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RNA binding proteins in intestinal epithelial biology and colorectal cancer

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Abstract

The intestinal epithelium is highly proliferative and consists of crypt invaginations that house stem cells and villus projections with differentiated cells. There exists a dynamic equilibrium between proliferation, migration, differentiation and senescence that is regulated by several factors. Among these are RNA binding proteins (RBP) that bind their targets in a both context dependent and independent manner. RBP:RNA complexes act as rheostats by regulating expression of RNAs both co- and post-transcriptionally. This is important especially in response to intestinal injury, to fuel regeneration. The manner in which these RBPs function in the intestine and their interactions with other pivotal pathways in colorectal cancer may provide a framework for new insights and potential therapeutic applications.

Keywords

RNA binding proteins; LIN28; Musashi (MSI); IGF2BP/IMP (Insulin-like growth factor 2 mRNA binding proteins); MEX3A; CELF1 (CUGBP Elav-Like Family Member 1); RBM3 (RNA binding protein 3) and HUR (Hu-Antigen R); intestinal stem cells; colorectal cancer

The proliferative and dynamic intestinal epithelium

Tissue homeostasis is a consortium of fundamental physiological processes involving proliferation, differentiation, apoptosis and senescence. There is a disparity in tissues that are proliferative with rapid turnover (e.g. intestine, skin) versus those that are largely quiescent (e.g. neurons, smooth muscle cells, endothelial cells, kidney). The small intestinal epithelium has proliferative crypt cells at its base. Daughter cells migrate to the luminal surface undergoing differentiation into cells that comprise the villus compartment. Thus, there is a proliferation-differentiation gradient from the crypt compartment to the villus compartment, which comprises two key lineages: absorptive enterocytes (most of the cells) and secretory cells (**Paneth, enteroendocrine, goblet**) [1]. The large intestinal epithelium

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differs from the small intestinal epithelium in terms of differences in the Paneth cells and the nature of the surface (absence of villi) (Figure 1). The intestinal proliferation-differentiation gradient and lineage specification is regulated to a large extent through two stem cell populations: the active crypt base columnar (**CBC**) cells and **reserve +4 cells** [2, 3]. A number of genes and pathways annotate these two populations, especially as related to Wnt signaling [4], Notch signaling [5], BMP pathway [6], amongst others.

Injurious agents include infectious organisms, inflammatory conditions that trigger immune mediated responses (e.g. inflammatory bowel diseases) and subversion through activation of oncogenes and inactivation of tumor suppressor genes that drive malignant transformation (especially colorectal cancer). An emerging node of regulation of intestinal epithelial homeostasis, response to injury and malignant transformation is through RNA binding proteins (RBP) [7]. We will focus on the published cohort of RBPs in the context of intestinal homeostasis, regeneration and colorectal cancer. These include LIN28, MSI (Musashi), IGF2BP/IMP (Insulin-like growth factor 2 mRNA binding proteins), MEX3A, CELF1 (CUGBP Elav-Like Family Member 1), RBM3 (RNA binding protein 3) and HUR (Hu-Antigen R).

RNA binding proteins and their functions

Broadly speaking, RNA binding proteins (RBPs) are vital for regulation of several essential cellular processes such as RNA splicing, modifications, transport, localization, stability, degradation and translation [10]. Several RBPs are expressed ubiquitously and are evolutionarily conserved [11] to maintain their roles in basic cellular functions. Any significant change or disturbance in the RBPs regulating these essential cellular functions can lead to different diseases, including cancer [10]. RBPs function by binding to their target RNA, forming ribonucleoprotein (RNP) complexes [12] and regulating gene expression post-transcriptionally in a plethora of ways. Since RBPs can regulate already transcribed RNAs, they act in a rapid and efficient manner to alter gene expression, especially during changes in the microenvironment. A single RBP can bind to hundreds, if not thousands of targets, and a combination of several RNP interactions contribute to cellular identity and response to stimuli [13]. RBPs can help recruit translation machinery to activate translation [14]. By contrast, RBPs involved in the RNA-induced silencing complex (miRISC) result in decapping, deadenylation and translational repression of the target mRNAs [15]. They can also suppress translation and induce degradation [16]. In some cases, two RBPs can bind to the same RNA target to stabilize it, either enhancing or repressing translation [17]. RBPs can also have dichotomous functions where they can both enhance [18, 19] or repress [20, 21] tumorigenesis depending upon the cellular context. Figure 2 shows a simplistic schematic of the functional consequences of RBP binding to mRNA targets (as the RBPs discussed in this review bind mainly to mRNAs).

Structure of RNA binding proteins

The functional effects of conventional RNA binding proteins are dependent upon their binding to their target RNAs and forming ribonucleoprotein (RNP) complexes. The RNP complexes help with RNA processing, translation, export and localization. Since RBPs have

multiple biological roles, their structures consist of multiple small domains. These consist of several types of RNA recognition and binding domains interspersed between catalytic domains to efficiently recognize a wide range of targets and regulate catalytic activity [22]. These catalytic domains include helicases, deaminases and RNAse III domains [23, 24]. Multiple RNA binding domains (RBDs) provide specificity to recognize and bind either long RNA sequences or sequences separated by many nucleotides or two different RNAs [22]. These can help form large complexes and regulate major signaling pathways. RBDs may comprise RNA recognition motifs (RRMs), dsRNA binding motifs (dsRBD), K-homology domain (KH), Zinc fingers, S1 domain, Piwi and PAZ (PIWI, AGO, and Zwill) domains amongst others. RRM is by far the most common and well-characterized domain and most RBPs have multiple RRMs to provide specificity. By contrast, RBPs involved in translation, such as initiation and elongation factors, bind all mRNAs and lack specificity [22]. RBPs can regulate subcellular localization of their targets due to nuclear and/or nucleolar localization signals (NLS/NoLS) or nuclear export signals (NES) depending upon their functional requirements [25, 26]. Overall, the structure of these conventional RBPs comprise multiple repeats of different RBDs with varying functional specificities and catalytic domains to regulate their target RNAs.

