifs (RRM), zinc finger domains or K-homology domains and thus are involved in their translational fate (Anderson, 2008). Some of these RBPs can inhibit the translation of mRNAs e.g. TIA-1, TIAR (Piecyk et al., 2000) and CUGBP2 (Mukhopadhyay et al., 2003), some of them can lead to the decay of the mRNA e.g. TTP (Carballo, 1998) while others can stabilize the mRNAs e.g. HuR (Peng, Chen, Xu, & Shyu, 1998). RBPs such as AUF1 have several isoforms (p37, p40, p42 and p45) and some of these variants promote (p37, p42) (Sarkar, Xi, He, & Schneider, 2003) or inhibit (p40, p45) (Raineri, Wegmueller, Gross, Certa, & Moroni, 2004) mRNA decay.

RBPs, upon binding, can recruit poly-A tail specific exonucleases and lead to the decay of mRNAs via de-adenylation of the poly-A tail (Gherzi et al., 2004; Webster, Stowell, & Passmore, 2019). In addition, RBPs can mediate 3'-5' exonuclease degradation of the bound mRNAs in exosomes (Mukherjee et al., 2002) or decay through de-capping (Gao, Wilusz, Peltz, & Wilusz, 2001). Stabilizing RBPs, on the other hand, promote translation of mRNAs by competing with microRNAs or by blocking the association of other RBPs with the same 3'UTR (Gardiner, Twiss, & Perrone-Bizzozero, 2015). RBPs do not only compete with each other but can interact cooperatively for the regulation of mRNA stability (Katsanou et al., 2005).

Since many mediators involved in acute inflammation are short lived and their expressions are highly temporal, regulation of their expression through both transcriptional and post-transcriptional mechanisms are prevalent. 3'UTR of many inflammatory mRNAs are targets of RBPs and therefore RBPs have critical roles in immune homeostasis (Mino & Takeuchi, 2018). Inflammatory mRNAs that are known to be targeted by RBPs include TNFα (Carballo, 1998), IL-23 (Lee et al., 2013), COX-2 (Young et al., 2009) and many more (Mino & Takeuchi, 2018). A previous study showed that expression of TTP, a de-stabilizing RBP is induced with HDAC inhibitors and led to the decay of its target, the COX-2 mRNA (Sobolewski, Sanduja, Blanco, Hu, & Dixon, 2015), suggesting that butyrate has a role in the regulation of RBPs and therefore in the mRNA stability. Accordingly, butyrate's effect on other RBPs for the stability of inflammatory genes came into prominence. In this study, we focused on the stabilizing RBP HuR since it recognizes the 3'UTR of many pro-inflammatory mediators that are involved in CRC.

1.4.1. HuR

HuR was first identified as a fourth member of the neuronal specific Elav-like family proteins but was found to be also expressed in various non-neuronal cell types (Ma, Cheng, Campbell, Wright, & Furneaux, 1996). HuR recognizes the ARE motifs

AUUUA, AUUUUA and AUUUUUA (Ma et al., 1996). This RBP can shuttle between the nucleus and cytoplasm; however, both endogenous and overexpressed HuR are found to be primarily localized in the nucleus (Fan & Steitz, 1998). HuR binds to the ARE regions of mRNAs in the nucleus and then it acts as an adapter that transports the bound mRNA to the cytoplasm (Gallouzi & Steitz, 2001), therefore the cytoplasmic localization of HuR is an indication of the stabilization function of this RBP. The cytoplasmic localization of HuR is reported to be increased in many cancer types (Cho, Han, Soh, Lee, & Son, 2007; Heinonen et al., 2005; Young et al., 2009) and is considered to be a prognostic marker of malignancy (Heinonen et al., 2005). In CRC cell lines, overall HuR expression as well as its cytoplasmic localization was shown to be higher than non-transformed colon cell lines and this was also concomitant with up-regulated COX-2 expression observed in these cell lines (Young et al., 2009).

1.4.1.1. Pathways Involved in HuR Activity

1.4.1.1.1. p38-MK2 Pathway

Mitogen-activated protein kinases (MAPKs) are activated by phosphorylation on their tyrosine and threonine residues upon cellular stress or inflammatory stimuli (Cuadrado & Nebreda, 2010; Cuenda & Rousseau, 2007). The protein p38 is an important member of this family that exists as four isoforms. The isoforms p38α and p38β are expressed in most cell types, p38γ is expressed in skeletal muscle while p38σ is expressed in the pancreas, kidney and small intestine (Goedert, Cuenda, Craxton, Jakes, & Cohen, 1997). Among the four isoforms, p38α has been characterized extensively. Together with its downstream target MAPK-activated protein kinase 2 (MK-2), p38α is involved in many cellular events such as cell migration through actin cytoskeleton reorganization (Rousseau, Houle, Landry, & Huot, 1997), regulation of checkpoint controls due to DNA damage (Manke et al., 2005) as well as inflammation (Freshney et al., 1994; Ridley et al., 1998). p38α was initially identified as a kinase

that is activated by IL-1β and then phosphorylates MK-2. This cascade leads to the phosphorylation of heat shock protein HSP27 as one of the targets (Freshney et al., 1994). The p38-MK2 signaling pathway also controls the post-transcriptional regulation of many inflammatory genes through their 3'UTR such as TNF and IL-6 (Neininger et al., 2002). In an MK2 knocked-down mice model, production of TNFα and IL-6 was decreased upon LPS stimulation (Kotlyarov et al., 1999). Similarly, p38-MK2 pathway was shown to be involved in the regulation of COX-2 expression (Ridley et al., 1998) and its mRNA stability (Lasa et al., 2000). Another target of p38 is the stabilizing RBP HuR which is phosphorylated at its T118 residue leading to its cytosolic translocation, this in turn leads to the stabilization of p21 mRNA leading to G1 phase arrest during cell cycle (Lafarga et al., 2009). Mutations at the T118 residue of HuR or blockage of the p38 pathway by using SB203580 (p38 MAPK inhibitor) was shown to decrease the expression of cPLA(2)α which, normally, HuR binds to and stabilizes upon IL-1β treatment (Liao, Wang, Chang, & Tseng, 2011). Therefore, the p38-MK2 pathway is critical for the regulation of inflammatory gene expression and response and this regulation might be through altered subcellular localization of HuR.

1.4.1.1.2. Chk2 Pathway

The serine/threonine kinase Chk2 is best described with its roles in DNA damage response involving in double strand break repair, base excision repair and cell cycle arrests (Zannini, Delia, & Buscemi, 2014). In addition to being a key component in DNA damage response, it participates in apoptosis, senescence and DNA structure modification (Zannini et al., 2014). Furthermore, Chk2 can phosphorylate HuR at S88, S100 and T118 and in fibroblasts subjected to oxidative stress, this phosphorylation was shown to promote apoptosis by leading to the dissociation of HuR from its target, the NAD+ dependent protein deacetylase sirtuin 1 (SIRT1), which enhanced survival of stressed cells (Abdelmohsen et al., 2007). In the colorectal cancer cell line HCT-

116, Chk2 activation was shown to cause a global dissociation of HuR from its target mRNAs when the cells were subjected to radiation and this dissociation enhanced cell survival (Masuda et al., 2011). The cross talk between Chk2 and HuR is not simply restricted to their roles in cell survival; Chk2 was also shown to protect HuR from degradation through ubiquitination due to heat shock and thus led to the stabilization of HuR itself (Abdelmohsen et al., 2009). These findings show that Chk2 is a crucial factor in regulating the activity and function of HuR and therefore it is likely that the same cross talk exists during an inflammatory response, too.

1.5. Aim of the Study

Butyrate can be found in mM amounts in the colon as a result of microbial metabolic activity. This SCFA can exhibit HDACi activity and transcriptionally regulate the expression of mammalian genes. However, some studies suggest that butyrate may also affect gene expression post transcriptionally. In this study, we aimed to establish mechanistically the role of butyrate on post transcriptional regulation of gene expression. We hypothesized that butyrate, by suppressing the expression or cytosolic translocation of RNA binding proteins, can regulate the expression of inflammatory genes. We examined the expression and subcellular location of several RBPs in the presence of butyrate. Focusing on the stabilizing RBP HuR, we analyzed how cells treated with butyrate showed altered signaling pathways that affected the cytosolic translocation of HuR. We also established whether the RNA binding of HuR and 3'UTR activity of many ARE sequences were affected in the presence of butyrate. Overall, our data suggest that butyrate producing bacteria may contribute to gut health not only through transcriptional regulation, but also through post transcriptional regulation of gene expression.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Culture

Caco-2 and HT-29 cell lines were purchased from Şap Enstitüsü (Ankara, Turkey) and HCT116 cell line was purchased from DKFZ (Heidelberg, Germany). Caco-2 cells were grown in Eagle's minimum essential medium (EMEM) (Gibco) supplemented with 20% fetal bovine serum (FBS), 2mM L-glutamine, 1mM sodium pyruvate, 0.1 mM non-essential amino acids and 1% penicillin/streptomycin. HT-29 and HCT116 cells were grown in RPMI 1640 without phenol red (Biological Industries, Israel) supplemented with 10% FBS, 2mM L-glutamine and 1% penicillin/streptomycin. All three cell lines were incubated at 37 °C with 5% CO_2. All cells were tested for mycoplasma contamination frequently and were treated with 2.5 µg/ml Plasmocin® (Invivogen, France) for prevention.

2.1.1. Caco-2 Cell Line

Caco-2 is a human colon cancer cell line that can undergo confluency dependent differentiation into enterocyte-like cells with characteristics such as apical-basolateral polarization, formation of tight junctions and domes and expression of hydrolases (Grasset, Pinto, Dussaulx, Zweibaum, & Desjeux, 2017). Differentiation of Caco-2 cells lead to decrease in glucose consumption whereas accumulation of glycogen increases (Rousset et al., 1985). Even when the cells are in a proliferative state, Caco-2 cells are highly heterogeneous with different subpopulations. This is primarily because the cells have been used in numerous laboratories with different culture

protocols for many decades; therefore, the obtained results by using this cell line can show variabilities (Sambuy et al., 2005)

2.1.2. HT-29 Cell Line

The human colon cancer cell line HT-29 can also exhibit enterocytic differentiation when grown to confluency under glucose deprivation (Zweibaum et al., 1985). Similar to Caco-2 cells, differentiated HT-29 cells show polarization of cell layer, formation of tight junctions and expression of sucrose-isomaltase; however, if these cells are grown in glucose containing medium they do not undergo differentiation even when they reach confluency (Zweibaum et al., 1985) . Moreover, HT-29 cells can de-differentiate when they are grown in medium with glucose after a few passages (Zweibaum et al., 1985).

2.1.3. HCT116 Cell Line

HCT116 cells were isolated from a primary single human colonic carcinoma as three subpopulations (Brattain, Fine, Khaled, Thompson, & Brattain, 1981). On mouse fibroblasts and in soft agarose, HCT116 cells are able to form colonies and when they are injected to nude mice, they can exhibit tumorigenic properties (Brattain et al., 1981). Compared to Caco-2 and HT-29, HCT-116 cells do not differentiate (Yeung, Gandhi, Wilding, Muschel, & Bodmer, 2010). These cells are shown to be very effective for gene transfections (Yamano, Dai, & Moursi, 2010).

2.2. Treatments

Cells were treated with 1mM, 3mM and 5mM sodium butyrate (NaBt, Sigma-Aldrich, Germany) or vehicle (PBS) for 3, 6, 9, 12 or 48 h. NaBt was purchased as a powder and was stored in desiccator. To prevent oxidation, the vial was filled with argon after

each use. NaBt was prepared as a 100mM stock solution in cell culture grade PBS for treatments and kept at -20 °C.

2.3. RNA Isolation and cDNA Synthesis

After treatments, cells were trypsinized and collected. The cell pellets were washed with PBS twice by centrifuging at 500 x *g* for 5 min. Total RNA isolation was carried out by using the NucleoSpin RNA Kit (Macherey Nagel, Germany) according to the manufacturer's guidelines and the RNAs were stored at -80 °C until use. cDNA was synthesized from 2µg of RNA by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). cDNAs were synthesized by using oligo-dT primers (same kit supply) to maintain the 3' untranslated region (3'UTR) of the mRNAs and stored at -20 °C.

2.4. RT-PCR and qRT-PCR

RT-PCR reactions were prepared in total of 20 µl volume of mixture containing 2 µM forward and reverse primers, 0.2 mM dNTP mix, 1X PCR Buffer (Applied Biological Materials (ABM), Vancouver, Canada) and 1 U *Taq* Polymerase (ABM). PCR reaction was carried out in a thermal cycler with initial denaturation (5 min) and denaturation (30 sec) at 94 °C, extension (30-45 sec) and final extension (7 min) at 72 °C. PCR products were run on agarose gel and visualized by staining the products with 1X loading dye and observation under UV light. For the PCR reactions that were carried out for cloning, Phusion DNA Polymerase (NEB, Ipswich, MA, USA) and Q5 High Fidelity DNA Polymerase (NEB) were used due to their higher fidelity. For these polymerases, initial denaturation (30 sec) and denaturation (10 sec) were at 98 °C, extension (30-60 sec) and final extension (7 min) were at 72 °C

qRT-PCR was carried out in Bio-RAD CFX Connect (Bio-Rad, USA) by using 0.2 ml 8-strip tubes. Standard amplification curves were generated firstly to determine the reaction efficiency of the primers and according to the standard curve, the dilution factors of the cDNAs were determined. Initially, cDNAs were diluted 1:500 with nuclease free water and the reaction mixture was prepared with 1 µl diluted cDNA, 5µl GoTaq qPCR Master Mix (Promega, Madison, WI, USA), 1 µM forward and reverse primers and up to 10 µl dH_2O. The primers and their reaction conditions are shown in Table 2.1. Threshold cycle (Ct) values were calculated after a 40 cycle reaction and the transcriptional expression changes were determined based on the relative standard curve method. Transcriptional level changes were calculated according to Pfaffl method (Pfaffl, 2001). MIQE guidelines were followed during the qRT-PCR reactions (Bustin et al., 2009).

