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Regulation by 3′-Untranslated Regions

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Abstract

3′-untranslated regions (3′-UTRs) are the noncoding parts of mRNAs. Compared to yeast, in humans, median 3′-UTR length has expanded approximately tenfold alongside an increased generation of alternative 3′-UTR isoforms. In contrast, the number of coding genes, as well as coding region length, has remained similar. This suggests an important role for 3′-UTRs in the biology of higher organisms. 3′-UTRs are best known to regulate diverse fates of mRNAs, including degradation, translation, and localization, but they can also function like long noncoding or small RNAs, as has been shown for whole 3′-UTRs as well as for cleaved fragments. Furthermore, 3′-UTRs determine the fate of proteins through the regulation of protein–protein interactions. They facilitate cotranslational protein complex formation, which establishes a role for 3′-UTRs as evolved eukaryotic operons. Whereas bacterial operons promote the interaction of two subunits, 3′-UTRs enable the formation of protein complexes with diverse compositions. All of these 3′-UTR functions are accomplished by effector proteins that are recruited by RNA-binding proteins that bind to 3′-UTR *cis*-elements. In summary, 3′-UTRs seem to be major players in gene regulation that enable local functions, compartmentalization, and cooperativity, which makes them important tools for the regulation of phenotypic diversity of higher organisms.

INTRODUCTION

Regulation of gene function through 3′-untranslated regions (3′-UTRs) is a relatively new field as only recent sequencing technology has provided us with the full landscape of 3′-UTRs across species and cell types. Before sequencing technology was available, detailed functional and mechanistic studies were performed only on a few model 3′-UTRs. Although these model 3′-UTRs have contributed substantially to our understanding of 3′-UTR biology, the conclusions drawn about their regulatory functions have focused primarily on the role of 3′-UTRs in gene regulation. 3′-UTRs determine protein levels through regulation of mRNA stability and translation mediated largely by AU-rich elements and miRNAs (6, 7, 21). 3′-UTRs also enable local translation through the regulation of mRNA localization (82, 98). In this review, some of the general principles that were learned from these model 3′-UTRs will be discussed, but I mostly focus on newer findings that may change our view about the functions of 3′-UTRs. This includes the discovery that 3′-UTR length can be regulated by alternative cleavage and polyadenylation (APA) (81, 87, 106), as well as the observation that 3′-UTRs may be cleaved off and act like long noncoding RNAs living their own life independent of the coding region (17, 69, 90). Lastly, 3′-UTRs were found to be able to mediate protein–protein interactions (PPIs), a discovery which has widespread consequences for protein complex formation, protein localization, and protein functions (9, 19, 31, 45).

3′-UTRs regulate gene expression through the binding of RNA-binding proteins (RBPs) (5, 15, 51, 93). RBPs bind to 3′-UTR *cis*-elements and mediate 3′-UTR functions through the recruitment of effector proteins. As RBPs interact with diverse effector proteins, each 3′-UTR regulatory element has the potential to carry out several different functions, depending on the cell type or cellular state. The cellular state also determines the RBPs that are able to access 3′-UTRs at a given moment. As such, 3′-UTR functions can be assessed only in the context of their bound RBPs. The composition of RBPs bound to a 3′-UTR at a given moment is dynamic and can change depending on the local environment, e.g., through addition of posttranslational modifications (PTMs), local expression of other RBPs, and interactions with membranes and cytoskeletal filaments (59, 60, 118). RBP binding is also influenced by secondary and tertiary RNA structure formation that regulates the accessibility of 3′-UTRs (2, 42, 68, 122). Importantly, RBPs cooperate with other bound RBPs to enable functional specificity in vivo. Answers to open questions will ultimately stimulate new avenues of 3′-UTR research in coming years.

In 1958, Francis Crick wrote, "Watson said to me, a few years ago, 'The most significant thing about the nucleic acids is that we don't know what they do.' By contrast the most significant thing about proteins is that they can do almost anything" (24, p. 138). In the same article, Crick published his hypothesis of the central dogma, in which he stated that the "transfer of information from nucleic acid to nucleic acid, or from nucleic acid to protein may be possible, but transfer from protein to protein, or from protein to nucleic acid is impossible" (24, p. 153). The simplicity of the idea was widely embraced and shaped the protein-centric view of cell biology for the next 50 years. However, in addition to the coding region—which is translated into protein—mRNAs also consist of untranslated regions at the 5′ and 3′ ends, which contain important regulatory elements (103). The novel finding that the nucleic acids located within 3′-UTRs can mediate PPIs, and thus can determine protein functions without altering the amino acid sequence (9), has the potential to lead to a less protein-centric view of cell biology.