The target RNAs for RBPs are quite diverse. While RBPs can bind different regions of mRNAs (exonic, intronic, UTRs), there is increasing evidence of interactions with other types of RNAs, including non-coding RNAs, namely microRNAs, t-RNAs, small interfering RNAs (siRNA), telomerase RNA, small nucleolar RNAs (snoRNAs), splicesomal small nuclear RNAs (snRNA), as well as the RNA moiety of the signal recognition particle (SRP RNA or 7SL RNA). These non-coding RNAs form extensive secondary structures to associate with proteins and regulate several processes like splicing, RNA modifications, protein localization and secretion as well as chromosomal maintenance [27].

In recent years, advanced structural-analysis studies have provided evidence of complex protein–RNA interactions that do not require canonical RBDs [27]. RNA interactome capture (RIC) [28] studies have identified ‘non-conventional’ RBPs in several organisms that do not have discernible RBDs and have no known relationship to RNA biology [27]. Further studies have also shown that disordered protein regions can also facilitate protein-RNA interactions that can be specific or non-specific [29]. These unorthodox interactions can regulate RNA metabolism and different RNA processes, both co- and post- transcriptionally [29].

In this review, we will discuss only those specific RBPs that have been published in the context of intestinal homeostasis and intestinal tumorigenesis. These RBPs fall under the ‘conventional’ RBP category (the basic domain structures are summarized in Figure 3) (The role of non-coventional or non-canonical RBPs in cancer has been beautifully reviewed in [30]).

LIN28

LIN-28 was first discovered in *C. elegans* as a heterochronic gene that plays a vital role in developmental events [31]. LIN28 has been studied in multiple species as a promoter of

pluripotency. It has been shown to be expressed highly in undifferentiated tissues and its expression is downregulated as differentiation and development progress [32]. Hence, LIN28 is evolutionarily conserved to promote pluripotency and act as a 'gatekeeper' of differentiation. The most well studied mechanism of LIN28B function is via its interaction with the let-7 miRNAs [33].

In mammals, there are two paralogs of LIN28; LIN28A and LIN28B that have mostly overlapping functions [34]. LIN28A and LIN28B have a cysteine cysteine histidine cysteine (CCHC) zinc finger domain and a cold shock domain [35]. LIN28B also contains an extended C terminal region with a nuclear localization signal (NLS) [36]. In mice, LIN28 proteins are expressed highly during embryonic development but their expression declines rapidly after E18.5 in the small intestine and colon correlating reciprocally with intestinal differentiation [37, 38]. In adult mice, LIN28B expression is limited to the crypt compartment [38]. This correlates with the reciprocal increase in the expression of the *Let-7* microRNAs. LIN28B expression is observed in the nucleus of undifferentiated cells whereas low expression of LIN28B can be seen in the cytoplasm of differentiated intestinal cells. The constitutive knockout of either *Lin28a* or *Lin28b* causes dwarfism and a growth retardation phenotype in mice [39]. The double knockout is synthetically lethal, and the mice do not survive past E12.5. This phenotype, however, is not observed when the genes are deleted in neonatal or adult mice [39]. The intestinal epithelium specific single or double knockouts of *Lin28a* and *Lin28b* show no obvious intestinal phenotype [40]. Furthermore, these mice also do not show any difference in susceptibility to colonic tumorigenesis with dextran sodium sulphate (DSS)/azoxymethane (AOM) when compared to their wild-type littermates [40].

Several studies have shown that LIN28B is overexpressed in about 30% of colorectal tumors [41, 42]. LIN28B overexpression correlates with invasive tumor phenotype, worse survival and increased tumor recurrence in colorectal cancer (CRC) [38, 40, 43]. In mice, intestinal epithelial cell (IEC) specific *Lin28b* overexpression is sufficient to transform the epithelium and give rise to adenomas and adenocarcinomas between 9–12 months of age, which is accelerated by the concurrent knockout of *Let7 b1/c2* with faster and greater formation of adenocarcinomas within 6 months [38, 43]. LIN28B cooperates with Wnt signaling to increase tumor formation in carcinogen-induced mouse model of colitis-associated tumorigenesis [40]. Furthermore, LIN28 overexpression increases tumor formation and decreases tumor latency in an $Apc^{+/min}$ model of colon cancer [40]. LIN28A, which is structurally similar to LIN28B [44], is upregulated in over 70% of CRC patients [45] and overexpression of LIN28A is functionally similar to LIN28B [40]. While silencing either LIN28 protein leads to increased apoptosis by targeting of anti-apoptotic BCL2L1 protein for degradation [46], LIN28A overexpression however, leads to increased chemosensitivity in CRC cells lines to 5FU (fluorouracil) treatment through induction of apoptosis [45]. In summary, LIN28B is critical in colorectal tumorigenesis and has been established to oncogenic effects in this context. While less studied in colorectal cancers, LIN28A has similar functions.

IGF2BPs/IMPs

The insulin-like growth factor-2 mRNA binding proteins (IGF2BPs or IMPs) belong to a conserved subfamily of RBPs. The IMPs have been studied for their roles in regulation of post-transcriptional processes such as mRNA localization, turnover, and translational control [47, 48]. In mammals, the canonical domain structure of IMPs is similar. IMP1 and IMP3 are more closely related and have 73% sequence similarity whereas IMP2 shares 56% similarity [49]. IMPs contain 2 RRMs in their N-terminal region and 4 KH domains in the C-terminal region [50]. The KH domains are the primary RBDs while the RRMs are involved in stabilization of IMP-mRNA complexes [51, 52]. The IMPs bind their targets in multiple low affinity higher-order complexes because KH domains allow recognition of only short stretches of RNA with relatively weak binding affinity [53].