2.5. Plasmids and Constructs

2.5.1. Cloning of HuR to pGWIZ RPS30M vector

For the overexpression of RNA binding protein (RBP) HuR, the coding sequence of the gene fused with MYC epitope tag was cloned into pGWIZ RPS30M vector. This vector contains a modified ribosomal protein RPS30M promoter which was shown to non-responsive to transcriptional stimuli (Hitti et al., 2010). The vector was a kind gift from Prof Khalid S. A. Khabar of the King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia. pGEX-6P-1 ELAVL1 vector was used as a template for the PCR reactions where the MYC tag was added to the 3' end of the HuR gene. These reactions were carried out by using Phusion DNA Polymerase (NEB). The PCR conditions are shown in Table 2.1. After addition of the MYC tag, the PCR product was used as a template to add a Kozak sequence to the 5' end and restriction sites to both 5' and 3' ends. After each PCR reaction, the product was run on 1.2 % agarose gel and gel isolation was done with NucleoSpin Gel and PCR Clean-up Kit (Macherey Nagel). The final PCR product (350 ng) and the vector (1.5 µg) were each cut with 1

µl *SalI* HF (NEB) and 1 µl *NotI* (NEB) in 1 µl NEB 3.1 Buffer in a 10 µl reaction mixture at 37 °C overnight (O/N). After clean-up, the cut insert and vector (50 ng) were ligated by using T4 DNA ligase with 1:5 and 1:10 vector to insert ratios for 16 °C, O/N. The ligation was inhibited by incubating at 65 °C for 20 min. Ligation products were stored at -20 °C.

2.5.2. Cloning of ARE regions to pGWIZ RPS30M SuperNanoLuc vector

Two canonical AU-rich regions (ARE) as Artificial ARE (Art. ARE) and HuR binding ARE (HuR ARE) as well as the ARE sequence from the 3'UTR of TNFα (Sakai, Kitagawa, & Hirose, 1999) were cloned into pGWIZ RPS30M SuperNanoLuc vector at its BamHI site. These ARE regions were purchased as oligos and the sequences are shown in Table 2.1. Oligo pairs were diluted with nuclease free water to a final concentration of 10 µg/ml. Each oligo was phosphorylated with T4 Polynucleotide Kinase (NEB). For the annealing of the oligos, 3 µg/ml of each oligo was used and the pairs were incubated in 1X annealing buffer (10X: 100mM Tris pH:8, 500Mm NaCl, 10mM EDTA pH:8) at 90°C for 4 min, 70 °C for 10 min, 37 °C for 20 min, 25 °C for 10 min and 10 °C for 10 min. 2.5 µg pGWIZ RPS30M SuperNanoLuc vector was cut with *BamHI* (Thermo) in NEBCutter 3.1 Buffer at 37 °C for 4 h beforehand and the cut vector was incubated with 1U CIAP phosphatase (Thermo) at 37 °C for 7 min. The annealed oligos and the phosphatase treated, *BamHI* cut vector were ligated by T4 DNA Ligase (NEB) at 16 °C, O/N. The ligation was inhibited by incubating at 65 °C for 20 min. Ligation products were stored at -20 °C.

2.5.3. Cloning of COX-2 ARE region to pGWIZ RPS30M SuperNanoLuc vector

The ARE rich region (first 190 bp) of the 3'UTR of COX-2 was cloned into pGWIZ RPS30M SuperNanoLuc vector at *BamHI* site. This region had previously been cloned into a psiCHECK-2 dual luciferase vector in our lab and was used as a template to

amplify the regions by PCR. The COX-2 ARE region was amplified by using Q5 HF DNA Polymerase and *BamHI* restriction sites were added to both ends in the PCR reaction. The PCR products were run on an agarose gel and gel isolation was carried out. The pGWIZ RPS30M SuperNanoLuc vector and the amplified regions were cut with *BamHI* (Thermo) in NEBCutter 3.1 buffer at 37°C for 4 hours. For both cut vector and the inserts, clean-up (NucleoSpin Gel and PCR Clean-up Kit, Macherey Nagel) was applied. The inserts and the vector were ligated by T4 DNA Ligase (NEB) at 16 °C, O/N. The ligation was inhibited by incubating at 65 °C for 20 min. Ligation products were stored at -20 °C.

Table 2.1. *Primers and oligos used for cloning*

Target gene	Forward 5'->3'	Reverse 5'->3'	Product length	Annealing temp (˚C)
Primers for Cloning				
HuR oex_1	GCCACCATGTCTAATGGTTATGAAGAC	TTACAGATCCTCTTCTGAG ATGAGTTTTTGTTCTTTGTGGGACTTGTTGG	NA	54
HuR oex_2	ACGCGTCGACACTAGTGCCACCATGTCTAATGG	GCGGGATCCGCGGCCGCTTACAG ATCCTCTTCTGAGAT	2 step PCR	NA
COX-2 ARE	GGGGGATCCAGAACGTTCGACTGAACT	GCGCGGATCCGTAGTGACATAAAAGTCT	170	53
HuR	ATGAAGACCACATGGCCGAAGACT	AGTTCACAAAGCCATAGCCCAAGC	189	66
Oligos for cloning				
Art. ARE (oligo)	GATCCATTTTATTTATATTATTTAA TTTTATTTATATTATTTAGCTAGCG	GATCCGCTAGCTAAATAATATAAATAAA ATTAAATAATATAAATAAAATG		
HuR ARE (oligo)	GATCCTTCCGATTTCCGATTTATTTCGGAT TTTTCCGATTTCCGATTTATTTCGGATTTGCTAGCG	GATCCGCTAGCAAATCCGAAATAAATCGGAA ATCGGAAAAATCCGAAATAAATCGGAAATCGGAAG		
TNF ARE (oligo)	GATCCTTATTATTTATTATTTATTTATTAT TTTTATTATTTATTATTTATTTATTATTTGCTAGCG	GATCCGCTAGCAAATAATAAATAAATAATAA ATAATAAAAATAATAAATAAATAATAAATAATAAG		

2.6. Protein Isolation

2.6.1. Total Protein Isolation

M-PER Mammalian Protein Extraction Kit (Thermo Fisher Scientific) along with 1X protease and phosphatase inhibitors (Roche, Germany) were used for whole cell extract (WCE) isolation. The collected cell pellets were washed with PBS twice and

centrifuged at 500 x *g* for 5 min at 4 °C. After adding the M-PER mixture, the cell pellets were vortexed for 15 sec and incubated on ice for 15 min. This procedure was repeated one more time. Lysed cells were centrifuged at 14000 x *g* for 10 min at 4 °C to collect supernatant containing proteins.

2.6.2. Cytoplasmic and Nuclear Protein Isolation

The collected cells were washed with PBS twice and centrifuged at 500 x *g* for 5 min at 4 °C. The cell pellets were lysed with 300 µl hypotonic buffer (10 mM HEPES pH:7.5, 4mM sodium fluoride, 10 µM sodium molybdate, 0.1 mm EDTA, 1X protease and phosphatase inhibitors). The lysed cells were incubated on ice for 15 min and then mixed with 75 µl 10% NP-40 (Pan-Reac AppliChem, Darmstadt, Germany). The mixture was centrifuged at the highest speed for 30 sec at 4 °C and the supernatant, which contains the cytoplasmic fraction, was transferred to a new Eppendorf tube. The remaining pellet was re-suspended in 80 µl nuclear extraction buffer (10mM HEPES pH: 7.9, 0.1mM EDTA, 1Mm EDTA, 1.5mM $MgCl_2$, 420mM NaCl, 10% glycerol, 1X protease and phosphatase inhibitors). The mixture was vortexed for 30 sec and incubated on ice on an orbital shaker for 15 min. This procedure was repeated twice. The mixture was centrifuged at 14000 x *g* for 10 min at 4 °C and the supernatant containing the nuclear fraction was collected in a fresh Eppendorf tube.

2.6.3. Quantification of Proteins

Concentrations of isolated proteins were measured by using Coomassie Protein Assay Reagent (Thermo Fisher Scientific) in disposable cuvettes. The isolated proteins were diluted 1:10 in dH2O and 1.5 ml of the Coomassie reagent was added and mixed. The absorbance was measured at OD_{595} with MultiSkan GO Microplate Spectrophotometer (Thermo Scientific). To determine the concentration of proteins, a

standard curve generated with different concentrations of bovine serum albumin (BSA) was used.

2.6.4. Western Blot

Proteins were separated in 10% SDS-PAGE gels by running at 100V and transferred to polyvinylidine fluoride (PVDF) membranes at 115V for 1 h and 15 min. The membranes were blocked in 5% Skim Milk (AppliChem) or 5% BSA (AppliChem) in 0.1% TBS-T for 1 h. at RT on an orbital shaker. Primary antibody incubation of the membrane was carried out at 4 °C O/N on a shaker followed by rinsing the membrane; incubation with the secondary antibody incubation was carried out at RT for 1 h. Protein bands were visualized either with WesternBright ECL HRP Substrate (Advansta) or Clarity ECL Substrate (Bio-Rad) by using ChemiDoc MP Imaging System (Bio-Rad). Band intensities were measured with ImageLab and the change in expression was determined with normalizing the band intensities to housekeeping proteins. All the antibodies used in the current study are shown in Table 2.2.

Table 2.2. *List of antibodies used in this study*

Antibody name	**Origin**	**Brand**	**Catalog No**
COX-2	Mouse	Cayman	CX229
β-Actin (C4)	Mouse	Santa Cruz	SC-47778
GAPDH (FL-335)	Rabbit	Santa Cruz	SC-25778
α-Tubulin	Mouse	ProteinTech	HRP-66031
TopoII-β (A-12)	Mouse	Santa Cruz	SC-365071
Lamin B1	Mouse	ProteinTech	66095-I-Ig
CUGBP2	Rabbit	Abcam	ab186430
ELAVL1/HuR	Mouse	ProteinTech	66549-I-Ig
AUF1	Rabbit	Novus	NBP1-88915
TIAR	Goat	Santa Cruz	SC-1749
p-p38 (T182)	Mouse	Santa Cruz	SC-7973
p38	Rabbit	Cell Signaling Technology	9212P
p-MK2 (T222)	Rabbit	Cell Signaling Technology	3316P
MK2	Rabbit	Cell Signaling Technology	30425
p-Chk2 (T68)	Rabbit	Cell Signaling Technology	2661S
Chk2	Rabbit	Santa Cruz	SC-9064
Goat α-mouse		Advansta	R05072 500
Goat α-rabbit		Advansta	R05072 500
Donkey α-goat		Advansta	R05077 500
Normal mouse IgG		Santa Cruz	SC-2025

2.7. Luciferase Assay

To determine the 3'UTR activity of target genes, a luciferase assay was carried out. Caco-2 cells that reached 50-60% confluency in 48 well plates were transfected with 40ng SuperNanoLuc vectors cloned with ARE sequences (Table 2.1) and 360ng firefly vector as an internal control. To determine the effect of HuR on ARE sequences in the presence of NaBt, Caco-2 cells at 50-60% confluency or HT-29 cells which were seeded at a density of $3x10^4$ cells per well in 48 well plates were transfected with 40 ng ARE fused SuperNanoLuc vectors, 80 ng HuR overexpression vector or 80 ng empty vector to keep the DNA amount constant and 280ng firefly vector. The

SuperNanoLuc empty vector was obtained as a gift from Dr Khalid S Khabar of King Faisal University, Saudi Arabia.

For all the transfections, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 1:2 DNA to reagent ratio was used according to the manufacturer's guidelines. The transfections were carried out in Opti-MEM reduced serum media (Thermo Fisher Scientific). After 6 h (for Caco-2 cells) or 24h (for HT-29 cells) incubation at 37 °C, the transfection medium was removed and the cells were washed with PBS. Next, the cells were treated with 5mM NaBt or vehicle (PBS) for 48 h. The cells were collected and processed for luciferase activity (NanoLuc and Firefly) with the Nano-Glo Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions using a Modulus luminometer (Turner Biosystems, Thermo Fisher Scientific)

2.8. RNA Immunoprecipitation (RNA IP)

RNA IP was carried out with HCT116 cells to determine whether the binding of endogenously expressed HuR to an HuR-binding ARE sequence was affected in the presence of NaBt. HCT116 cells were transfected with the vector containing the HuR binding ARE sequence in a T25 flask using 3.5 µg plasmid and 10 µl Lipofectamine 2000 (Invitrogen) for 6h at 37°C. After transfection, the transfection medium was removed and the cells were either treated with 5mM NaBt (Sigma) for 48 h or were not treated and allowed to grow in complete medium. RNA IP was performed with the transfected HCT116 cells as described previously (Peritz et al., 2006). For immunoprecipitation, $5x10^6$ cells, and for input, $3x10^5$ cells were used. Lysis of cells were carried out with 1 ml polysome lysis buffer (PLB) (100 mM KCl, 5mM $MgCl_2$, 10mMm HEPES, pH 7.0, 0.5% Nonidet P-40, 1mM DTT, 100 Uml^{-1} RNasin RNase inhibitor (Promega), 2mM vanadyl ribonucleoside complexes solution (Sigma), 25 µl ml^{-1} protease inhibitor cocktail (Roche)). Protein A/G magnetic beads were used for the pull down. Firstly 50% beads slurry was equilibrated in PLB by washing with 500

µl PLB twice and then restoring the original volume again with PLB. The bead slurry was aliquoted to three tubes with two of them used for pre-clearing and the third one for incubation with antibody bound cell lysate. Firstly, one pre-clearing aliquot of bead slurry was incubated with the cell lysate for 1h at 4°C with rotation. After the incubation, the mixture was placed on a magnetic rack to separate magnetic beads and the cell lysate. The supernatant was pooled and transferred to the second pre-clearing aliquot and incubated for 1h at 4°C with rotation. Next, the supernatant was incubated with 4 µg HuR antibody (ProteinTech, Manchester, UK, Cat. NO: 66549-1-Ig) or 4 µg mouse IgG antibody (Santa Cruz, Cat.NO: sc-2025) O/N at 4°C with rotation. The following day, the antibody incubated cell lysates were incubated with the remaining bead slurry aliquot for 4 h. at 4°C with rotation. After the incubation, the beads and supernatant were separated with the magnetic rack and the supernatant was discarded. The beads were washed firstly with 500 µl PLB for 5 min at 4°C with rotation four times and next with 500 µl PLB containing 1M Urea for 5 min at 4°C with rotation four times. Next, the beads which were re-suspended in 100 µl PLB with 0.1%SDS and 30 µg proteinase K were incubated at 50 °C for 30 min and then one volume (100µl) of phenol-chloroform-isoamyl mixture was added to the suspension. After vortexing the suspension, it was centrifuged to separate the phases. Upper water phase which contains the RNA was withdrawn and put into a fresh eppendorf tube. 100 µl of the water phase was mixed with 5 µl glycogen (20 mg/ml), 12 µl 3M sodium acetate and 250 µl %100 ethanol and ethanol precipitated at -20 °C overnight. The samples were centrifuged at 4°C for 20 min at 16000 x g. Ethanol was removed and the pellet was allowed to dry in air. The RNA pellet was dissolved in RNase free water and stored at -80 °C.