EXPANSION OF 3′-UTRs DURING EVOLUTION OF MORE COMPLEX ORGANISMS

3′-UTRs are ancient components of mRNAs and are found in all three domains of life—bacteria, archaea, and eukaryotes. Despite the compact genomes found in unicellular organisms, sequencing

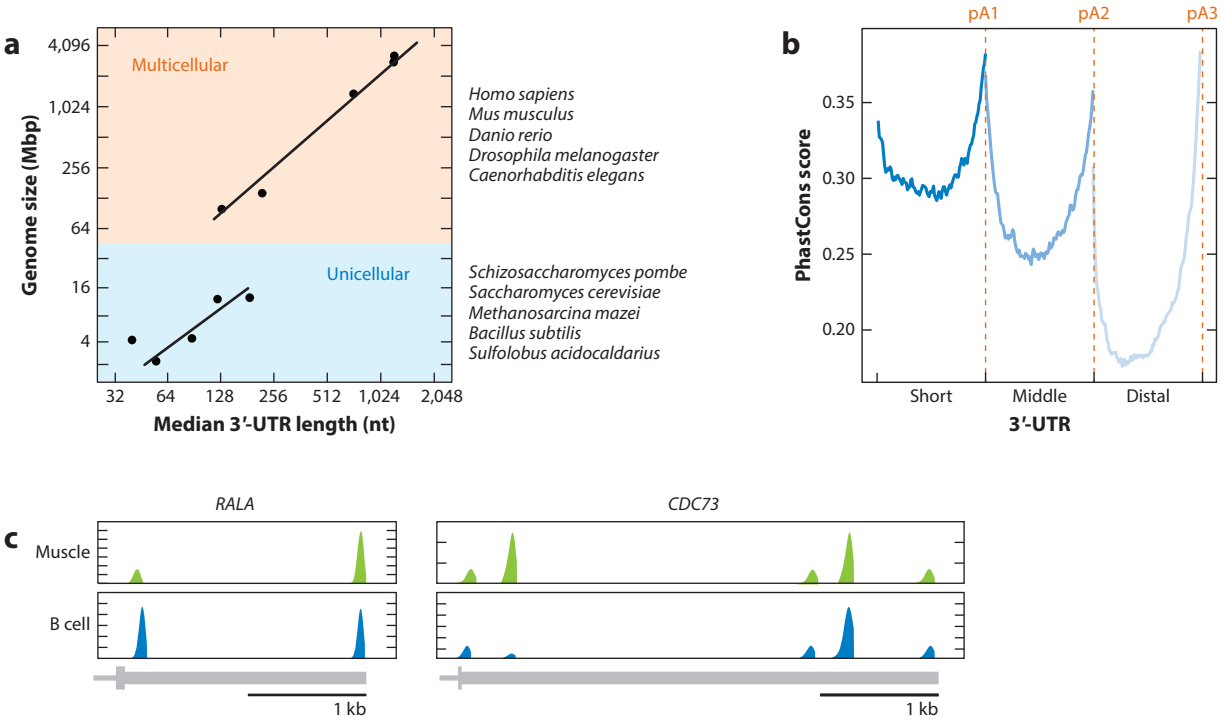


Figure 1

Characteristics of 3′-UTR isoforms. (*a*) Correlation between genome size and median 3′-UTR length in uni- and multicellular organisms. Genome size was obtained from Reference 97. 3′-UTR length was obtained from References 26–28, 58, 81, 107, 130, and 139. (*b*) Sequence conservation shown as PhastCons scores for genes that generate three 3′-UTRs ($n = 1{,}939$) for the regions between the stop codon and the first pA site (short 3′-UTR), the first to the second pA site (middle 3′-UTR), and the second to the last pA site (distal 3′-UTR). (*c*) Alternative 3′-UTRs are expressed in a cell type–specific manner. Shown are 3′-seq data for skeletal muscle (*top*) and naive B cells (*bottom*) obtained from Reference 81. The gene model (*gray*) is drawn to scale and depicts the last exon. Abbreviations: 3′-UTR, 3′-untranslated region; Mbp, millions of base pairs; nt, nucleotide; pA, polyadenylation.

technology has revealed that their mRNAs contain 3′-UTRs (26, 27, 107, 139). In unicellular genomes, medium 3′-UTR length correlates with genome size (**Figure 1*a***) and is approximately 60 nucleotides (nt) in the bacterial and archaeal species sequenced so far and approximately 150 nt in yeast. Characteristic of the evolution of more complex multicellular organisms is a substantial expansion in genome size that also strongly correlates with increased 3′-UTR length (28, 58, 81, 130) (**Figure 1*a***). For example, median 3′-UTR length increased from approximately 140 nt in worms to approximately 1,200 nt in humans (86). Intriguingly, the increase in 3′-UTR length correlates with organismal complexity when measured as the number of cell types found in an organism (20).

Notably, although they are less conserved than coding regions, 3′-UTRs still show a high degree of sequence conservation across species (117, 143), which is exemplified by the distribution of highly conserved elements. The percentage of base pairs in vertebrate genomes that are highly conserved is 0.14%; however, these elements are enriched 22-fold in coding regions and 11-fold in 3′-UTRs (117). Furthermore, 3′-UTRs often contain many islands of sequence conservation that are similar in conservation to coding regions, and these islands often contain binding sites for miRNAs or RBPs (34, 143). Taken together, the increase in 3′-UTR length during the

development of higher organisms and the enrichment of conserved sequence elements within 3′-UTRs suggests a critical role for 3′-UTRs in the regulation of biological complexity (86).