Imp proteins, especially Imp1, are expressed highly during development but expression is reduced drastically after post-natal day 12 in the small and large intestine. The adult mice retain low expression of IMP1 in the crypts [54]. IMP3, an isoform of IMP1, also follows a similar pattern of expression in the intestine [55]. IMP2, by contrast, has been shown to be expressed postnatally [56] and is mainly found in **Processing bodies** (P bodies) in the cytoplasm [57]. Similarly, *Imp1* null mice show significant growth retardation at E17.5 and more than 50% of the mice do not survive past post-natal day 3. The mice show impaired intestinal morphology and development [54]. By contrast, *Imp2* null mice have no growth retardation but are highly resistant to diet induced obesity [58]. In colorectal cancer cell lines and fibroblasts, *Imp2* deletion results in reduced proliferation [59].

IMP1 plays a functional role in the RNA stability by binding and shielding several mRNAs that play critical roles in cell growth and proliferation from proteolytic degradation [60]. IMP1 also regulates cell cycle progression and migration in human CRC cells [61]. *IMP1* is overexpressed in more than 80% of human CRC [62] and correlates with invasion, lymph node metastasis, and worse prognosis [18, 38, 63]. IMP1 overexpression in CRC cell lines causes a significant increase in tumor volume in xenograft models [18]. By contrast, *Imp1* loss in the stroma is associated with increased tumor number in a AOM-DSS model of colonic carcinogenesis. This dichotomous role of Imp1 is seen in other instances where IMP1 stabilizes *β-catenin* mRNA in breast cancer cells [64] and is in turn activated by it in a feedback mechanism [65]. In others studies, IMP1 was shown to bind and stabilize *beta-TRCP1*, a *β-catenin* antagonist in CRC cell lines [66]. *IMP2* gene is amplified at a higher frequency in several solid tumors. IMP2 depletion inhibits proliferation of mouse embryonic fibroblasts (MEFs) and well as several human cancer cell lines. It is also shown to stabilize oncogenic transcriptional regulator HMGA1 in MEFs [59]. IMP3 expression has been shown to correlate with worse prognosis and increased recurrence in colon cancer patients [67]. It has also been associated with low progression-free survival in small-intestinal neuroendocrine neoplasms [68] and studied as an immunohistochemical marker in small intestinal adenocarcinomas [69].

These studies imply divergent roles for IMP1, depending upon whether one considers the epithelial vs. stromal compartment.

Musashi

The Drosophila *musashi* gene was discovered in 1994 as a regulator of asymmetric cell division of Drosophila sensory organ precursor (SOP) cells [70]. Since then, the Musashi (Msi) proteins have been shown to be expressed in the stem cell compartments of different tissues such as brain, intestine and blood and are known to be upregulated in cancers [71–73]. They function as regulators of stem cell renewal, cell cycle progression and metabolism [72, 73]. The *msi* gene is evolutionarily conserved and humans have two related genes, Musashi-1 (MSI1) and Musashi-2 (MSI2) with 75% amino acid identity in structure [74]. Both MSI1 and MSI2 contain 2 N-terminal RRM RBDs. Biochemical and structural studies show that the RRM1 contributes the majority of the binding energy and specificity, while RRM2 has a more supportive role [74]. They usually bind to 3′ ends of target RNAs [75]. MSI1 also contains domains to interact with other RBPs such as PABP1 and LIN28B [76].

In the intestinal epithelium, the MSI family of proteins are expressed in the crypts in mice [73]. Their expression is observed in adult mice in both the active and reserve stem cell compartments [72, 77]. The MSI family of proteins consist of the functionally redundant MSI1 and MSI2. Ablation of *Msi* proteins (*Msi1* or 2) in IEC, either individually or together, showed no changes in morphology, proliferation or differentiation [78]. Although, intestinal epithelial specific double knockout of *Msi1* and *Msi2* ($Msi1^{\Delta IEC}$ $Msi2^{\Delta IEC}$) in mice does not show any overt phenotype under basal conditions [78]. However, following 12Gy radiation injury, $Msi1^{\Delta IEC}$ $Msi2^{\Delta IEC}$ mice show a significant impairment in the regenerative response. MSI proteins are also up-regulated during activation of reserve intestinal stem cells and are required for lineage tracing from these cells under basal conditions by enabling their S-phase entry [78]. MSI1 overexpression has been shown to induce tumorigenesis by activation of Wnt and Notch pathways in primary intestinal cells and xenograft models [79]. The overexpression of either MSI is sufficient to transform the intestinal epithelium and form tumors [72, 73, 78] via activation of the mTORC1 complex with inhibition of Pten [72, 73]. In patients with small intestinal adenocarcinomas, MSI1 is overexpressed in 71% of the tumors as compared to the normal tissue and correlated with depth of wall invasion [80]. In patients with Irritable bowel syndrome (IBS) the density of MSI1+ cells is significantly reduced and correlates with dysfunctional stem cell potential [81]. MSI1+ cells have been shown to be involved in repair of the intestinal epithelium induced by 5-FU [82].

Due to their roles in EMT, stem cell identity, and oncogenesis, the MSI proteins have increasingly been linked to therapeutic resistance in cancer treatments [83–85]. This has resulted in efforts to develop inhibitors of MSI proteins as potential therapeutic targets [86, 87].

HuR

HuR, a member of ELAV family of RBPs (reviewed extensively in [88]) consists of 2 RRM domains, a hinge region and a third RRM [89] that helps it bind to adenylate uridylate (AU) rich regions in 3′ UTRs of target RNAs involved in cell survival and tumorigenesis [90]. HuR is mainly expressed in the nucleus but can shuttle between the nucleus and cytoplasm

due to the nucleo-cytoplasmic shuttling sequence present in the hinge region of the protein [91].

HuR is expressed throughout the intestinal epithelium in mice [92] [93]. Although mice with intestinal epithelial cell (IEC) knockout specific *HuR* show signs of mucosal atrophy, there are no changes in body weight or other abnormalities [92]. These mice show reduction in proliferating cells in the intestine and shorter crypts and villi but are otherwise healthy and reproduce normally [92] [93].