Immuno-precipitated RNA samples were converted to cDNA as described above. Primers used in qRT-PCR are shown in Table 2.3. To determine the binding of HuR to the HuR ARE construct, primers recognizing the Super NanoLuc sequence in the vector were designed and used. For determining binding of HuR to 3'UTR of eIF4E

(positive control), primer sequences of eIF4E gene were taken from a previous study (Topisirovic et al., 2009). According to the generated standard amplification curve with input cDNA, the dilution factor was determined as 1:500. The input Ct value was obtained by extracting the transformation of dilution factor in logarithmic scale base 2 from the measured input Ct value of both treated and untreated samples. The ΔCt value determined by extracting the obtained input Ct value from the Ct values of immune-precipitated samples. For untreated samples, untreated input Ct value was used and for treated samples, treated input Ct value was used. Mean of ΔCt values of IgG precipitated samples was calculated and ΔΔCt value was calculated for treated and untreated samples separately by extracting the mean value from ΔCt of IgG and HuR precipitated samples. The fold change of HuR precipitated samples compared to IgG precipitated samples was calculated for both untreated and NaBt treated samples.

Table 2.3. *Primers used for RNA IP analysis*

Target gene	Forward 5'->3'	Reverse 5'->3'	Product length	Annealing temp (˚C)
Primers for RNA IP				
NanoLuc	GCACTACGGCACACTGGT	TGTTGCCGTTCCACAGGG	131	60
eIF4E	TGTGGCGCTGTTGTTAATGT	ATTGCTTGACGCAGTCTCCT	252	59

2.9. Cell Cycle Analysis

Since the signaling pathway leading to the cytosolic translocation of HuR is known to be cell cycle dependent (Atasoy, Watson, Patel, & Keene, 1998; Wang, Caldwell, Lin, Furneaux, & Gorospe, 2000), HT-29 and Caco-2 cells were synchronized by overnight starvation. To confirm the synchronization of the cells, cell cycle analysis was carried out with flow cytometry. Firstly, O/N starved HT-29 and Caco-2 cells were released into complete medium for 12 h. Following this, HT-29 cells were treated with 5mM butyrate for 3, 6, 9 and 12 h while Caco-2 cells were treated with 5mM butyrate for 6h. Untreated, starved, released and butyrate treated cells for each time point were

collected and washed with PBS. Each sample was fixed by adding 1ml 70% ethanol dropwise followed by vortexing. The samples were incubated at -20 °C overnight. The next day, the samples were centrifuged at 400 x *g* for 5 min and the ethanol was removed. The samples were washed with PBS and centrifuged. Cells were incubated with PI staining solution (0.1% Triton X-100, 2mg/ml RNase A and 20mg/ml Propidium Iodide (Sigma)) for 30 min in dark at RT. After staining, the cells were analyzed for cell cycle assay by using Accuri C6 Flow Cytometry (BD Biosciences, USA). The samples were analyzed at FL-3 channel.

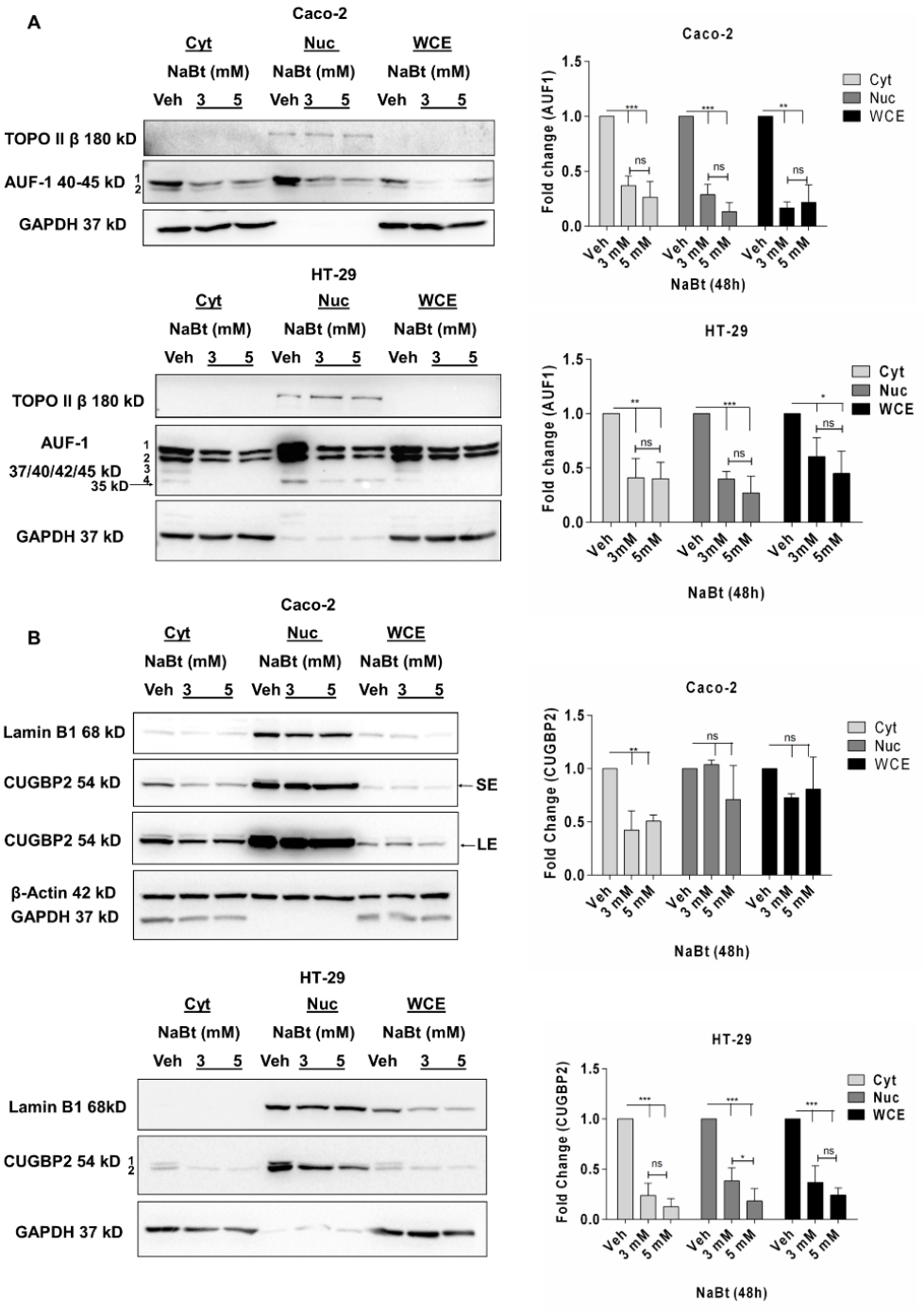
CHAPTER 3

RESULTS

3.1. Down-regulation of RBPs with NaBt Treatment of Colorectal Cancer Cell Lines

Inflammation is a closely regulated process where the turnover of inflammatory genes has a critical role during the progression and resolution of inflammation. Therefore, RNA binding proteins (RBPs) are likely to play a critical role in the regulation of inflammatory genes to determine their stability by binding to their 3'UTRs. Since butyrate is known to have anti-inflammatory functions and is known to affect gene expression (Berni Canani, Di Costanzo, & Leone, 2012), we sought to examine the effect of butyrate on the expression of several RBPs. We determined the cytoplasmic, nuclear and total protein amounts of the de-stabilizing RBPs, AUF1, CUGBP2 and TIAR; and the stabilizing RBP HuR change with two different concentrations (3mM & 5mM) of sodium butyrate (NaBt) treatment for 48h. in both HT-29 and Caco-2 cell lines. These RBPs were selected as they are known to bind to the 3'UTR of inflammatory mRNAs (Carpenter, Ricci, Mercier, Moore, & Fitzgerald, 2014; Ivanov & Anderson, 2013). We observed that treatment with NaBt reduced the overall protein levels (nuclear, cytoplasmic and whole cell extract) of AUF-1 and CUGBP2 in both cell lines (Fig 3.1A & 3.1B). In Caco-2 cells, the cytoplasmic and nuclear levels of TIAR decreased with NaBt treatment; however, in HT-29 cells, we did not observe a significant change in the cytoplasmic levels, although a decrease in the nuclear and whole cell levels was observed (Fig 3.1C). Interestingly, for HuR, we did not observe any change in the expression of the protein in the presence of NaBt, rather the cytoplasmic translocation of protein was decreased (with a concomitant increase in the nuclear levels) with butyrate in HT-29 cells (Fig 3.1D). The cytosolic HuR levels remained unchanged in Caco-2 cells treated with butyrate, although a decrease in the

nuclear and whole cell extract was seen (Figure 3.1D). These data suggest that butyrate might play a critical role in the activity of RBPs since it can reduce their protein expression and can affect their subcellular localization. We focused on HuR as our candidate RBP because its cytoplasmic translocation is known to be important for this RBP's activity on 3'UTR (Young et al., 2009). Moreover, several signaling pathways that regulate the cytosolic translocation of HuR have been described. Finally, a role of butyrate in the altered cytosolic translocation of HuR has not been described previously.


A
Caco-2
Cyt
Nuc
WCE
NaBt (mM)
Veh 3 5
TOPO II β 180 kD
AUF-1 40-45 kD
1
2
GAPDH 37 kD
HT-29
AUF-1
37/40/42/45 kD
35 kD
3
4
Fold change (AUF1)
Cyt
Nuc
WCE
Veh
3mM
5mM
NaBt (48h)
B
Lamin B1 68 kD
CUGBP2 54 kD
SE
LE
β-Actin 42 kD
GAPDH 37 kD
Lamin B1 68kD
Fold Change (CUGBP2)

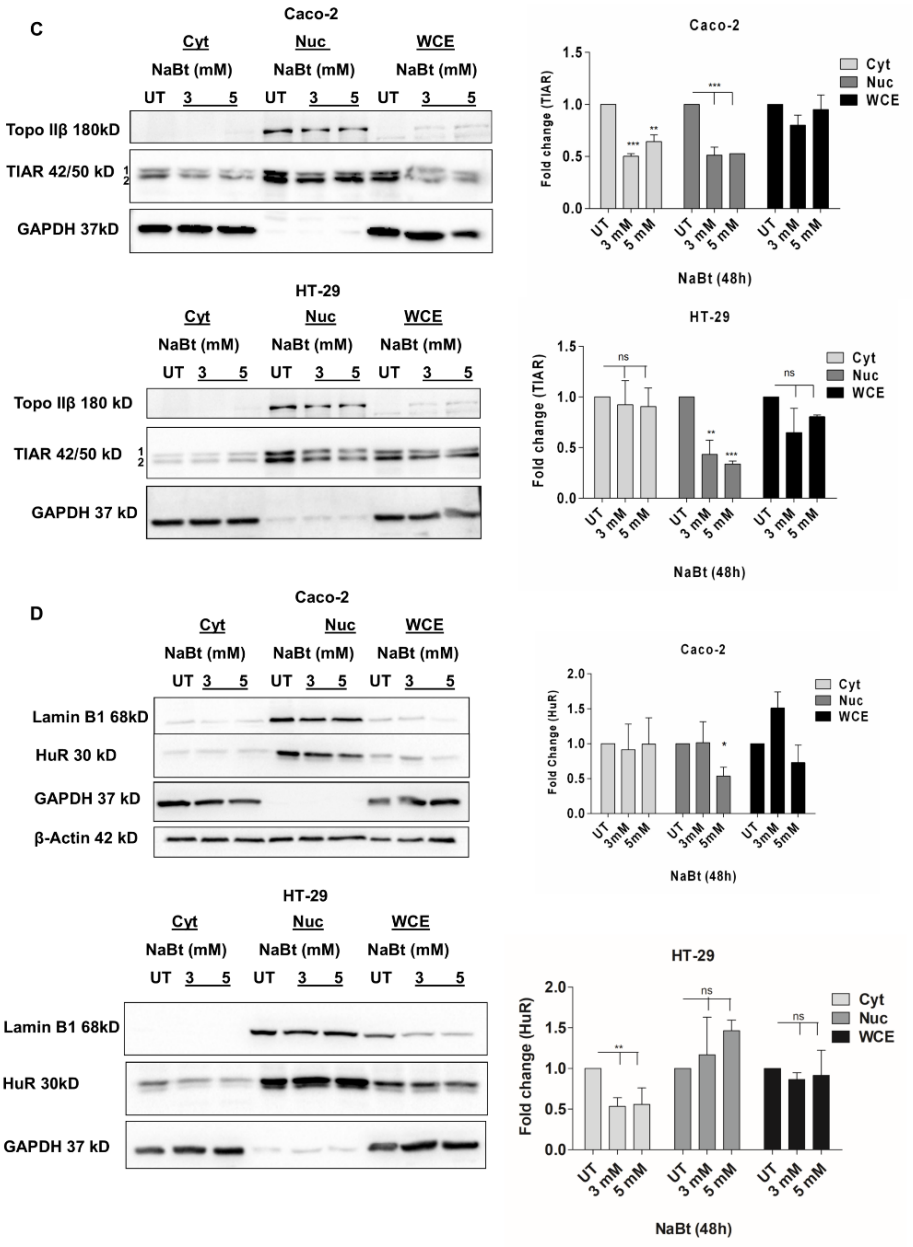


Figure 3.1. Expression of RBPs in Caco-2 and HT-29 cells treated with NaBt.