ALTERNATIVE CLEAVAGE AND POLYADENYLATION REGULATES THE PRESENCE AND ACCESSIBILITY OF REGULATORY ELEMENTS IN 3′-UTRs

Further evidence for the importance of 3′-UTRs is the fact that the presence of functional elements is regulated through the process of APA. In archaea, 30% of genes generate alternative 3′-UTRs with a median length difference of 54 nt (26). In humans, the median 3′-UTR length of genes that produce alternative 3′-UTR isoforms is 2,462 nt, and the majority of genes (51–79%) express alternative 3′-UTRs (28, 81; I. Singh & C. Mayr, unpublished data**)**. Thus, the number of genes that generate alternative 3′-UTRs has expanded alongside the increase in 3′-UTR length during the evolution of multicellular organisms.

Intuitively, one would think that longer 3′-UTRs mediate more regulatory functions as they contain more regulatory elements. However, this is only partially the case, as it was found that especially long 3′-UTRs are less responsive than expected to miRNA regulation (2, 68). One reason for this relationship seems to be a decreased accessibility of regulatory elements because of occlusive structures in long 3′-UTRs. As was shown several years ago, miRNA-binding sites located in the middle of 3′-UTRs mediate less repression than do sites at the ends of 3′-UTRs (42), which is also true for alternative 3′-UTRs (50). Better accessibility is supported by cross-linking immunoprecipitation (CLIP) studies demonstrating an enrichment of RBP-binding sites toward the very ends of 3′-UTRs (95, 136). Interestingly, increased accessibility correlates with increased sequence conservation in the last few hundred nucleotides toward the ends of alternative 3′-UTR isoforms (**Figure 1*b***) (I. Singh & C. Mayr, unpublished data). The general association between RNA sequence conservation and accessibility in 3′-UTRs is reminiscent of what was also found in introns and ribozymes (109, 122). The generation of alternative 3′-UTRs seems to enable increased accessibility of 3′-UTR regulatory elements that are otherwise buried within long 3′-UTRs.

In addition to facilitating the accessibility of regulatory elements, alternative 3′-UTRs can also differentially regulate gene expression. Most genes that generate alternative 3′-UTR isoforms have evolved ways to regulate 3′-UTR ratios (81). Embryonic tissues tend to express shorter 3′-UTRs than differentiated tissues (63, 73, 115, 130). Across differentiated tissues, alternative 3′-UTRs are usually expressed in a cell type–specific manner (**Figure 1*c***) (33, 81) and can change through activation of signaling pathways (32, 106) or disease (83, 87, 125). Usage of the most proximal polyadenylation (pA) site in these 3′-UTRs results in mRNA transcripts with rather short 3′-UTRs (median length of 400 nt). These are similar in length to mRNA transcripts generating only one 3′-UTR isoform (median length of 505 nt). This indicates that usage of proximal pA sites can result in the loss of the majority of regulatory elements present in longer 3′-UTRs.

BIOLOGICAL FUNCTIONS OF 3′-UTRs

3′-UTRs are best known to determine the fate of mRNAs through the regulation of mRNA stability, translation, and mRNA localization. 3′-UTR functions are mediated by RBPs that bind to 3′-UTRs in a sequence- or structure-dependent manner. However, RBPs act only as adaptors that connect 3′-UTRs to effector proteins. Thus, the biological consequences that are mediated by 3′-UTRs depend on the functions of the effector proteins. For example, mRNA destabilization is achieved through RBPs that recruit a deadenylase (**Figure 2*a***) (147), whereas translational

repression is accomplished by RBPs that recruit decapping enzymes (22). 3′-UTRs also determine the fate of newly translated proteins. This is facilitated by RBPs that recruit effector proteins that are transferred in a cotranslational manner onto the nascent peptide chain (9) (**Figure 2*b***). This indicates that 3′-UTRs can mediate PPIs, and thus, regulate both protein localization and function (9).