High HuR protein expression is found in both the nucleus and cytoplasm of human colon cancers [94]. While low HuR protein expression is observed in the normal colon [95], it is increased significantly in the cytoplasm of colorectal tumors [95]. Mice with intestinal specific *HuR* deletion ($HuR^{\Delta IEC}$) show increased injury in a doxorubicin induced acute injury model [92]. These mice also show increased regeneration and compensatory proliferation during the peak damage phase. Furthermore $HuR^{\Delta IEC}$ mice show more than 60% attenuation in the polyposis phenotype in the $Apc^{min/+}$ mice [92]. By contrast, $HuR^{\Delta IEC}$ mice show increased protection in the AOM-DSS model of tumorigenesis [92]. In intestinal cell lines, *HUR* inhibition causes a significant decrease in Wnt signaling, thereby suggesting a potential role in the regulation of the Wnt pathway [93]. HuR also has tumor suppressive functions via the regulation of tumor suppressors p21 and Wnt family protein Wnt-5a [96]. HuR is known to mediate post-transcriptional regulation of its target mRNAs and is critical for neoplastic transformation and cancer development. Furthermore, HuR is activated in response to various stressors [97].

HuR is being explored as a therapeutic target and small molecule inhibitors are being developed [90, 97, 98]. One such molecule, MS-444, has been shown to inhibit HuR that in turn decreases GI tumorigenesis and the proliferation of colon cancer cells [98, 99].

Mex3A

Mex-3 protein was discovered as a translational regulator in *C. elegans* that helps to maintain germline totipotency. In humans, MEX3 has 4 homologous isoforms MEX3A-3D [100]. The MEX3 proteins consist of 2 KH domains at the N terminal and a RING finger module domain at the C terminal end. The KH domain helps bind target RNAs whereas the nuclear export signal (NES) helps in shutting between the nucleus and cytoplasm [100]. Recently, MEX3C has been identified as a E3 ubiquitin ligase [101], whereas a variant of MEX3D has been shown to negatively regulate the anti-apoptotic protein BCL-2 in HeLa cells [102]. MEX3B (as well as MEX3A) has been shown to be a novel component of the RNA granules called P bodies [103].

In mice, MEX3A is expressed in the crypt base and labels a slowly cycling subpopulation of Lgr5+ intestinal stem cell population that can give rise to all lineages [104]. These MEX3A-high cells appear to resist the deleterious effects of chemotherapy or irradiation and play an important role in regeneration of damaged crypts [104]. Previous studies have shown that MEX3A regulates CDX2 in human colon cancer and correlates with “stemness” [105]. *Mex3a* deletion in IEC does not cause any changes in reproduction and intestinal

morphology in mice [104]. In Caco2 cells that can spontaneously differentiate into an enterocytic-like phenotype upon reaching confluence [106], inhibition of endogenous *MEX3A* using siRNA resulted in higher *CDX2* expression [105]. MEX3A overexpressing Caco2 cells show increased RNA expression of stem cell markers [105].

Mex3a is overexpressed in cancers like bladder urothelial carcinoma [107] and Wilm's tumor [108] whereas knockdown of MEX3A in human gastric cancer cells has been shown to significantly reduce cell proliferation [108] thus indicating its role in carcinogenesis and potential as a therapeutic target. This indicates the potential to study Mex3a in colorectal cancer.

CELF1

CUG binding protein 1 (CUBP1) or CELF1 is a multifunctional RBP studied primarily for its role in RNA metabolism related processes like decay, translation and splicing. CELF1 is known to bind GU rich elements in 3′UTR of target RNAs to regulate RNA stability [109]. CELF1 contains three highly conserved RRMs, two near the N terminal and one at the C terminal region. The three RRMs help recognize different motifs and form conformational changes to dictate specificity and range of binding partners [110].

In mice, CELF1 is expressed throughout the small intestinal epithelium [111] and can be repressed by mir-503 and recruited to P bodies [112]. mRNAs are localized to these cytoplasmic RNP foci and sorted for degradation and/or translational repression. CELF1 is also known to recruit certain target mRNAs like occludin to these P bodies and partially repress their translation [111]. Although CELF1 expression is found to increase proliferation and progression of several cancers [113–115], increased CELF1 causes G1 phase growth arrest in intestinal epithelial cells. By contrast, CELF1 silencing enhances cell proliferation, with an increase in cells residing in S-phase, and elevated cell number. CELF1 silencing enhances MYC translation by releasing MYC RNA from RNP complexes [111]. HUR is found to competitively repress this CELF1-MYC interaction [111]. CELF1 is mainly studied for its role in regulation of splicing in myotonic dystrophy [116, 117]. In the context of cancer, CELF1 can act as a tumor suppressor (in liver cancer [118]), increase caspase activity/apoptosis (in hepatocellular carcinoma [119] and esophageal cancer [120]) and act as a central node in post transcriptional regulatory programs underlying EMT (in breast cancer [121]) indicating its diverse role in carcinogenesis.

RBM3

RNA-binding motif protein 3 (RBM3), a glycine rich RBP [122], is an important cold shock protein that is upregulated during environmental stimuli such as hypothermia, ischemia, and hypoxia [123]. It binds to RNAs via its RRM domain and alters the secondary structure of the RNA affecting the access of mRNA initiation factor to the ribosome subunit [124], which modulates the potential activity of kinases in tumors.

RBM3 deficient mice show no overt phenotype or growth changes and are fertile [125]. RBM3 overexpression in HCT116 and DLD1 colon cancer cells increases proliferation and engenders chemotherapy resistance. These cells also exhibit increased stem cell markers via

an increase in B-CATENIN activity. Therefore, the *B-CATENIN* signaling pathway may be regulated through alterations in expression of RBM3 [126]. In colon cancer, RBM3 is upregulated in a stage dependent manner and its overexpression is capable of inducing oncogenic transformation [127]. RBM3 is shown to increase the stability and translation of rapidly degraded mRNAs such as cyclooxygenase 2 (*COX-2*), interleukin-8 (*IL-8*), and vascular endothelial growth factor (*VEGF*) [127]. Like the other RBPs, the role of RBM3 can be dichotomous in different contexts. In breast cancer, higher RBM3 expression correlates with increased disease-free survival [128]. RBM3 expression is upregulated in, and correlates with, good prognosis in several cancers, including ovarian, prostate, bladder, gastric, and colorectal cancer [129–132]. RBM3 causes cellular differentiation and apoptosis in these cancers.