*Caco-2 (upper panels) and HT-29 (lower panels) were treated with 3mM or 5mM NaBt for 48h. Cytoplasmic (Cyt), nuclear (Nuc) fractions and whole cell extracts (WCE) were isolated. (A) Protein expression of two different AUF1 isoforms in Caco-2 and four different isoforms in HT-29 are shown. Expression of (B) CUGBP, (C) TIAR, (D) HuR are shown. The experiments were repeated at least three times independently and fold changes in expressions in the NaBt treated cells with respect to vehicle treated cells are shown in densitometric analyses. Statistical analyses were carried out using ANOVA followed by Tukey's multiple comparison test. GAPDH was used as cytoplasmic loading control while TopoIIβ or Lamin B1 were used as nuclear loading controls. *p<0.05,**p<0.001, ns: not significant. (Experiments in this figure were carried out by Shabnam Enayat, PhD).*

3.2. Effect of Butyrate on 3'UTRs Activity

To determine the effect of butyrate on 3'UTR activity, we designed a number of reporter assay systems. The first one contained an artificial ARE sequence (Art. ARE) to which many RBPs such as HuR, HuB and TTP can bind, the second contained an artificial ARE to which only HuR can bind (HuR ARE), the third was the ARE region (first 190bp) from the 3'UTR of COX-2 and the fourth was the ARE region from the 3'UTR of TNFα (described in Section 2.5). These ARE regions were cloned to a reporter vector which has a modified ribosomal protein RPS30 promoter (RPS30M) that was shown to be non-responsive to many inflammatory stimuli (Hitti et al., 2010).

Previous studies in our lab indicated that the SV40 promoter found in the reporter vector psiCHEK2 was responsive to butyrate. Cells transfected with the empty vector showed increased luciferase activity when treated with butyrate compared to empty vector transfected cells treated with vehicle (PBS) (data not shown). Therefore, we first tested whether the RPSM30 promoter would respond to NaBt. For this, we treated the empty vector transfected Caco-2 cells with 5mM NaBt for 48h and observed that the RPS30M promoter was not affected by NaBt treatment (Fig 3.2A).

We cloned the ARE sequences indicated above downstream of the Super NanoLuciferase sequence (please see Appendix A). First, we determined the effect of NaBt treatment on the canonical ARE sequences (Art. ARE and HuR ARE). In the vehicle treated cells, both vectors showed high 3'UTR activity suggesting that these sequences (Art. ARE and HuR ARE) were stabilized by endogenous RBPs found in Caco-2 cells. The increase in reporter signal observed with the HuR ARE construct was greater compared to the construct containing the Art. ARE sequence (Fig 3.2B) indicating that for HuR ARE, binding of only HuR stabilized the Nanoluc mRNA. The Art. ARE sequence, on the other hand, was predicted to bind to both stabilizing and destabilizing RBPs, suggesting that competition between RBPs for binding could have resulted in a weaker signal. When the transfected cells were treated with butyrate,

there was a decrease in 3'UTR activity suggesting that butyrate suppressed the endogenous RBPs that are involved in the stabilization of the mRNAs.

Since butyrate had a suppressive effect on endogenous RBPs, we next investigated whether the stabilizing effects of HuR when it is overexpressed could be inhibited in the presence of butyrate. Therefore, we transfected HT-29 and Caco-2 cells with a HuR overexpression vector along with the ARE constructs and treated the cells with NaBt. HuR overexpression alone led to an increase in the 3'UTR activity confirming its stabilizing effect; however, this stabilizing effect of HuR was lost when the cells were treated with NaBt (Fig 3.2C) suggesting that butyrate could repress the stabilizing effect of HuR on the 3'UTRs. To ensure that this effect was not cell line specific, we also transfected HT-29 cells with the HuR ARE reporter construct. Similar to Caco-2 cells, HuR overexpression increased the Nanoluc activity, however it was decreased when the cells were treated with NaBt for 48h even in the presence of HuR (Fig 3.2D, right panel). As with Caco-2 cells, HT-29 cells transfected with the empty vector did not show any change in luciferase activity when the cells were treated with butyrate or vehicle. This indicated that the RPS30M promoter was not responsive to NaBt in HT-29 cells, too (Fig 3.2D, left panel).

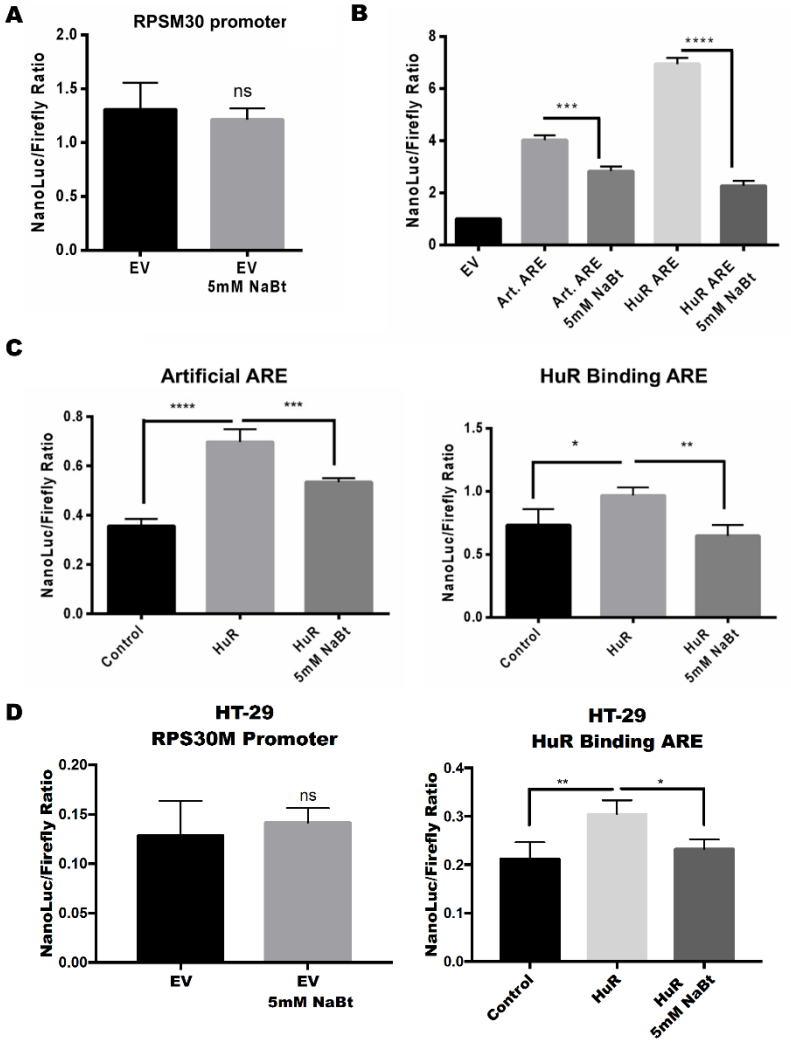


Figure 3.2. Luciferase assay showing the effect of NaBt on 3'UTR activity of canonical ARE sequences.

*Caco-2 or HT-29 cells were transfected with Art. ARE, HuR binding ARE (HuR ARE) constructs or empty vector separately and treated with 5mM NaBt for 48h. The cells were lysed for luciferase activity. pGWIZ RPS30M Firefly vector was used as a normalization control. (A) Cells transfected with empty vector containing RPS30M promoter showed no change in luciferase signal with butyrate treatment. (B) Cells transfected with Art. ARE or HuR ARE showed decrease in the 3'UTR activity with butyrate treatment. (C) Cells transfected with Art ARE (left panel) and HuR ARE (right panel) showed stabilization with HuR overexpression. Butyrate treatment suppressed this stabilization for both constructs. (D) HT-29 cells were transfected with empty vector (left panel) or HuR ARE (right panel) constructs separately. RPS30M promoter was not affected by butyrate (left panel). HuR overexpression stabilized HuR ARE and this effect was lost with butyrate treatment (right panel). Two independent replicates were carried out for statistical analysis. (ANOVA with Tukey's post hoc test, *p<0.05, **p<0,005, ***p<0,001, ****p<0.0001, ns: not significant)*

Next, we carried out an RNA-IP assay to determine directly whether butyrate treatment affected the binding of HuR to its target RNA sequences. For this we used the HCT116 cell line which has high transfection efficiency (Yamano et al., 2010). We first treated HCT-116 cells with NaBt for 48h and determined the protein levels of HuR in the cytoplasmic and nuclear fractions. As seen with HT-29 cells, the cytoplasmic HuR levels decreased dramatically with butyrate treatment in HCT116 cells as well (Fig 3.3. A).

For RNA-IP, we transfected HCT116 cells with the HuR ARE construct, treated the cells with NaBt for 48h and processed the cells for RNA-IP followed by PCR. We observed that the HuR antibody could pull down the HuR ARE sequences suggesting the binding of endogenous HuR (Fig 3.3B, second bar). When the cells were treated with 5Mm NaBt, the binding of HuR to the same target ARE was abolished (Fig 3.3B, fourth bar). The binding of HuR to eIF4E was used as a positive control (Fig. 3.3C). Together with the luciferase assay, these data suggest that butyrate mediated a suppressive effect on HuR mediated stabilization that resulted from reduced cytosolic translocation and therefore reduced binding of HuR to its target mRNA.

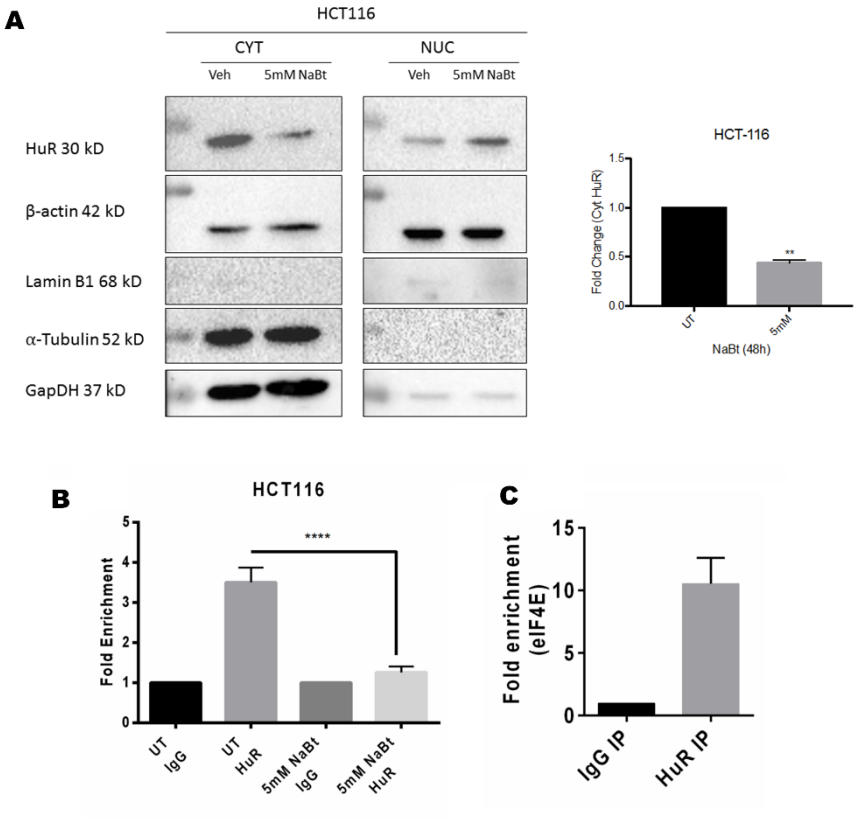


Figure 3.3. Effect of butyrate on binding of endogenous HuR to its target ARE sequence.

*(A) HCT116 cells were treated with NaBt for 48h to determine the nucleo-cytoplasmic localization of HuR. Cytoplasmic levels of HuR decreased significantly with butyrate treatment. Statistical significance was determined with t-test (**p<0,005). (B) HCTT16 cells were transfected with HuR ARE construct and treated with 5mM NaBt for 48h. The cells were collected and processed for RNA IP. RNA IP showed decrease in the binding of HuR to the ARE sequence in the presence of butyrate compared to vehicle treated cells. (C) The binding of HuR to the 3'UTR of eIF4E was used as positive control. RNA IP showed enrichment of binding of HuR to eIF4E 3'UTR. Two independent replicates were carried out for statistical analysis. (ANOVA with Tukey's multiple comparison test, ****p<0.0001)*

Next, we aimed to examine whether butyrate affected HuR mediated stabilization of well-known HuR targets, such as COX-2 and TNFα. We transfected Caco-2 cells with either the ARE sequences of COX-2 or TNFα along with the HuR overexpression vector and determined the Nanoluc reporter activity in the presence of butyrate. As seen with the artificial ARE sequences, we observed significant increases in reporter activities of both COX-2 and TNFα in cells overexpressing HuR; however, this

increase was inhibited by NaBt (Fig 3.4). Since butyrate can inhibit the binding of HuR to its target and considering HuR's role in stabilization of inflammatory genes, butyrate can have functional roles for resolution of inflammation through affecting post-transcriptional regulation of these genes.