3′-UTRs Mediate Protein–Protein Interactions

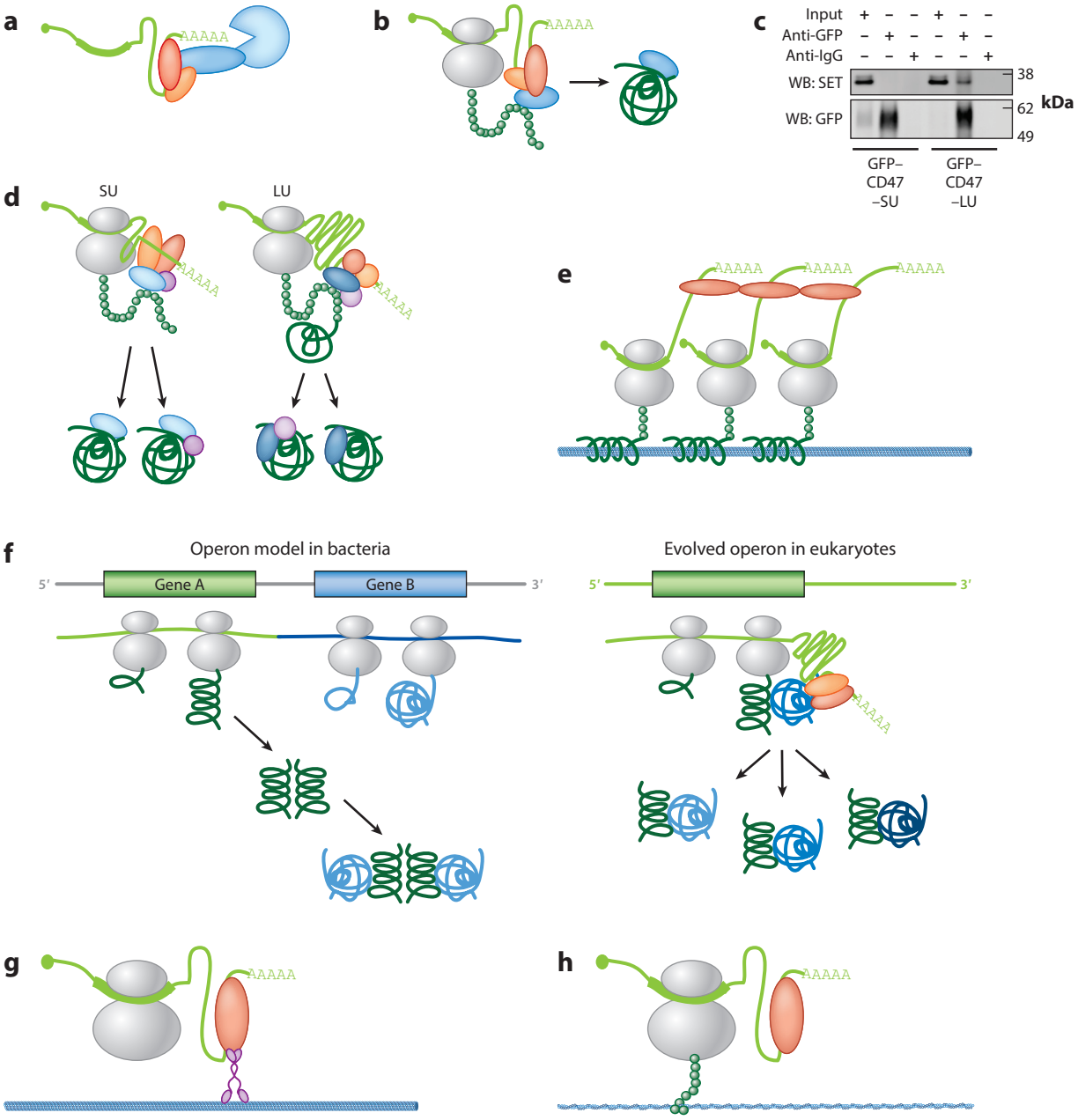
During translation of mRNAs, the nascent peptide chain and the 3′-UTR come into close proximity which seems to enable the transfer of proteins from the vicinity of the 3′-UTR onto the nascent peptide chain. Recruitment of protein interaction partners (PIPs) to the nascent peptide chain by 3′-UTRs may increase the association rate of the interacting proteins, and thus, may enable 3′UTR-dependent PPIs.

3′-UTRs can regulate protein localization independently of mRNA localization. It is commonly thought that the information for the formation of PPIs is fully contained within the interacting proteins themselves. This is illustrated in experiments studying PPIs by the use of constructs that contain only the coding regions (54). It is also generally assumed that proteins find their binding partners through diffusion and random collision. However, Berkovits & Mayr (9) made an observation that is not consistent with this prevalent model through their discovery that the 3′-UTR of *CD47* is required for the PPI between CD47 and SET. CD47 is known as a plasma membrane receptor that acts as a don't-eat-me signal (55), and the *CD47* gene uses APA to produce mRNA transcripts with either a short or a long 3′-UTR. Berkovits & Mayr called the protein that was generated from the short *CD47* 3′-UTR isoform CD47-SU and called the protein that was made from the long 3′-UTR isoform CD47-LU. Although CD47-SU and CD47-LU have identical amino acid sequences, Berkovits & Mayr demonstrated that, of the two, only CD47-LU interacts with SET (**Figure 2*c***). This indicated that the formation of the PPI between CD47-LU and SET required recruitment of SET by the long *CD47* 3′-UTR (9). This PPI has functional consequences, as it promotes plasma membrane localization of CD47-LU, whereas CD47-SU is predominantly expressed intracellularly.

Importantly, Berkovits & Mayr demonstrated that the difference in CD47 protein localization was not due to differences in mRNA localization. Instead, a model was proposed and experimentally verified in which the RBP HuR binds only to the long, not the short, *CD47* 3′-UTR. HuR binding was responsible for the recruitment of SET to the long 3′-UTR (9, 13). During translation of the CD47 polypeptide chain, SET seems to be transferred onto the newly made CD47-LU protein, where SET binds to positively charged amino acids in the cytoplasmic domains of CD47-LU. SET also interacts with RAC1, and active RAC1 mediates efficient surface localization of CD47-LU (9, 124). In contrast, SET is not recruited to the short *CD47* 3′-UTR and does not interact with CD47-SU, leading to inefficient localization of CD47-SU to the plasma membrane. Thus, CD47-LU largely represents the well-known surface CD47 protein (55).