Identifying the RNA targets of RBPs

RNA-binding proteins are a rapid and efficient way to alter gene expression. RBPs can bind to their target mRNAs and regulate everything from developmental transitions to response to injury or stress. These RNA-protein interactions can alter gene expression on both the post-transcription and translation levels. In recent years, high throughput assays have been developed to identify RBP binding sites and enumerate their target mRNAs. Therefore, in order to elucidate the functional dynamics of RBPs, it's important to identify the repertoire of RNAs that stably or transiently interact with the RBPs in a context-dependent and independent manner.

Large scale, high-throughput sequencing techniques, as well as mass spectrometry, have been used to identify mRNA targets and the functional effects of protein-RNA interactions [133, 134]. The widely used method for identifying RBP binding sites and partners consist of **C**ross**L**inking the RNP complexes followed by **I**mmuno**P**recipitation and then deep **SEQ**uencing of the bound RNA fragments also known as **CLIP-Seq** [135]. Several variations of CLIP or HITS-CLIP (High Throughput Sequencing - CLIP) have been described, including PAR-CLIP (photoactivatable-ribonucleoside-enhanced CLIP) [136], iCLIP (individual nucleotide resolution CLIP) [137], eCLIP (enhanced CLIP) [138], cross-linking analysis of cDNA (CRAC) [139], Fully Automated and Standardized iCLIP (FAST-iCLIP) [140] and cross-linking, ligation, and sequencing of hybrids (CLASH) [141, 142]. The main features, as well as potential advantages and disadvantages of the techniques are described in Table 1.

The targets of several of the RBPs mentioned in this paper have been discovered through these high throughput sequencing techniques (Table 2). In PAR-CLIP experiments done in HEK293 cells, LIN28A and LIN28B bound to a largely overlapping set of ~3000 mRNAs at ~9500 sites located in the 3′ untranslated region (UTR) and coding DNA sequence (CDS). The binding stabilizes target mRNAs to a certain degree and increases protein abundance mainly in cell cycle regulatory genes [35, 136]. CLIP-Seq studies done in CRC cell lines and in the mouse intestinal epithelium overexpressing LIN28B indicated an enrichment in RNAs for genes regulating metabolism, protein processing in the ER, the actin cytoskeleton, mRNA processing, and focal adhesion with most of the targets being epithelial specific or associated with the translation machinery [38].

Similarly, CLIP-Seq analysis for both endogenous and overexpressed MSI1 and MSI2 proteins in the intestine reveal that MSI1 and MSI2 drive common gene expression programs and interact with common target transcripts [72, 73, 78]. As high as 72% of gene expression changes resulting from Msi1 induction also occurred upon Msi2 induction [72, 73, 78]. The pathways found to be upregulated by gene ontology (GO) and pathway analysis were genes involved in ribosome biogenesis, signal transduction, and ErbB signaling, whereas oxidative phosphorylation and mitochondrial activity genes were downregulated [72, 80].

eCLIP studies have shown that there is substantial overlap between IMP1 and IMP2 binding but not between IMP1 and IMP3 [138]. During development and cancer, all IMP isoforms are highly expressed and might share redundant regulatory roles. IMP1 binds to and regulates genes associated with cell cycle, cell and focal adhesion and cellular integrity [138]. In HEK293 cells, separate studies with overexpression and depletion of IMP isoforms followed by PAR-CLIP showed a significant overlap of target transcripts that were mainly stabilized by IMP proteins [136].

Using PAR-CLIP, another group identified highly conserved HUR binding sites enriched for HUR binding motifs and mainly located in 3′ untranslated regions. Furthermore, the presence of some binding sites in the intronic regions suggests HuR's role in mRNA processing. Upon *HuR* knockdown, both mRNA expression and protein synthesis of thousands of target genes were downregulated, thereby suggesting a role in RNA stability and translation [143].

The increased density of RBM3 binding sites (seen via PAR-CLIP) near polyadenylation sites, especially those regulating genes that show strong circadian oscillations, has indicated the role of RBM3 in circadian gene expression [144]. In addition, CLIP-Seq analysis of alternate polyadenylation (APA) sites has elucidated the role of RBM3 in response to thermal stimuli [145]. Finally, several large-scale studies have been carried out for CELF1. HITS-CLIP has shown its preferential binding to the 3′ UTR and its role in destabilization of target mRNAs, specifically myogenic differentiation factors and RNA-binding proteins [146]. Another study has shown the role of CELF1 in stabilization and localization of developmentally regulated genes in skeletal muscle and heart cells [147]. In mice hindbrains, CELF1 binding sites were enriched in UG repeats [148] and bound to intronic and 3′UTR regions validating its role in splicing.

The CLIP studies show that all these RBPs have a huge number of targets that might overlap. Although these RBPs bind mainly to mRNAs, they can bind them at different regions and regulate a multitude of functions. Since the RBP function depends on which target RNAs are present in the microenvironment, the RBPs can function differently in different cellular contexts. In recent years, ribosome profiling studies [149] are being investigated as a means to study genome-wide translational effects of RBP overexpression or deletion in different model systems. This will help us gain a better insight into the functional effects of RBPs at the translational or protein level.

Concluding Remarks and Future Directions

The intestinal epithelium illustrates a proliferation-differentiation gradient with a rapid renewal and turnover of cells. This dynamic equilibrium can be disturbed during inflammation or injury that result from cellular stresses mediated by infectious organisms, radiation, and autoimmune diseases. These trigger a rapid protective and regenerative response that is regulated by several factors. Prolonged inflammation together with genetic alterations can result in malignant transformation. RBPs including LIN28, MSI, IMP1, MEX3A, CELF1, RBM3 and HUR-constitute a new set of regulatory proteins that play an important role in intestinal homeostasis, adaptation to injury and participation in malignant transformation. The effect of overexpression and deletion of these RBPs on intestinal development, homeostasis, response to injury and carcinogenesis is increasingly being studied both *in vitro* and in mouse models.