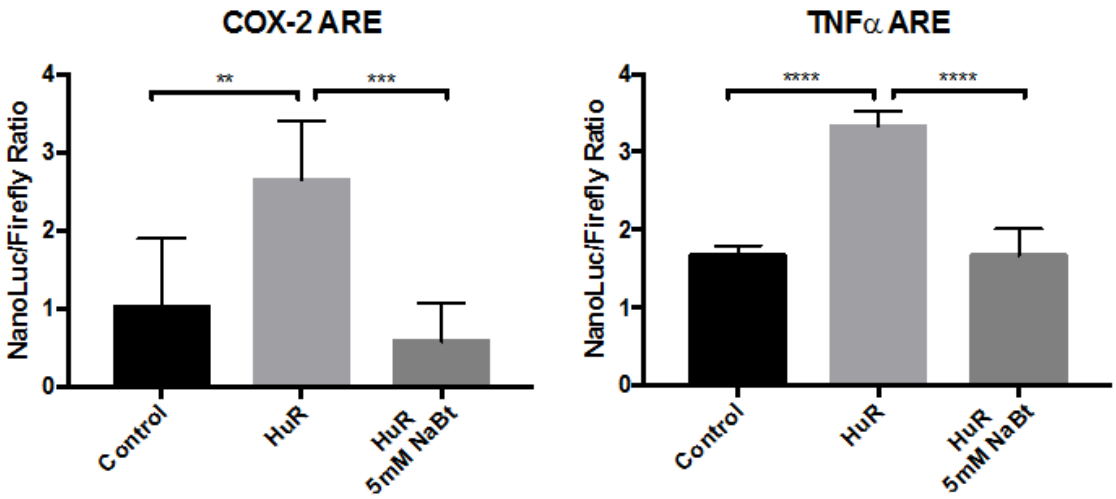


Figure 3.4. Luciferase assay showing the effect of butyrate on regulation of 3'UTR activity of inflammatory genes.

*Caco-2 cells were transfected with COX-2 ARE (left panel) and TNFα ARE (right panel) constructs and processed for luciferase assay. Both ARE sequences showed stabilization with HuR overexpression and this signal was lost with butyrate. Statistical analyses were carried out for two independent replicates with ANOVA followed by Tukey's multiple comparison test. (**p<0.005, ***p<0.0005, ****p<0.0001)*

As the 3' UTR reporter activity of the COX-2 ARE could be inhibited in the presence of butyrate, we next examined whether HuR overexpression in the presence or absence of butyrate would alter the protein expression of COX-2. Therefore, we transfected Caco-2 and HT-29 cells with a HuR overexpressing construct and simultaneously treated the cells either with either vehicle or 5mM butyrate for 48 h. HuR alone increased COX-2 protein expression, most likely due to mRNA stabilization, however; this stabilization was lost when the cells were treated with butyrate (Fig.

3.5). These results were consistent with the luciferase data showing the effect of butyrate and HuR on the COX-2 ARE (Fig. 3.4) suggesting that butyrate can reverse the stabilizing effect of HuR and lead to a decrease at the protein level, too.

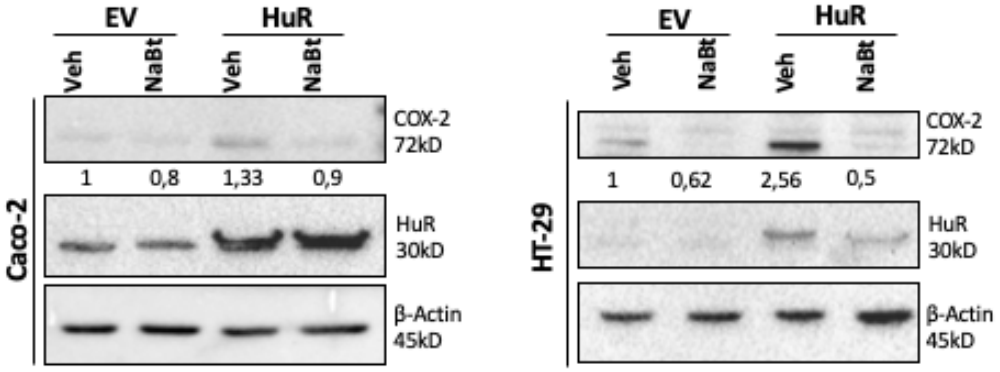


Figure 3.5. Western Blot showing the effect of butyrate on HuR stabilization of COX-2 protein.

Caco-2 (left panel) and HT-29 (right panel) cells were transfected with the HuR overexpressing vector and treated with 5mM butyrate for 48 h. Cells transfected with empty vector (EV) were used as control. HuR transfection increased the COX-2 protein levels in both cell lines. When the transfected cells were treated with butyrate, COX-2 protein levels were reduced. Densitometric analysis was carried out with respect to empty vector transfected and vehicle treated cells and normalized to the loading control β-actin.

3.3. Role of NaBt in Signaling Pathways Determining Nucleocytoplasmic Shuttling of HuR

We established that butyrate reduced the stabilization of mRNAs by inhibiting the cytoplasmic translocation and binding of HuR (Fig 3.3A). Therefore, we next focused on the pathways that are involved in the cytoplasmic translocation of HuR. Chk2 is a DNA damage response kinase protein and has roles in cell cycle regulation by inducing cell cycle arrest at S/G2 phase upon DNA damage (Kim, Abdelmohsen, & Gorospe, 2010; Zannini et al., 2014). Moreover, when activated, Chk2 was shown to phosphorylate HuR and lead to the dissociation of HuR from its target mRNA (Masuda et al., 2011). Since we observed an inhibition of HuR binding to its target sequences in cells treated with butyrate, we examined the phosphorylation of Chk2

mediated phosphorylation in the presence of butyrate. HT-29 cells were synchronized by overnight starvation and then transferred to complete medium for 12 h. Starved cells were arrested at G1 phase as expected and when they were released in complete medium, the proportion of cells at the G1 phase decreased, suggesting that the cells had started to cycle again (Fig. 3.6A, left panel). When the synchronized HT-29 cells were treated with NaBt for 3, 6, 9 and 12 hours, a larger proportion of the cells were found in the S/G2 phase compared to the vehicle treated cells (Fig 3.6A, right panel). This suggested that butyrate mediated arrest of cells at S/G2 phase could have resulted from enhanced Chk2 activity. Indeed, we observed that in concordance with S/G2 accumulation of the treated cells, enhanced phosphorylation of Chk2 in the presence of butyrate was observed at each time points (Fig. 3.6B). Phosphorylation of Chk2 was highest with 6 h of treatment with NaBt, after which there was a reduction (Fig 3.6B, lanes 4&5). Next, we determined the cytoplasmic levels of HuR in the synchronized cells. We observed that at the 6th hour time point where the highest phosphorylation of Chk2 was observed, HuR translocation to the cytoplasm was reduced the most (Fig. 3.6C) suggesting that activated Chk2 could inhibit the cytoplasmic translocation of HuR. However, we did not observe any significant change of Chk2 phosphorylation in synchronized Caco-2 cells treated for 6h with NaBt (Fig. 3.6D). This either suggests that the 6h of treatment may not have been appropriate for Caco-2 cells, or that another signaling pathway may be involved in the regulation of HuR in these cells.

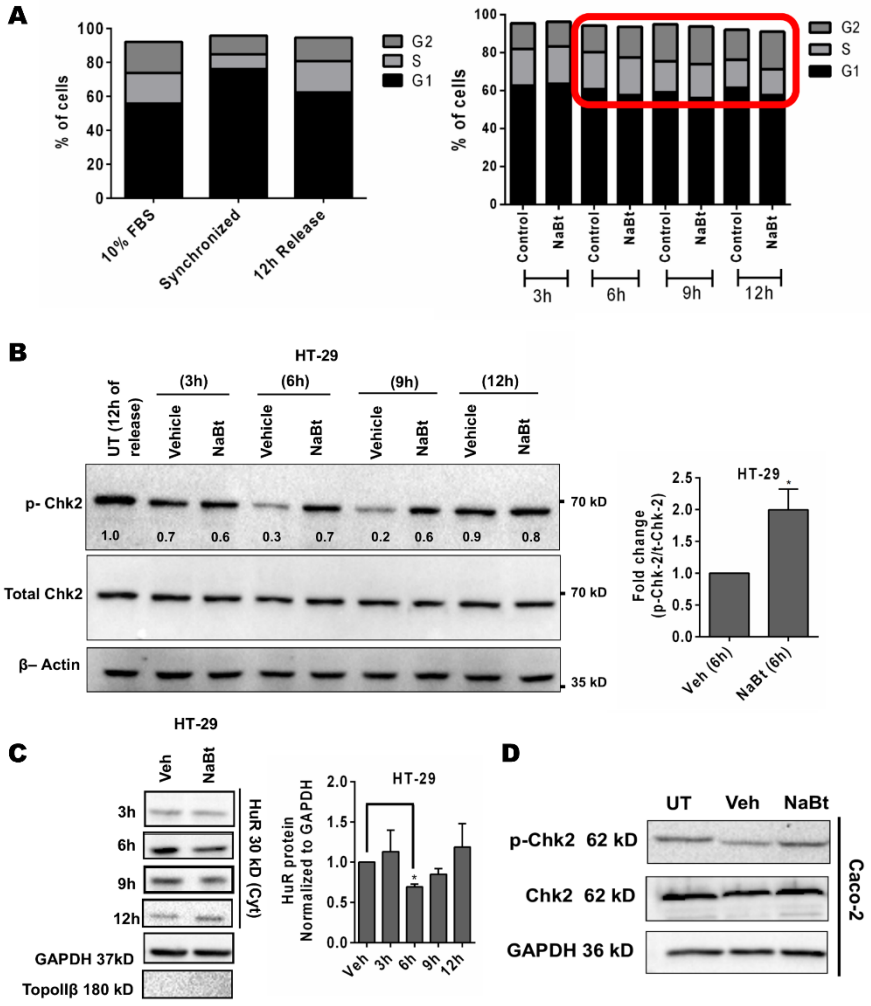


Figure 3.6. Chk2 activation for the reduced cytoplasmic translocation of HuR with butyrate treatment.

*HT-29 cells were synchronized with O/N starvation and treated with butyrate for 3,6,9 and 12 h. The cells were collected and processed for cell cycle analysis by flow cytometry or for protein isolation. (A)HT-29 cells were incubated overnight in serum free medium and then released in medium containing 10% FBS. The cells were treated with 5mM NaBt for four different time points. The cell cycle profile at each time point is shown. The cells were arrested after starvation at G1/S phase, as expected (left panel). NaBt treatment resulted in increased proportion of cells at the S/G2 phase. (B) Phosphorylation of Chk2 increased with NaBt treatment for 6 and 9 h in HT-29 cells. (C)Cytoplasmic levels of HuR in 3,6,9 and 12 h NaBt treated synchronized HT-29 cells are shown. At 6 h, cytoplasmic HuR levels showed a significant decrease. (D) Phosphorylation of Chk2 did not change in 6h NaBt treatment in synchronized Caco-2 cells. Densitometric analyses were carried out with respect to vehicle treated cells and normalized to the loading control. Statistical analyses were carried out with t-test (*p<0.05).*

As Caco-2 cells did not show any difference in the phosphorylation of Chk2 upon treatment for 6h, we focused on whether the p38-MK2 signaling pathway was affected in these cells with butyrate treatment. The p38-MK2 signaling pathway is also involved in the shuttling of HuR and activation of this pathway leads to enhanced cytoplasmic translocation of HuR and stabilization of its target mRNAs (Lafarga et al., 2009). We observed that butyrate treated cells showed reduced cytoplasmic translocation of HuR in HT-29 and HCT116 cells (Fig 3.1D; lower panel & Fig 3.3A). When treated with butyrate for 48 h, in both Caco-2 and HT-29 cell lines, phosphorylated p38 and MK2 levels were decreased compared to untreated cells (Fig 3.7). This decrease was more dramatic with increasing concentrations of NaBt. These data were in concordance with our finding that increasing butyrate amount decreased the cytoplasmic translocation of HuR (Fig 3.1). These data suggest that butyrate is an important determinant of HuR cytoplasmic shuttling by inhibiting the activation of p38 and subsequently MK2.

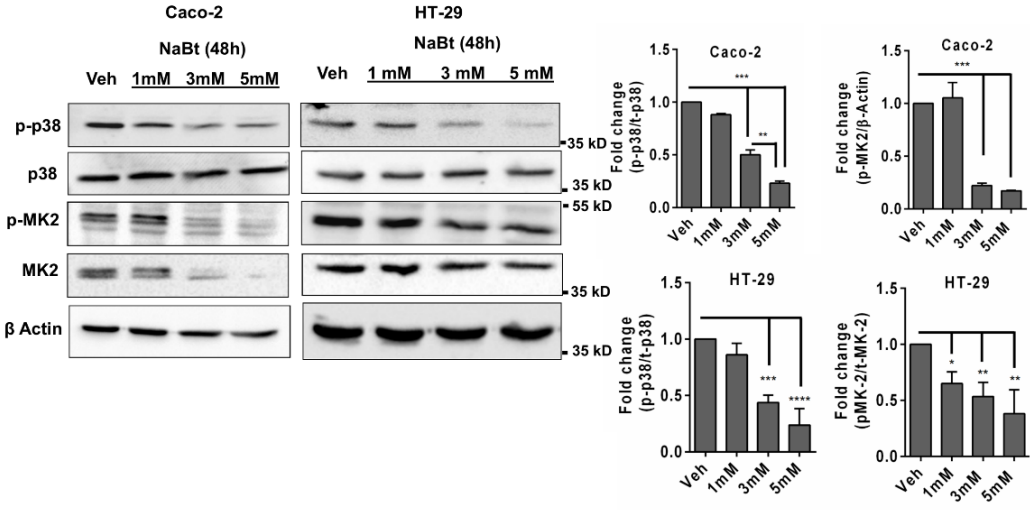


Figure 3.7. Activation of the p38-MK2 pathway leading to reduced cytoplasmic translocation of HuR in the presence of NaBt.