The study also expanded these findings to show that long 3′-UTRs of genes encoding other plasma membrane proteins, such as CD44, Integrin α1, and the BAFF receptor, were also able to increase cell surface expression of their cognate proteins. These candidates were investigated because they had the minimal requirements necessary for 3′-UTR-dependent protein localization. These requirements included HuR-binding sites in their long 3′-UTRs that facilitate SET recruitment and positively charged amino acids in their cytoplasmic protein domains necessary for SET binding (9). Therefore, 3′-UTRs can mediate PPIs, and thus they enable subcellular localization of proteins independently of mRNA localization.

3′-UTR-mediated prerecruitment of the signal recognition particle to the ribosome. 3′-UTR-mediated recruitment of proteins to the nascent peptide chain was recently confirmed in yeast using ribosome profiling of ribosome subpopulations. The 3′-UTRs of membrane protein-encoding genes, but not the 3′-UTR of a gene encoding a cytosolic protein, can recruit the signal recognition particle (SRP) to the ribosome before the polypeptide chain encoding the signal sequence has become exposed (19). The prevailing model of SRP recruitment to ribosomes has



been that translation of mRNAs encoding secretory proteins begins in the cytoplasm and, following exposure of the hydrophobic signal sequence at the exit channel of the ribosome, SRP binds to the signal sequence. This leads to halted translation and transfer of the SRP-bound ribosome to the surface of the endoplasmic reticulum. Once the ribosome is localized to the endoplasmic reticulum, translation resumes and the membrane protein is folded into the membrane (135). However, using co-immunoprecipitation (co-IP) of SRP72 followed by ribosome profiling, Chartron and colleagues (19) showed that this model is only true for a minority of secretory proteins. For the vast majority of proteins, SRP is prerecruited to ribosomes in a 3′-UTR-dependent manner before the signal sequence has been translated and before the nascent peptide chain has become exposed (19). These findings demonstrate that the selective interaction of SRP with ribosomes that translate secretory proteins is for the majority of cases not contained within the hydrophobic amino acids of the nascent peptides, but rather is mediated by elements in the 3′-UTRs of the mRNAs that are being translated. This suggests that specific 3′-UTRs recruit to the site of translation proteins that subsequently bind the nascent peptide chain.

Widespread occurrence of cotranslational cognate mRNA–protein complexes. For both SET and SRP, which are transferred onto the nascent peptide chain in a 3′-UTR-mediated manner, the model contains a complex that consists of a translating ribosome, an mRNA that encodes the nascent protein, and the nascent protein with an mRNA-recruited cotranslational PIP (**Figure 2*b***). Such complexes seem to be widespread in yeast. For example, purification of the SET1 histone methyltransferase complex revealed an enrichment of *SET1* mRNA. *SET1* mRNA copurified with four of the eight proteins of the SET1 histone methyltransferase complex in a process that was dependent on cytoplasmic expression of the mRNA and on translation (45). Therefore, it was concluded that a SET1 subcomplex assembles on the nascent SET1 protein in a cotranslational manner. Using RNA-IP on proteins that do not contain RNA-binding domains, Duncan & Mata (31) showed that such cotranslational complexes occur in 38% of tested cases (**Figure 2*b***). These data strongly suggest that the protein used for RNA-IP binds to the nascent polypeptide chain and that cotranslational PPIs that involve the corresponding mRNAs are widespread.

Figure 2

Models of 3′-UTR-mediated functions. (*a*) RBPs (*red*, *orange*) bound to 3′-UTRs recruit effector proteins (*blue*) that determine the fate of mRNAs. (*b*) RBPs (*red*, *orange*) bound to 3′-UTRs recruit effector proteins (*blue*) that are transferred onto the nascent peptide chain (*dark green*), resulting in a 3′-UTR-dependent protein complex. (*c*) Co-IP of endogenous SET using GFP-trap after transfection of GFP-CD47-SU or GFP-CD47-LU in HEK293 cells. Two percent of input was loaded. (*d*) Model of alternative PIPs recruited by alternative 3′-UTRs despite the sequence of the short 3′-UTR being fully contained within the long 3′-UTR. It is possible that the mRNA structure occludes the regulatory elements in the short 3′-UTR within the context of the long 3′-UTR. Effector proteins are shown in light blue, dark blue, and purple. (*e*) Model of *peripherin* mRNA clustering by an RBP that binds to the *peripherin* 3′-UTR and clusters the mRNAs through multimerization. This brings nascent peripherin proteins together and may allow them to oligomerize using microtubules (*blue*) as scaffold. (*f*) In bacterial operons, genes encoding subunits of protein complexes are in close proximity and are translated simultaneously. This facilitates protein complex formation. In evolved operons in eukaryotes, the 3′-UTR recruits various PIPs to the site of translation. This has the advantage of being independent of simultaneous translation of the PIPs and allows the recruitment of different PIPs that enable formation of substoichiometric protein complexes with diverse compositions. (*g*) mRNA localization through a microtubule motor protein (*purple*) that connects the 3′-UTR to cytoskeletal filaments using an RBP (*red*) as adaptor. (*h*) An actin-binding domain in the N terminus of Abp140 binds to actin filaments and transports the cognate mRNA using actin retrograde flow. Abbreviations: 3′-UTRs, 3′-untranslated regions; co-IP, co-immunoprecipitation; GFP, green fluorescent protein; IgG, immunoglobulin G; LU, long 3′-untranslated region; PIP, protein interaction partner; RBP, RNA-binding protein; SU, short 3′-untranslated region; WB, Western blotting. Panel *c* adapted from Reference 9.