In addition to the phenotypic effects, the understanding of RBP-RNA interactions is critical. This involves identification of target RNAs, the interaction mechanism and the effect it has on the RNA metabolism. This is done initially through high-throughput approaches but mandates functional validation in model systems and in tissues. Furthermore, in RBPs that target mRNAs, the effect on translation needs to be further investigated using emerging techniques like ribosome profiling. The role of these RBPs in regulating key signaling processes and malignancies has led to their emergence as targets for therapeutics with potential implications in colorectal cancer.

Glossary

Paneth cells
Secretory cells in the intestinal epithelium that secrete antimicrobial peptides and proteins and help maintain the stem cell niche

Enteroendocrine cells
Secretory cells in the intestinal epithelium that secrete gastrointestinal hormones and peptides

Goblet cells
Mucus secreting intestinal epithelial cells

Enterocytes
Absorptive cells in the intestinal villi that aid in digestion and transport of molecules

Crypt base columnar cells
The radiosensitive, actively dividing stem cell population in the intestinal crypt base

Reserve +4 cells
The radio-resistant, quiescent stem cell population in the intestinal crypt base. It is activated during injury

Processing bodies/P bodies

The distinct foci in the cytoplasm of eukaryotic cells consisting of RNA-protein complexes that help in mRNA turnover

Crosslinking Immunoprecipitation and Sequencing/CLIP-Seq
Technique used for genome-wide profiling of protein-RNA interactions as well as RNA modifications.

RNA binding proteins/RBP
The proteins that bind different types of RNAs and regulate their function in one way or another

RNA binding domains/RBD
Structural motifs present in RBPs that help them bind to RNA

Ribosome profiling
A high throughput technique that provides a global snapshot of actively translating RNAs in the genome by sequencing RNA sites protected by ribosomes. This technique can be used to identify translated mRNA regions, observe protein folding patterns, and measure the amount of specific proteins that are synthesized.

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Box 1

The intestinal cell types

The intestinal epithelium consists of invaginations of crypts and protrusions villi that increase its surface manifold. The crypt base is the niche for two types of stem cells; the actively dividing crypt base columnar stem cells (CBCs) that are radiosensitive and the quiescent +4 stem cells that are radioresistant [2, 3]. All intestinal lineages (absorptive and secretory) arise from the stem cells and migrate upwards towards the villi except for Paneth cells that migrate towards the base [8]. The secretory cells consist of Paneth cells (secreting anti-microbial peptides and maintaining the stem cell niche), goblet cells (secrete mucus), enteroendocrine cells (secreting hormones) and tuft cells (secrete cytokines). The absorptive enterocytes make up the majority of the epithelium and absorb micronutrients, water and electrolytes. The transit amplifying cells are immediate progeny o stem cells. The colon epithelium is similar to the small intestinal epithelium but lacks villi and serves to absorb remaining water and provide a barrier against micro-organisms [9]. (Figure 1)

Highlights

- Intestinal epithelial cells harbor proliferation-differentiation gradient spanning crypts to villi. This is regulated by two stem cell populations.
- RNA binding proteins (RBPs) provide a nexus of regulation of intestinal epithelial homeostasis, adaptation to injury and contribution to malignant transformation
- These specific RBPs that have been reported in the published literature in the context of intestinal epithelial biology and colorectal cancer include: LIN28, Musashi (MSI), IGF2BP/IMP (Insulin-like growth factor 2 mRNA binding proteins), MEX3A, CELF1 (CUGBP Elav-Like Family Member 1), RBM3 (RNA binding protein 3) and HUR (Hu-Antigen R).
- These specific RBPs play important roles in intestinal regeneration following injury.
- These specific RBPs are overexpressed in human colorectal cancer and overexpression of some of them have been shown to be sufficient to transform the intestinal epithelium in mouse models.

Outstanding questions

More than one RBP can bind to a transcript and regulate its function. The intestinal epithelium expresses a range of RBPs that bind common transcripts. This necessitates the need to develop high-throughput techniques to study combinatorial effects of RBPs in the intestine. Are there distinct functional effects of RBP-binding to a particular region of the RNA transcript (*eg.* Binding to the 3′UTR *vs* the coding region)?

RBPs have a range of functional effects that can regulate both the transcriptome and the translatome. What is the effect of RBP binding, not only at the RNA level, but also on protein translation and proteins? This will aid functional annotation of the RBPs.

How can RBPs be targeted therapeutically in colorectal cancer? The intestinal epithelium is highly proliferative and dynamic and the changes in microenvironment are rapid and complex. Since the function of RBPs is dependent upon which target RNAs are expressed in the microenvironment, it is necessary to study them in model systems that mimic stress and injury conditions.

Clinician's Corner

- RNA binding proteins (RBPs) represent a newly appreciated family that serve as regulatory networks of intestinal epithelial homeostasis, adaptation to injury to enable regeneration, and contributions to malignant transformation, the latter as evident in colorectal cancer.
- Many RBPs have conserved structural domains through which a repertoire of RNAs is targeted.
- These RBP:RNA complexes enable functional diversity in cellular processes and a rapid response to cellular stress.
- The protective role of some of these RBPs in response to injury can help inform strategies for chemotherapy and radiation therapy in colorectal cancer.
- The aberrant expression and function of certain RBPs in colorectal cancer might provide the impetus for the development of inhibitors of these RBPs.