*Caco-2 cells and HT-29 cell were treated with 3 and 5mM NaBt for 48h. Cells are collected and processed for protein isolation. Phosphorylation of p38 and MK2 was reduced with butyrate in both cell lines. Densitometric analyses was carried out with respect to the vehicle treated cells and normalized to loading control. Statistical significance was determined with t-test. (*p<0.05, **p<0.005, ***p<0.001,****p<0.0001*). (*Experiments in this figure were carried out by Shabnam Enayat, PhD).*

CHAPTER 4

DISCUSSION

The short-chain fatty acids (SCFAs) butyrate, propionate and acetate are products of microbial metabolism in the gut that can reach a total concentration of 50-150mM in the human colon (Louis, Hold, & Flint, 2014). Production of butyrate in the gut is dependent on dietary consumption of non-digestible carbohydrates (Louis et al., 2014). Butyrate is used as an energy source by the gut epithelial cells; however, it has many other functions in the gut. Butyrate functions as an HDACi and induces the hyper-acetylation of histones leading to increased expression of transcription factors and proteins (Fung, Cosgrove, Lockett, Head, & Topping, 2012). Through crosstalk with signal transduction pathways via its HDACi activity, butyrate was shown to downregulate several pro-inflammatory cytokines including IL-6 and IL-12 in the colon (Chang, Hao, Offermanns, & Medzhitov, 2014). Butyrate can regulate the activity of regulatory T (Treg) cells in the colon, which in turn can help mediate an anti-inflammatory role (Smith et al., 2013). Two studies have shown that butyrate is involved in the post transcriptional regulation of target mRNAs in colorectal cancer through its HDACi activity (Krishnan et al., 2010; Sobolewski et al., 2015). The first study showed that butyrate reduced the expression of claudin-1 through mRNA destabilization, however, the RBPs were not identified (Krishnan et al., 2010). The second study showed the HDACi mediated upregulation of the destabilizing RBP TTP in butyrate mediated downregulation of COX-2 expression (Sobolewski, Sanduja, Blanco, Hu, & Dixon, 2015). However, it is currently unknown whether butyrate mediated regulation gene expression is through altered nucleo-cytoplasmic shuttling of RBPs. We have shown that butyrate reduced the overall expression of several

different RBPs (but not all were checked), or their cytoplasmic translocations in Caco-2 and HT-29 colorectal cancer cell lines (Fig.3.1).

Among the RBPs that we tested, we chose HuR for further analysis because it targets mRNAs that participate in important cellular functions including proliferation, apoptosis, differentiation and inflammation (Srikantan & Gorospe, 2015). Additionally, our data showed that the overall expression of HuR did not decrease with butyrate in HT-29 cells; rather butyrate treatment resulted in reduced cytoplasmic translocation of the protein. This was particularly interesting as it would implicate butyrate-mediated regulation of an RBP independent of its effects on gene expression via its HDACi activity.

We first established via luciferase reporter assays and RNA IP that the decrease in the cytoplasmic translocation of HuR in butyrate treated cells could reduce the binding and stabilization of targets of HuR. Previously defined targets of HuR such as COX-2 and TNFα were destabilized in butyrate treated cells; additionally, the ARE sequence specifically designed for binding to HuR and no other RBP showed the most drastic decrease compared to the other ARE sequences (Fig. 3.2B). This also corroborated the well-known paradigm that RBPs can compete for binding to the same mRNA target (Dassi, 2017). Importantly, when HuR was overexpressed, stabilization of all ARE target sequences tested was seen; however, this stabilization was lost when the cells were treated with butyrate (Fig3.2C.). Moreover, HuR overexpression stabilized the COX-2 mRNA and led to an increase in the COX-2 expression at the protein level, however butyrate treatment resulted in the loss of this stabilization (Fig. 3.5). Therefore, overexpression of HuR could not reverse the inhibitory effect of NaBt on reporter activity as well as on the protein expression. Overexpressed HuR was reported to be localized in the nucleus (Fan & Steitz, 1998); this suggests that butyrate may affect signaling pathways that are responsible for the cytoplasmic translocation

of HuR even when excess of the protein is available. Moreover, nuclear HuR was shown to auto-regulate its expression by binding to its own 3' UTR (Dai, Zhang & Makeyev, 2011). High levels of nuclear HuR, which is the case for the overexpression of this protein, led to the degradation of HuR protein in the cytoplasm (Dai, Zhang & Makeyev, 2011). Therefore, overexpression of HuR might not contribute the stabilization of its target ARE sequence (Fig. 3.2 C&D) as much as endogenous HuR does (Fig. 3.2B) and this stabilization is easily lost in the presence of butyrate which can further inhibit cytoplasmic translocation of HuR.

The p38-MK2 pathway is such a signaling cascade in which HuR is phosphorylated at T118 residue by the kinase MK2. This phosphorylation event results in increasing the cytoplasmic translocation of HuR and thus stabilization of its targets (Lafarga et al., 2009). Although we could not show specifically a reduction in the phosphorylation of HuR due to the unavailability of a commercial antibody, we showed that butyrate decreased the phosphorylation of p38 and MK2 in both Caco-2 and HT-29 cell lines (Fig. 3.7). This implies that the observed decrease in the cytoplasmic translocation and activity of HuR might be due to butyrate mediated inhibition of p38 and MK2 phosphorylation. Interestingly, overexpression of HuR could not reverse the destabilization of target ARE sequences seen in cells treated with butyrate. This may be because the overexpressed HuR was sequestered in the nucleus (Fan & Steitz, 1998), which in turn would not be able to translocate to the cytoplasm due to the inhibition of the p38-MK2 pathway in the presence of butyrate.

Another protein that was reported to be critical for the activity of HuR is Chk2. It is an important DNA damage response protein involved in many DNA repair mechanisms (Zannini et al., 2014). In addition to its roles in DNA damage response, Chk2 is an important regulator of HuR. It phosphorylates HuR at S88, S100 and T118 and leads to the dissociation of HuR from its target mRNAs (Abdelmohsen et al.,

2007; Masuda et al., 2011). Since Chk2 is a cell cycle dependent protein which induces cell cycle arrest at S and G2/M as a DNA damage response (Kim et al., 2010), we firstly synchronized HT-29 cells and then showed that butyrate treatment led to accumulation of cells at S and G2/M phases whereas untreated cells were accumulated at the G1 phase (Fig. 3.6A). Phosphorylation of Chk2 increased significantly with butyrate treatment and cytoplasmic HuR levels decreased the most (Fig 3.6). Together with our findings, this suggests that by enhancing Chk2 phosphorylation, butyrate treatment leads to an arrest S/G2 phase at which Chk2 phosphorylates HuR and prevents its translocation to the cytoplasm. This in turn promotes instability of target mRNAs. We did not observe a significant change in the phosphorylation of Chk2 in Caco-2 cells. This signaling axis might not be involved in the regulation of HuR in this cell line but p38-MK2 signaling cascade might be the primary pathway for it. Since two different pathways are involved in the regulation of nucleo-cytoplasmic shuttling of HuR in HT-29 cells, a more significant decrease in the cytoplasmic levels of HuR was seen in HT-29 cells compared to Caco-2 cells (Fig.3.1D).

In this study, we have shown that butyrate can post-transcriptionally regulate the expression of pro-inflammatory cytokines by decreasing the cytoplasmic localization of HuR in addition to its previously well-established transcriptional regulation of pro-inflammatory genes. Additionally, we have identified two independent pathways that could regulate the activity of HuR in butyrate treated cells. Inflammation is a hallmark of CRC and inflammatory bowel diseases in which chronic inflammation is observed can lead to CRC (Janakiram & Rao, 2014). Very-well coordinated chain of events is known to control the inflammatory response of the body. Acute inflammation is followed by the resolution of the inflammation in which pro-inflammatory cytokines are not released anymore and anti-inflammatory mediators take place. In CRC, there is chronic inflammation and by using NSAIDs, tumor development is reduced (Friis et al., 2015) suggesting that inflammation is a triggering factor for the tumorigenesis in CRC. Therefore, control of expression of inflammatory genes is important for the

development of CRC. It was shown that patients with CRC have lower levels of butyrate producing bacteria (Wu et al., 2013) and they have low levels of butyrate in their fecal samples (Marchesi et al., 2007). Additionally, rats fed with fiber supplemented diet showed an increase in the production of butyrate in colon and anti-inflammatory effect was observed (Rodríguez-Cabezas et al., 2018). These studies together with our finding that butyrate is involved in post-transcriptional regulation of inflammatory genes by regulating HuR suggest that butyrate as a dietary supplement might be very important for reducing the inflammation observed in CRC and reducing the tumorigenesis in CRC. In this study only the effect of HuR on inflammatory genes in the presence of butyrate was checked whereas we have shown that expression of other RBPs decrease with butyrate, too. It is not yet known what targets of these RBPs are affected by butyrate treatment. Since their expressions decrease with butyrate, what cellular response it can lead to remains to be seen. Further studies should focus on the particular effect of butyrate on these RBPs and how they might be involved in the inflammation in colorectal cancer.

CHAPTER 5

CONCLUSION

In the current study, we observed that butyrate suppressed the expression of several RBPs in several different colorectal cancer cell lines. Analysis of publicly available microarray data from work with butyrate treated HT-29 showed that there is an overall suppression of RBPs with butyrate treatment. Considering that RBPs can bind to 3'UTR of inflammatory genes, we aimed to examine whether butyrate has an effect on the post-transcriptional regulation of inflammatory genes by suppressing RBPs instead of transcriptional regulation through its HDACi activity.

In our study, butyrate treatment decreased cytoplasmic localization of the tested RBPs in addition to reducing their expression. The change in expression of RBPs is perhaps expected, considering the fact that butyrate can modify chromatin structure through its HDACi activity. However, data from our lab suggests that another HDACi, suberanilohydroxamic acid (SAHA), did not inhibit the p38 MK2 signaling pathway as butyrate did suggesting that HDACi independent functions of butyrate may also be functional in the post transcriptional regulation of genes. We have therefore chosen to examine the role of butyrate in the activity of the stabilizing RBP HuR since the cytosolic localization, rather than the expression of this RBP was inhibited in the presence of butyrate. We show that 3'UTR activities of many ARE sequences which can be bound and stabilized by HuR were down-regulated by butyrate. Mechanistically, we showed that activation of Chk2 and decrease in the phosphorylation of p38 and MK2 were likely to be involved in the diminished cytoplasmic translocation of HuR.

Consequently, our findings indicate that butyrate, via alterations in Chk2 and p38-MK2 pathways, reduced the cytoplasmic levels of HuR and affected the binding of the RBP to several ARE sequences including inflammatory genes such as COX-2 and TNFα. This study shows for the first time that butyrate can post-transcriptionally regulate inflammatory genes by inhibiting HuR.

CHAPTER 6

FUTURE STUDIES

Next questions to be answered are as follows:

- The effect butyrate on other HuR target inflammatory genes.

- The biological consequences of the down-regulation of other RBPs on functional characteristics (such as inflammatory response) of Caco-2 and HT-29.

- Whether the *in vitro* observations on the role of butyrate on the expression and activity of RNA binding proteins are replicable with *in vivo* studies needs to be tested. Rodent models can be administered with butyrate and the localization of HuR as well as expression of targets of HuR can be examined from the gut tissue.

- RBPs can often compete with each other or with miRNAs for binding to the same target sequence. Since the expression of many RBPs, both stabilizing and destabilizing, were affected in the presence of butyrate, it will be interesting to examine whether the reduced binding of HuR was because of competition with other factors.