Alternative 3′-UTRs mediate the multifunctionality of proteins. In the case of CD47, the alternative 3′-UTRs not only regulate protein localization but also determine alternative functions of CD47. When cells that expressed either CD47-LU or CD47-SU were exposed to γ-irradiation, the cells that expressed CD47-LU survived whereas the cells that expressed CD47-SU died (9). The functional difference between CD47-SU and CD47-LU was due to alternative 3′-UTR-dependent PIPs (**Figure 2*d***). As discussed earlier, SET binds to CD47-LU, but not to CD47-SU. Consequently, the binding domains in CD47-SU are available to interact with proteins other than SET. The CD47-SU-specific PIPs were shown to be responsible for cell death induction after γ-irradiation (85). These findings demonstrate that the information that determines protein complex formation as well as protein function is not always contained within the amino acid sequence; it can be encoded in the noncoding parts of mRNAs, specifically within 3′-UTRs.

3′-UTR-mediated cotranslational complexes may facilitate homopolymerization of filaments. Taking these new findings into account, earlier experimental evidence can be reinterpreted. For example, Chang and colleagues (16) studied the assembly of intermediate filaments in mammalian cells using peripherin as a model. Like the previously mentioned yeast studies, they identified cotranslational cognate mRNA–protein complexes together with a clustering of *peripherin* mRNAs. Interestingly, they showed that mRNA clustering depends on the presence of the *peripherin* 3′-UTR, translation of peripherin protein, and polymerized microtubules. Thus, a model can be proposed in which mRNA clustering facilitates cotranslational homopolymerization of filaments (**Figure 2*e***). mRNA clustering can be achieved through an RBP that binds and connects different *peripherin* 3′-UTRs. This brings the ribosomes translating *peripherin* mRNAs into proximity with one another so that nascent peripherin subunits can assemble on microtubules. Such a model is consistent with early reports on the association of nascent peptides, including vimentin and myosin, with the cytoskeleton (36). As vimentin, myosin, and peripherin are built through homotypic coiled-coiled interactions, it is possible that polymerization of intermediate filament chains takes place cotranslationally and involves the coordinated synthesis of mRNAs that closely associate through their 3′-UTRs.

3′-UTR-mediated protein–protein interactions; open questions and future directions. 3′-UTR-mediated cotranslational protein transfer is a conserved and widespread mechanism in both yeast and human cells. An emerging picture from the studies described above is that 3′-UTRs can assist cotranslational multiprotein complex assembly, protein oligomerization, and the formation of PPIs (9, 16, 19, 31, 45). These data suggest that although PPIs take place between two proteins, the initiation of binding is facilitated through recruitment of a PIP by a 3′-UTR. However, the number of edges of the cotranslational mRNA–protein network seems to be larger in human cells than in yeast. In yeast, mRNAs recruit only a few mRNA-dependent PIPs, and the recruited PIPs generally interact with only one to three mRNAs (31, 45). By contrast, in human cells dozens or even hundreds of 3′-UTR-dependent PIPs have been found (79, 85).

The recruitment of many different 3′-UTR-dependent PIPs may enable the multifunctionality of proteins, even in the absence of alternative 3′-UTRs. For example, a single 3′-UTR could assist in the cotranslational formation of several substoichiometric protein complexes or subcomplexes. For processes that require the presence of several proteins in a specific location, the assumption that proteins are able to freely diffuse makes it unlikely that all of them are present simultaneously at the required location. However, through 3′-UTR-dependent formation of subcomplexes that contain the majority of these factors, only one protein localization event would be necessary. This suggests that 3′-UTRs may preorganize ligand binding sites of a protein complex. Preorganization reduces the freedom for binding events of individual components, which, in turn, decreases the number

of nonproductive configurations and reduces entropy. Such 3′-UTR-dependent preorganization may even increase reaction rates, as it reduces the freedom of motion of individual proteins (141). Thus, 3′-UTR-dependent PPIs enable cooperativity.

The topology of the cotranslational complexes that enable 3′-UTR-dependent PPIs is very reminiscent of translational operons in bacteria. Operons organize genes that encode distinct subunits of protein complexes to facilitate cotranslational protein complex assembly (116, 140). Cotranslational 3′-UTR-dependent protein complex assembly can be viewed as an evolved eukaryotic operon because the recruitment of PIPs by 3′-UTRs has several key advantages: A lack of dependency on simultaneous translation of the PIPs makes it more flexible, and the ability to recruit several different PIPs enables the formation of 3′-UTR-dependent protein complexes with diverse compositions (**Figure 2*f***).