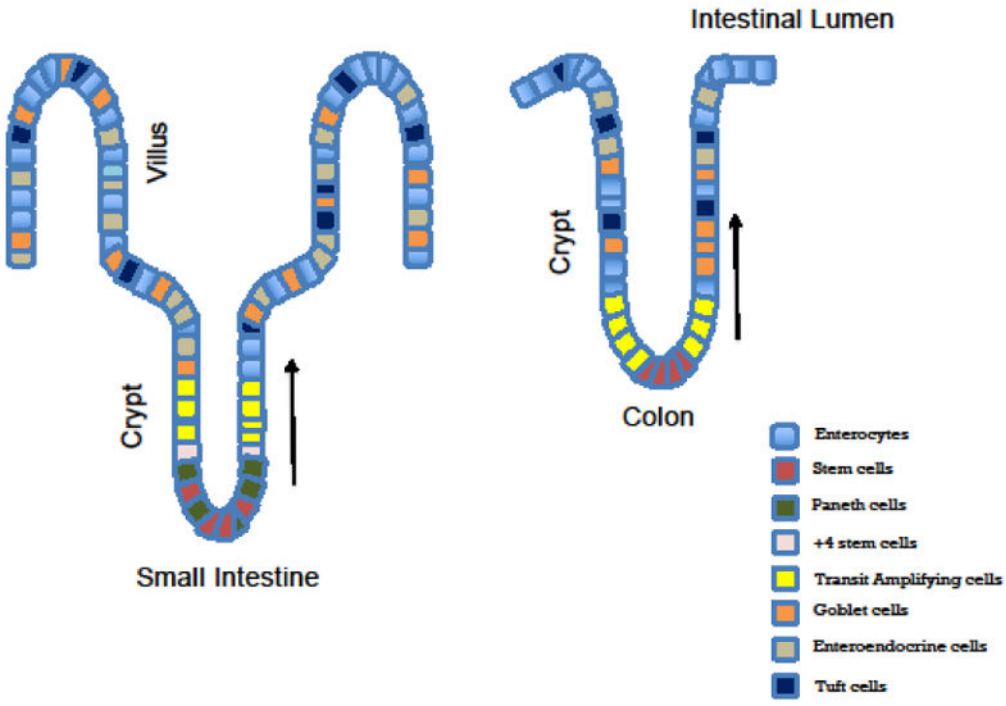

Figure 1.

Schematic representation of the crypt-villus axis and the major intestinal cell types for small intestine and colon

The figure depicts the major cell types in the small intestine and colon. The stem cells reside at the crypt base and proliferate (transit amplifying cells) and differentiate into secretory (Paneth, enteroendocrine, goblet, tufts cells) and absorptive lineages (enterocytes). These differentiated cells migrate towards the villi (in the small intestine).

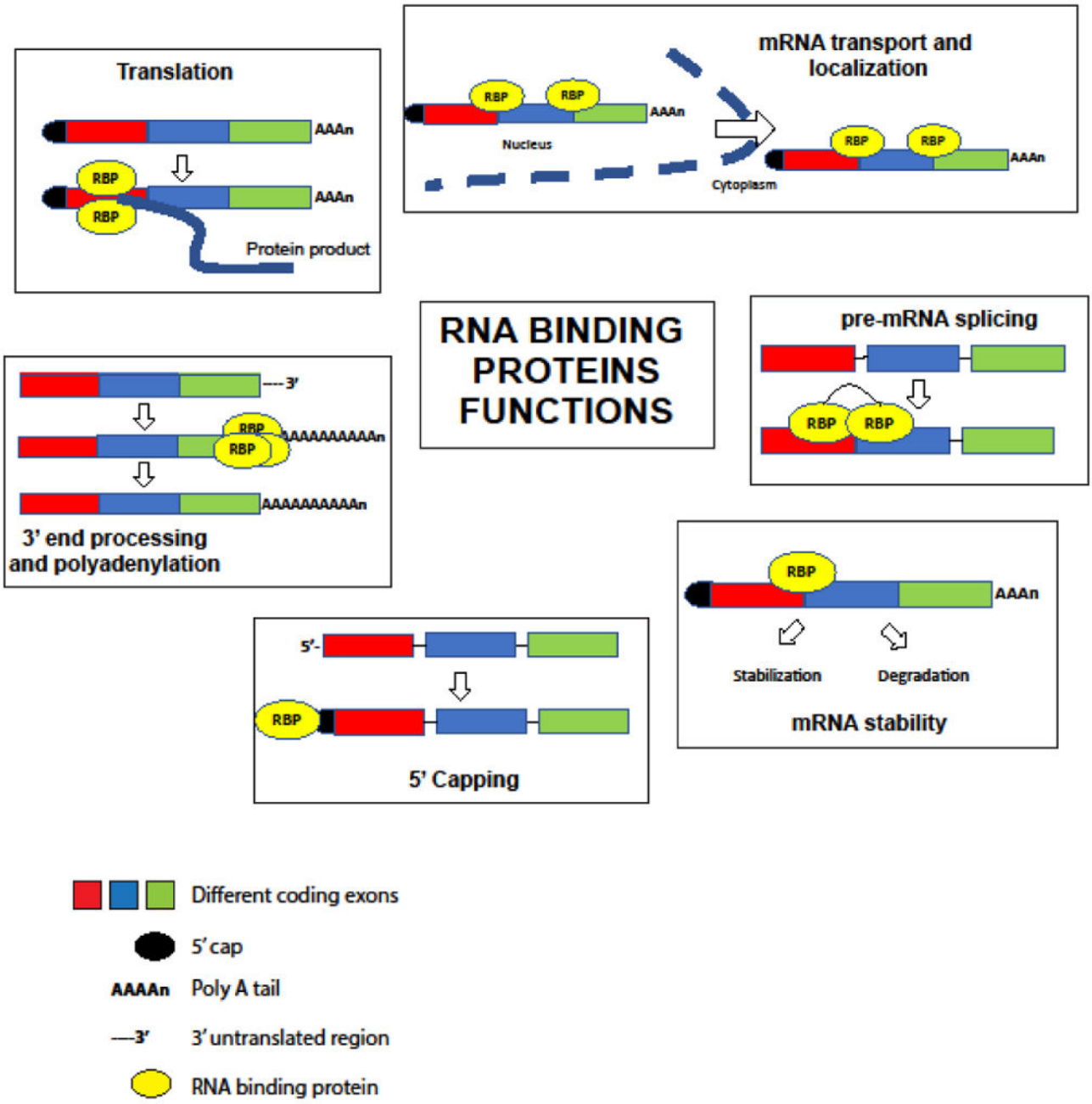

Figure 2.