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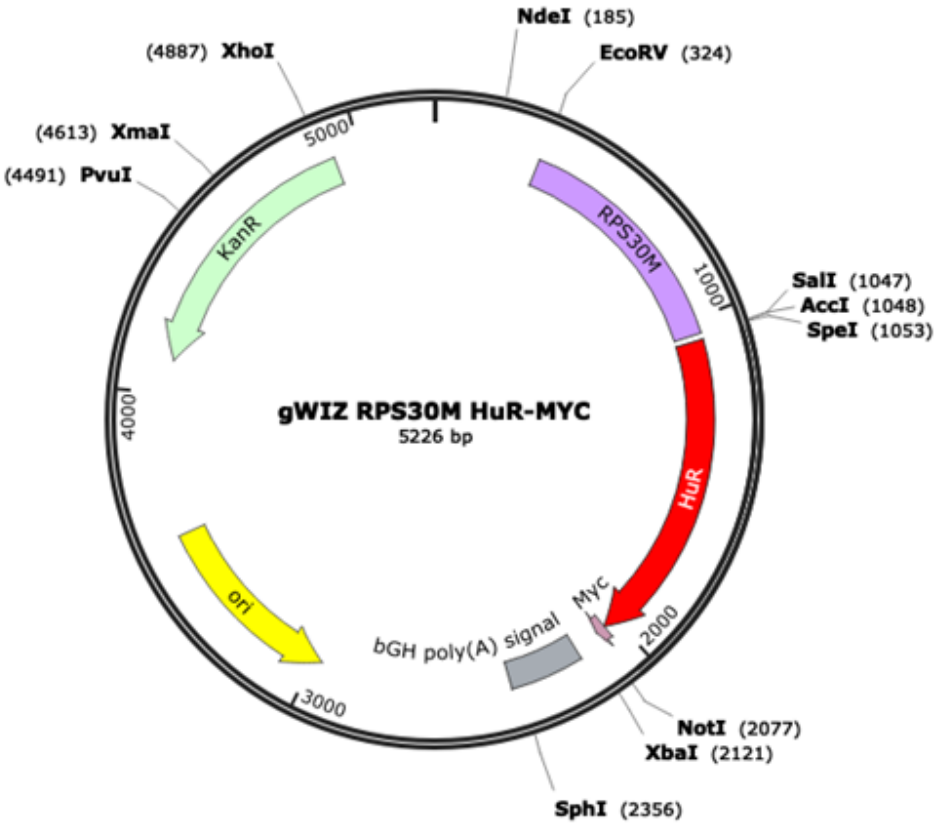
APPENDICES

A. MAPS OF VECTORS CLONED AND USED IN THIS STUDY

A



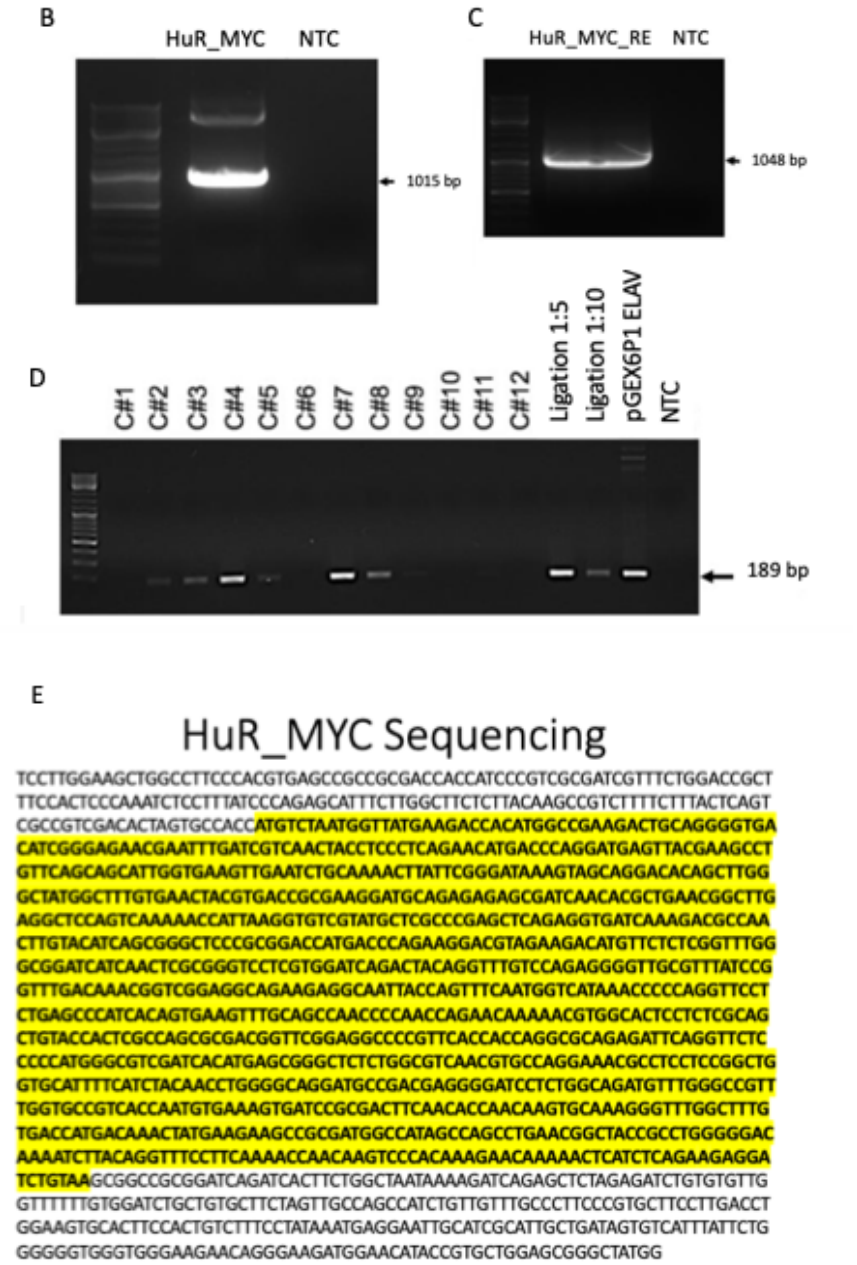
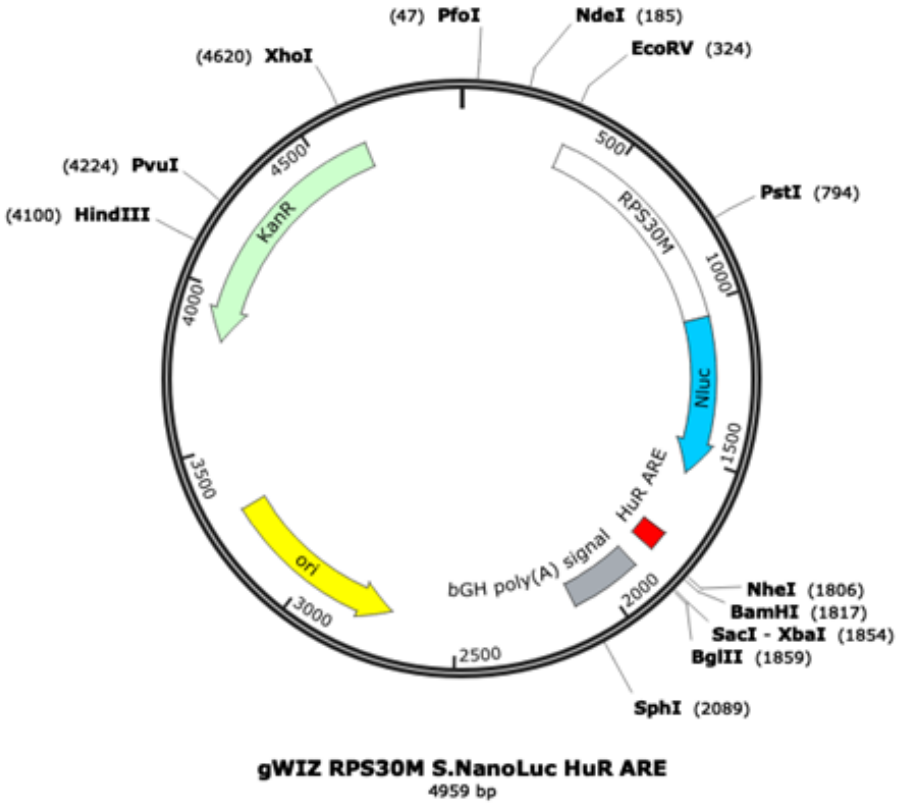


Figure A.1. Vector map and cloning of pGWIZ RPS30M HuR_MYC construct.

A) Vector map of pGWIZ RPS30M HuR_MYC is shown. B) PCR with HuR oex_1 primers (Table 2.1) to add MYC sequence to the HuR gene resulted in a 1015bp product. This product was cut and gel-purified. The resulting insert DNA was used as a template for the addition of restriction enzyme sites. C) Restriction sites were added to the HuR_MYC insert DNA by using HuR oex_2 primers (Table2.1). Products size is 1048bp. D) Positive colonies were checked with HuR primers (Table 2.1). Colony #7(C#7)) was selected. E) The sequencing result of C#7 is shown. HuR ARE sequence is highlighted. GeneRuler DNA Ladder Mix SM0331 (Thermo) was used as a marker.

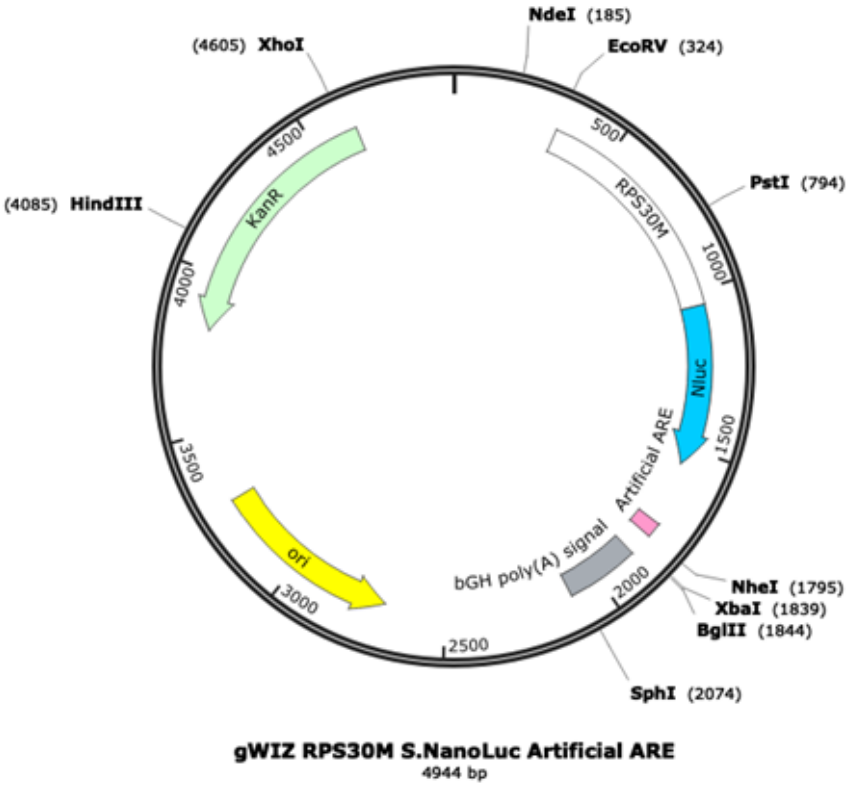


HuR ARE Sequencing

AGACTTGTCGGGACGGCGACAACAGCCGCTACAACTGGACCAAGTCCTGAACAGGAGGTGGTCCGTCT
GTCCCAGAATCTCGGGTGTCCGTGACTCCGATCCAAAGGATCGTCTGAGCGGTGAAATGGGCTGAAGAT
CGACATCCATGTCATCATCCCGTACGAAGGTCTGAGCGCGACCAAATGGGCCAGATCGAAAAAATCTTCA
AGGTGGTGTACCCTGTGGATGATCATCACTTCAAGGTGATCCCTGCACTACGGCACACTGGTCATCGACG
GGGTCACGCCGAACATGATCGACTACTTCGGACGGCCGTACGAAGGCATCGCCGTGTTCGACGGCAAAA
AGATCACTGTGACAGGGACCCTGTGGAACGGCAACAAAATCATCGACGAGCGCCTGATCAACCCCGACG
GCTCCCTGCTGTTCCGAGTGACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATCCTGGCGC
TGCTCAGCGTGTACGAGGAAAGCCCCGAGGACTCCCCCGAGAGCCCCGTCTCCGAGGAAGGCACCGAC
AACTCTCACGGCTTCCCGCCTGAGGTCGAAGAGCAAGCCGCCGGAACACTGCCCATGTCCTGCGCACAA
GAAAGCGGCATGGACCGGCACCCAGCCGCGTGTGCGTCAGCTCGCATCAACGTCTGAGGATCTTCCGAT
TTCCGATTTATTTCGGATTTTTCCGATTTCCGATTTGTTTCGGATTTGCTAGCGGATCCAGATCACTTCTGG
CTAATAAAAGATCAGAGCTCTAGAGATCTGTGTGTTGGTTTTTTGTGGATCTGCTGTGCCTTCTAGTTGCC
GTGCGATCTGTTGTTTGCCCCTCCCCCGTGCCTTCGGGGAGACCCTGGAAGGTGCCACTCCCCCTGTCCT
TTCCCTTAAGATAAAGGT

Figure A.2. pGWIZ RPS30M SuperNanoLuc HuR ARE construct.

The sequencing results are shown (Below panel). HuR ARE sequence is highlighted.