3′-UTRs Regulate mRNA Localization

Although it was initially thought that mRNA localization happens only in the case of a few select mRNAs in polarized cells, it seems that mRNA localization is actually very widespread. Large-scale localization experiments in *Drosophila* embryos showed that 71% of mRNAs have nonuniform distribution within cells (77).

mRNA localization through association with motor proteins. Localization of mRNAs is usually mediated by localization signals in 3′-UTRs that are bound by RBPs. These RBPs in turn bind motor proteins, which result in movement of the mRNAs along filaments of the cytoskeleton (**Figure 2*g***). One of the best-characterized examples of this is the asymmetric distribution of *ASH1* mRNA to the bud tip during cell division in yeast, which is required for the suppression of mating type switching in the daughter cells (98). *ASH1* mRNA contains four *cis*-elements, including one located in the 3′-UTR, which are required for proper localization. These elements are bound by two RBPs, She2p and She3p. She3p also binds to a myosin motor protein, Myo4p, which transports the mRNA–protein complex along actin microfilaments to the bud tip.

Other mRNAs, including *oskar* mRNA in *Drosophila* oocytes, are transported along microtubules. Local function of Oskar is required to promote the localization of *nanos* mRNA, which is translated in the embryo and forms a gradient important for proper embryo patterning (82). *oskar* mRNA enters the oocyte from the nurse cells, a step that requires a 3′-UTR element and the microtubule motor protein dynein (57). Dynein is connected to the 3′-UTR through the RBPs Egl and BicD, which serve as adaptors (29). After oocyte entry, *oskar* mRNA uses a different 3′-UTR element, bound by Tropomyosin1-I/C, which connects *oskar* mRNA to kinesin-1, a microtubule motor protein that moves toward the plus end. This results in localization of *oskar* mRNA to the posterior pole of the oocyte (37, 56, 152). 3′-UTR-dependent recruitment of motor proteins, which is mediated by RBPs, has been described for the majority of localized mRNAs, including *CAMK2A*, *bicoid*, and *β-actin* (82, 91, 112).

Additional mechanisms of mRNA localization. In some cases, RBPs that bind to mRNAs do not directly interact with motor proteins, but they are connected through additional RBPs that serve as adaptors, such as BicD or She3p (98). Interestingly, such an adaptor can also be an early endosome, as was shown for RRM4 in a fungus (8, 70). Another unconventional mRNA transport mechanism was described for Abp140, which localizes to the distal pole in the yeast mother cell. It forms a ternary complex consisting of the ribosome, the mRNA, and the nascent polypeptide chain. The actin-binding domain located in the N terminus of the nascent peptide chain binds to actin microfilaments and uses actin retrograde transport for localization (**Figure 2*h***) (67).

mRNA association with membranes and the cytoskeleton. mRNAs and their bound RBPs often seem to be associated with cytoskeletal filaments or membranes (30, 59). They associate with cytoskeletal filaments during nucleocytoplasmic transport, but also while mediating their functions in the cytoplasm. For instance, the RBPs HuD and RAVER1 were shown to interact with actin-binding proteins and with microtubule-associated proteins, respectively (35, 52). mRNAs also bind to several components of vesicle coats, including COPI and COPII vesicle coat complexes (127, 146), and were found to localize to the surface of different organelles, such as mitochondria, endosomes, or the endoplasmic reticulum (25, 38, 60, 105). These examples show an intimate association between mRNAs, membranes, and the cytoskeleton. Intriguingly, repression by siRNAs or miRNAs also seems to happen on the surface of the endoplasmic reticulum (120). However, our understanding of the interactions among mRNAs, membranes, and the cytoskeleton is still rudimentary. The association of subgroups of mRNAs through their RBPs with different parts of the cytoskeleton may not only help to organize their localization but also enable compartmentalization of 3′-UTR functions (40).

3′-UTRs Play a Role as Independent Information Units

Most often, mRNAs are viewed as the templates for protein synthesis. However, mRNAs or parts of mRNAs, including cleaved fragments of 3′-UTRs, can also act as regulatory RNAs.

mRNAs act like long noncoding RNAs. For some of the well-studied model mRNAs, it was shown that the mRNA itself carries out a specific function that is independent of the encoded protein. This indicates that mRNAs can act as independent information units with functions similar to long noncoding RNAs. This was first demonstrated for *VegT* mRNA, which localizes to the vegetal pole in *Xenopus* oocytes (47). Its deletion impaired anchoring of other vegetal pole–associated mRNAs, including *BicC* and *Wnt11* mRNAs. This function was specific to *VegT* mRNA, as inhibition of VegT translation did not have such an effect. A protein-independent role was also found for *oskar* mRNA (61). *oskar* mRNA, but not Oskar protein, is required for the completion of oogenesis, as deletion of *oskar* mRNA results in an eggless phenotype (61). Importantly, expression of the *oskar* 3′-UTR was sufficient to rescue this phenotype, but the mechanism of action is currently unknown. It was speculated that *oskar* mRNA may localize or sequester an RBP. Alternatively, it may play a structural role, as was suggested for *VegT* mRNA (47, 61).