Schematic representation of the different functional roles of RBPs

The figure depicts the major functional roles of the RBPs discussed in this review. The majority of the RBPs discussed here bind to mRNAs and regulate their processing (5′ capping, 3′ end processing, splicing), stability, localization and translation. This figure does not depict the non-conventional RBPs.

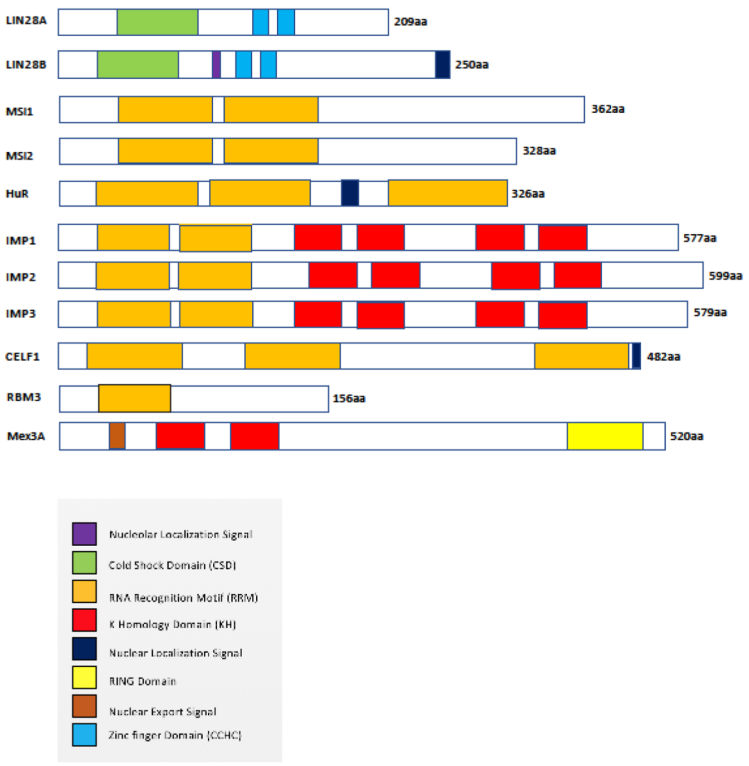

Figure 3.

Schematic representation of the structural domains of the RBPs

The figure shows a simplistic representation of the different structural domains of the RBPs discussed in this review that help them bind to their target RNAs and regulate their function. The amino acid length of the RBPs are also mentioned. The RBPs contain different types of RNA binding and catalytic domains including RRMs, KH domains, zinc finger domains and cold shock domains. Some of these RBPs also contain localization signals to help them shuttle in and out of the nucleas.

Table 1

Key features of the different CLIP techniques

Technique	Crosslinking method	Key feature	Advantages	Disadvantages
HITS-CLIP[150]	UV 254nm	Induction of covalent crosslinks between protein and a directly bound (within ~ 1 Å) RNA by UV irradiation	Takes advantage of the natural photoreactivity of nucleic acid bases	Less efficient for small and micro RNAs
PAR-CLIP[136]	UV 365nm	4-thio-uridine incorporation into RNA	High resolution due to T->C mutations	Expensive
iCLIP[137]	UV 254nm	Circularization of reverse transcribed product instead of 5′ adapter ligation	Efficiency, enables identification of the cross-linking site at nucleotide resolution	Technically difficult
eCLIP[151]	UV 254nm	5′ DNA adaptor ligation to the truncated cDNA	1,000-fold increased efficiency and shorter sample-preparation times	Expensive
FAST-iCLIP[140]	UV 254nm	Uses biotin-streptavidin affinity purification	Does not require CLIP grade antibody	The RBP needs to be tagged and overexpressed.
CRAC[139]	UV 254nm	Use of affinity resins that are independent of protein–peptide interactions	Mimics more native conditions by increasing stringency	Tagging and overexpressing RBP might cause binding changes
CLASH[141, 142]	UV 254nm	Affinity purification and RNA-RNA intermolecular ligation	Does not require CLIP grade antibody, helps study RNA-RNA interactions	Modification of RBP might have non-physiological effects
iCLAP[152]	UV 254nm	Uses biotin-streptavidin - Histidine affinity purification and circularization of reverse transcribed product	Individual nucleotide resolution	Modification of RBP might have non-physiological effects
irCLIP[153]	UV 254nm	Use of thermostable reverse transcriptase followed by circularization of the cDNA	Increased specificity, no radioactivity, quicker method	Expensive, technically challenging

Table 2

CLIP studies in the RBPs of interest

RBP	Technique Used	Cell Type	Reference
CELF1	CLIP/SoliD Sequencing	Human Hela Cells	[154]
CELF1	HITS-CLIP	mouse myoblast cell line C2C12	[146]
CELF1	CLIP-Seq	Murine skeletal and heart cells	[147]
CELF1	CLIP-SEQ	Mice hindbrains	[148]
HUR	PAR-CLIP	Human Hela Cells	[143]
HUR, IMP1	fRIP-Seq	K562 cells	[155]
MSI2	CLIP-Seq	Mouse epithelium	[73]
MSI1/MSI2	CLIP-Seq	Mouse epithelium	[72]
RBM3	RNA-Seq/CLIP-Seq	MEFs	[144]
LIN28A	HITS-CLIP	hESCs (H9, HUES6), 293 cells	[156]
LIN28A	CLIP-Seq, MS	BL21 Rosetta cell colonies	[157]
LIN28A	CLIP-Seq, Ribosome footprinting	Mouse embryonic stem cell A3-1	[158]
LIN28	HITS-CLIP	*C. elegans*	[159]
LIN28B	iDo-PAR-CLIP	Flp-In 293 T-REx cells	[160]
LIN28A, LIN28B	PAR-CLIP	HEK293 cells	[35]
LIN28B	CLIP-Seq, RNA Seq	LoVo cells, DLD1 cells, Mouse intestine	[38]
IMP1	eCLIP	iPSCs	[138]
IMPs	PAR-CLIP	HEK293	[136]