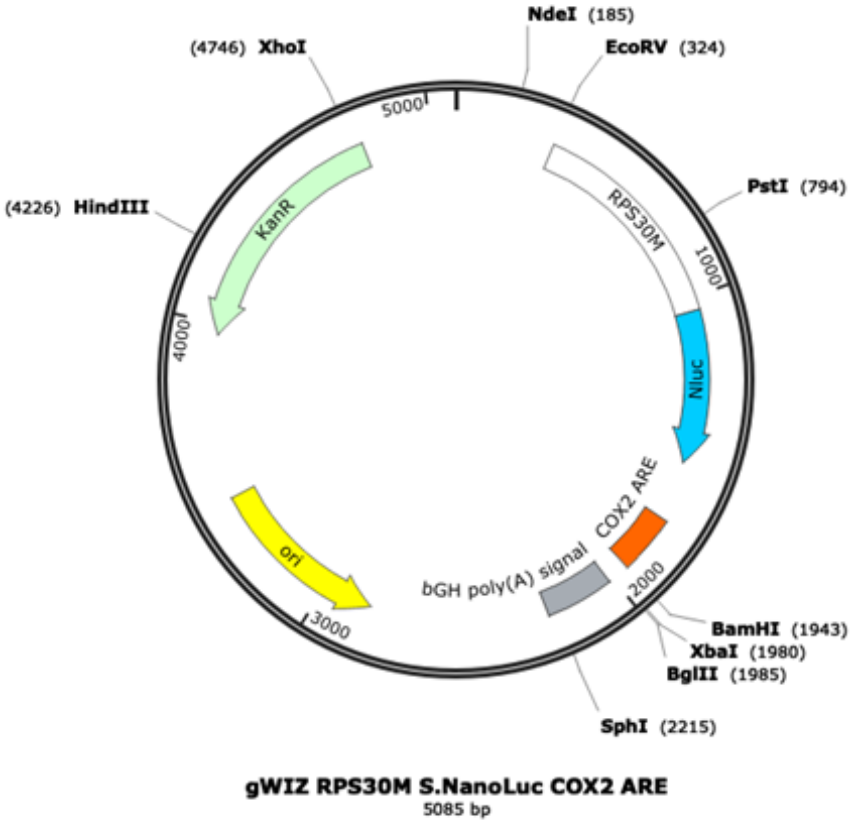


Artificial ARE Sequencing

CCACATGTCTGCCATGAGATAGTCGGGACTGCGACAGACAGCCGCTACAACCTGACCAGTCTGGACAGG
AGGTGTGTCCAGTCCGTCAGCATCTCGGGGTGTCGTGACTCCGATCCAAAGGATCGTCCTGAGCGGTGA
AAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTACGAAGGTCTGAGCGGCGACCAAATGGGCCA
GATCGAAAACATCTTCAAGGTGGTGTACCCTGTGGATGATCATCACTTCAAGGTGATCCTGCACTACGGCA
CACTGGTCATCGACGGGGTCACGCCGAACATGATCGACTACTTCGGACGGCCGTGCTGAAGGCATCGCC
GTGTTCGACGGCAAAAAGATCACTGCAACAGGGACCCTGTGGAACGGCAACAAAATCATCGACGAGCG
CGCTCGCAACCCCGACGGCTCCCTGCTGTTGCGAGTGACCATCAACGGAGTGACCGGCTGGCGGCTGTG
GGAACGCATGCCGGCGCTGCCCGGCGTGGACGAGGAAAGCCCCGAGGAGTCCCCCGAGAGCCCCGGG
TCCGAGGAAGGCACGGACAACTCTCACGGCTTCCCGCCTGAGGTCGAAGAGCAAGCCGCCGGAACACT
GCCCATGTCCTGCGCACAAGAAAGCGGCATGGACCGGCACCCAGCCGCGTGTGCGTCAGCTCGCATCAA
CGTCGGAGGATCCATTTTATTTATATTATTTAATTTTATTTATATTATTTAGCTAGCGGATCGAGATCACTTCT
GGCTAATAAAAGATCAGAGCTCTAGGGTATGTGTGTTGTTGGTTTTTTGTGGATCTGCTGTGCCTTCTAGTT
GCCGAGCCGATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCATGACCCTGGAAGGTGCCACTCCCGACCGG
CGATTCCCTTAAAAAA

Figure A.3. pGWIZ RPS30M SuperNanoLuc Artificial ARE construct.

The sequencing results are shown (Below panel). Artificial ARE sequence is highlighted.



COX-2 ARE Sequencing

GAGCGGCGACCAAATGGGCCAGATCGAAAAATCTTCAAGGTGGTTGTACCCTGTGGATGATCATCACTTC
AAGGTGATCCTGCACTACGGCACACTGGTCATCGACGGGGTCACGCCGAACATGATCGACTACTTCGGAC
GGCCGTACGAAGGCATCCGCTGTGTTCGACGGCAAAAAGATCACTGTGACAGGGACCCTGTGGAACGG
CAACAAAATCATCGACGAGCGCCTGATCAACCCCGACGGCTCCCCTGCTGTTCCGAGTGACCATCAACGC
AGTGACCGGCTGGCGGCTGTGCGAACGCATCCTGGCGCTGCCCAGCGTGGACGAGGAAAGCCCCGAG
GACTCCCCCGAGAGCCCCGTGTCCGAGGAAGGCACCGACAACTCTCACGGCTTCCCGCCTGAGGTCGA
AGAGCAAGCCGCCGGAACACTGCCCATGTCCTGCGCACAAGAAAGCGGCATGGACCGGCACCCAGCCG
CGTGTGCGTCAGCTCGCATCAACGTCTGAGGATCCAGAACGTTCGACTGAACTGTAGAAGTCTAATGATC
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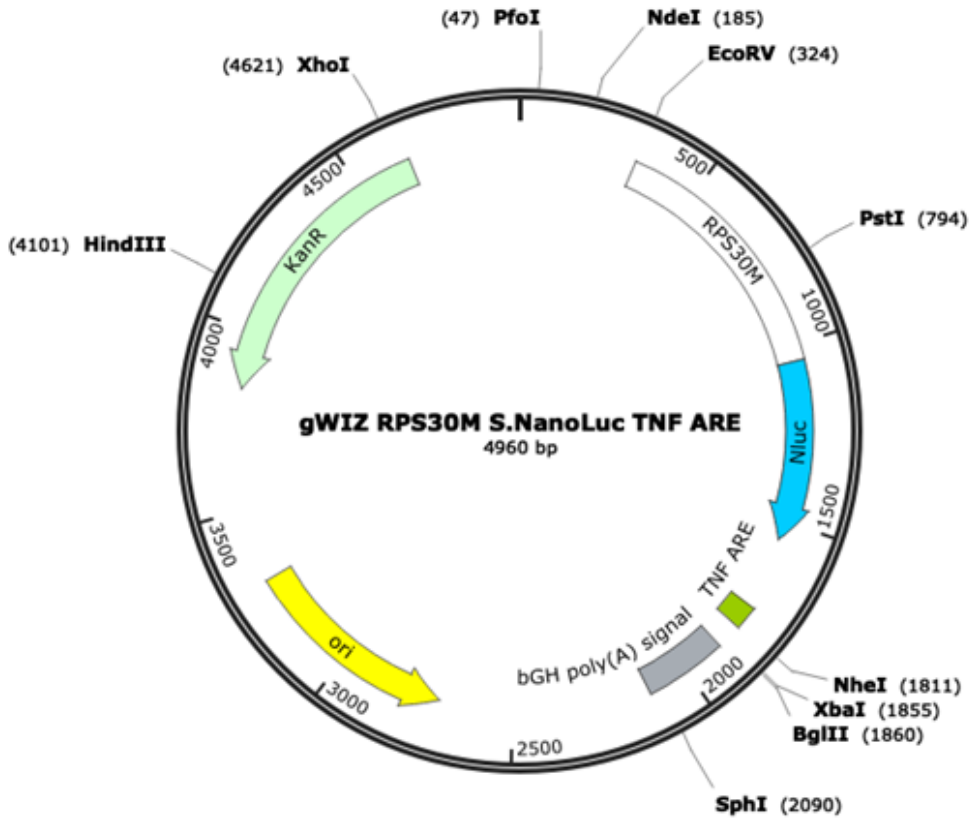
TAACATCTTCTGTAACAGAAGTCAGTACTCCTGTTGCGGAGAAAGGAGTCATACTTTGTGAAGACTTTTAT
GTCACTACGGATCCAGATCACTTCTGGCTAATAAAAGATCAGAGCTCTAGAGATCTGTGTGTTGGTTTTTT
GTGGATCTGCTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTGACCCTGG
AAGGTGCCACTCCCACTGTCCCTCCTTAAAAAAAGAGG

Figure A.4. pGWIZ RPS30M SuperNanoLuc COX-2 ARE construct.

The sequencing results are shown (Below panel). COX-2 ARE sequence is highlighted.



TNF ARE Sequencing

ACATGTCTTCACACTGAAACTTGTCGGGACTGGCGACAGACAGCCGGCTACAACCTGGACCAAGTCCTC
GAACAGGGAGGTGTGTCCAGTCTGTTCCAGAATCTCGCGTGTCCGTGACTCCGATCCAAAGGATCGTCCT
GAGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTACGAAGGTCTGAGCGGCGACCA
AATGGGCCAGATCGAACAAATCTTCAAGGTGGTGTACCCTGTGGATGATCATCACTTCAACGTGATCCTGC
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GCATCGCCGTGTTCGACGGCAAAAAGATCACTGTGACAGGGACCCTGTGGCACGGCCACAAAATCATCG
ACGAGCGCCTGATCAACCCCGACGGCTCCCTGCTGTTCCGAGTGACCATCAACGGAGTGACCGGCTGGC
GGCTGTGCGAACGCATCCTGGCGCTGCCCAGCGTGGACGAGGAAAGCCCCGAGGACTCCCCCGAGAGC
CCCGTGTCCGAGGAAGGCACCGACAACTCTCACGGCTTCCCGCCTGAGGTCGAAGAGCAAGCCGCCGG
AACACTGCCCATGTCCTGCGCACAAGAAAGCGGCATGGACCGGCACCCAGCCGCGTGTGCGTCAGCTCG
CATCAACGTCTGAGGATCCTTATTATTTATTATTTATTTATTATTTTTATTATTTATTATTTATTATTTGCTA
GCGGATCCAGATCACTTCTGGCTAATAAAAGATCAGAGCTCTAGAGATCTGTGTGTTGGTTTTTTGTGGAT
CTGCTGTGCCTTCTAGTTGCCGAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTGCAAATGACCCTGGAA
GGTGCCACTCCCGCCATTTTCTTTCCTTAATAAGT

Figure A.5. pGWIZ RPS30M SuperNanoLuc TNF ARE construct.

The sequencing results are shown (Below panel). TNF ARE sequence is highlighted.

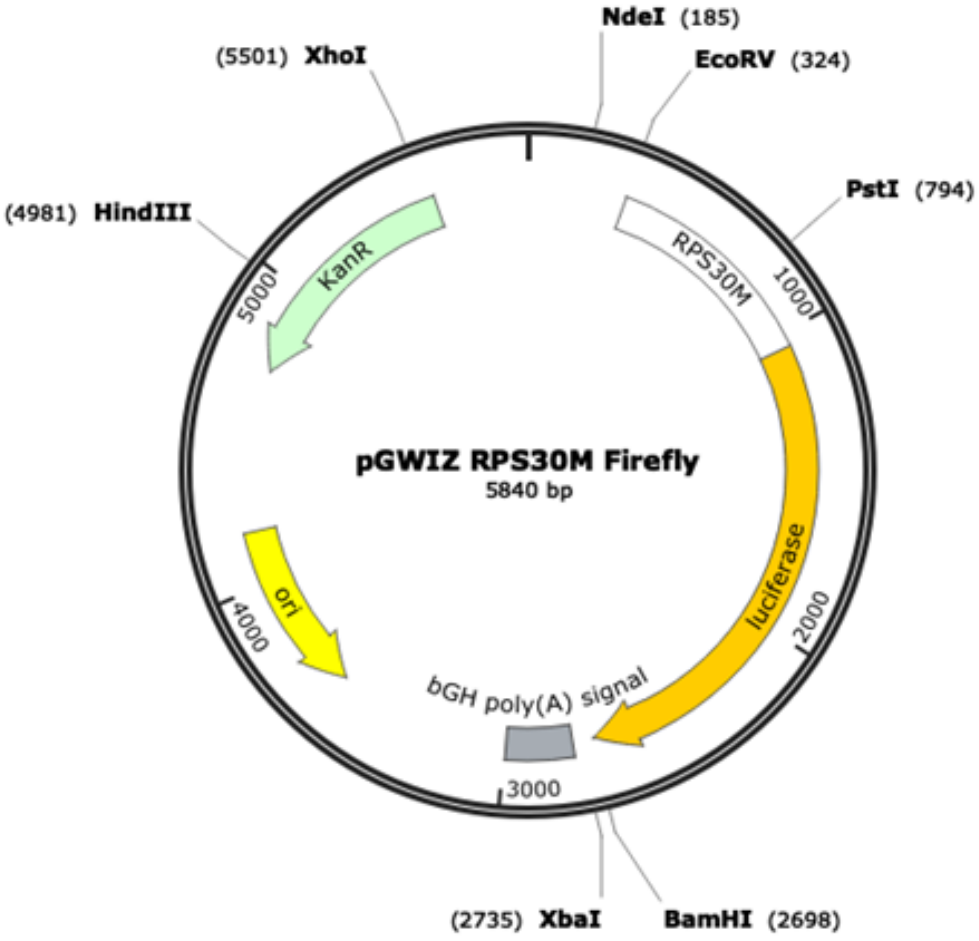

Figure A.6. pGWIZ RPS30M Luciferase (Firefly) construct.

The firefly vector was a gift from Dr Khalid S Khabar of King Faisal University, Saudi Arabia.

B. MATERIALS USED FOR WESTERN BLOT

10X TRANSFER BUFFER

0.25 M Tris

1.92 M Glycine

pH 8.3 in 1L dH_2O

1X TRANSFER BUFFER

100 ml 10X Transfer Buffer

200 ml Methanol

700 ml dH_2O

SDS-PAGE RUNNING BUFFER

25 mM Tris

190 mM Glycine

0.1% SDS

10% SEPARATING GEL MIX

4.1 ml dH_2O

2.5 ml 4X Separating Buffer (10% SDS, 1.5M Tris, pH 8.8)

3.33 ml Acrylamide/Bis Solution (SERVA, Germany)

100 µl 10% Ammonium Persulfate (APS)

10 µl TEMED

4% STACKING GEL MIX

3.1 ml dH_2O

1.25 ml Stacking Buffer (10% SDS, 1.5M Tris, pH 6.8)

650 µl Acrylamide/Bis Solution (SERVA)

50 µl 10% APS

5 µl TEMED

MILD STRIPPING BUFFER

15 g GLYCINE

1 g SDS

10 ml Tween-20

pH 2.2 in 1L dH_2O

TBS-T

50mM Tris-HCl pH 7.4

150mM NaCl

0.1% Tween-20 is added to 1X TBS before use

6X SAMPLE LOADING DYE

12% SDS

30% β-Mercaptoethanol

30% Glycerol

0.012% Bromophenol Blue

0.375 M Tris-HCl pH 6.8