Expression of 3′-UTRs independent from the coding region. While analyzing CAGE libraries from human, mouse, and fly tissues, Mercer et al. (90) found 3′-UTRs that contained 5′ caps. Although this was surprising, it was not a rare event: Thousands of genes have CAGE tags in their 3′-UTRs, including a subset of genes conserved between mouse and human transcripts (90). Several capped 3′-UTRs displayed discordant expression with the coding regions, which means that their expression is likely autonomous. Discordant expression was also seen in an independent study that analyzed polysome-derived mRNAs from mouse neurons (69). For several candidates, RNA-seq data revealed higher expression of the coding region than of the 3′-UTR, a finding likely explained by 3′-UTR shortening due to APA in this cell type. However, other candidates showed the opposite pattern, as they had substantially higher expression of the 3′-UTR compared to the coding region. *Sox11* was one of the most striking examples, with 6,000 reads mapping to the 3′-UTR and only eight reads mapping to the coding region. The expression difference of additional transcripts was less dramatic but still more than 20-fold (69). Differential expression of

a substantial number of candidates was confirmed using in situ hybridization and gave credence to the findings.

Dual function mRNAs were also recently described in bacteria, where stable 3′ by-products of mRNA turnover act as small RNAs (sRNAs) (17). One of the sRNAs, CpxQ, was found to be produced from endonucleolytic cleavage by RNase E, which leaves a 5′ monophosphate. The cleavage occurred a few nucleotides downstream of the stop codon and produced a 58-nt-long sRNA derived from the 3′-UTR (17). The 3′-UTR cleavage product is nearly completely conserved in sequence and acts as an Hfq-dependent repressor during the inner membrane stress response (17). Thus, a transcribed 3′-UTR can be converted into a sRNA through endonucleolytic cleavage.

Also in mammalian cell lines, researchers identified cleavage products with a 5′ monophosphate that were generated by endonucleolytic cleavage. Transcriptome-wide degradome sequencing was initially performed to uncover the cleavage sites of the endonucleases of the miRNA pathway, i.e., Ago2 and Drosha (64). Many cut sites were not dependent on these two endonucleases, but the enzymes responsible are currently unknown (10, 92, 108). Intriguingly, there is substantial overlap between the degradome and the 3′-UTR fragment data sets (64, 69, 90). Approximately 40% of the genes that were identified by degradome sequencing to harbor cut sites were found to generate capped 3′-UTR fragments (64, 90). This suggests that a subgroup of degradation products is de novo capped in the cytoplasm, an occurrence previously shown in mouse and human cell lines (94, 102). Current data suggest that the 5′ fragments that are produced through endonucleolytic cleavage are rapidly degraded by the exosome and are usually not detectable (69, 108). However, the 3′ fragments are protected at the 3′ end by the poly(A) tail (90) and may be stabilized at the 5′ end through a strong structural element like a pseudoknot (18), through the binding of an RBP, or when XRN1 levels are low (108).

3′-UTRs Regulate Protein Abundance

One of the first motifs discovered in 3′-UTRs was AU-rich elements leading to rapid mRNA decay (6, 21). AU-rich elements are preferentially found in genes whose expression requires tight regulation such as cytokines, proto-oncogenes, and immune-regulatory factors (6, 21). These genes are rather special, as their mRNA half-lives are shorter than 30 min, compared to the median half-life of the transcriptome which is 7 h (113). The importance of these regulatory elements in restricting protein expression is highlighted by the fact that their deletion is associated with cancer, chronic inflammation, and auto-immune disease (6).

Because of the drastic effects of AU-rich elements on the regulation of acute phase genes, it was suspected that 3′-UTRs may regulate protein abundance genome-wide. However, genome-wide analyses of 3′-UTR-mediated regulation of protein abundance generally showed widespread, modest changes (4, 111, 119). One explanation for the small effect may be that during unperturbed steady-state growth of cell lines, 3′-UTR elements do not substantially regulate protein abundance. However, in certain biological contexts, miRNAs and RBPs are important for the control of protein levels. Proto-oncogenes belong to the class of genes with short half-lives, and their expression levels are controlled by AU-rich elements and miRNA-binding sites (21). Removal of these elements through 3′-UTR shortening in cancer cell lines resulted in upregulation of their protein levels. This turned proto-oncogenes into oncogenes, as the increased protein expression was sufficient for oncogenic transformation (87).

In specific biological contexts, miRNAs and RBPs also degrade mRNA transcripts that do not belong to the class of short-lived acute phase proteins. In early animal development, gene expression is accomplished by maternal mRNAs. However, at the onset of zygotic transcription,

the maternal mRNAs need to be degraded. During zebrafish development, miR-430 is highly expressed at the onset of zygotic transcription and is the predominant regulator of maternal mRNA decay (41).

Another beautiful example of regulating protein abundance by RBPs occurs in the control of mitochondrial biogenesis after glucose depletion in yeast (128). When yeast cells are grown in high-glucose conditions, mitochondrial biogenesis is repressed; there is no need for oxidative phosphorylation because yeast cells prefer to obtain their energy from glycolysis (78). In *Saccharomyces cerevisiae*, nuclear-encoded mitochondrial transcripts are preferentially bound by the RBP Puf3 (38, 128). Under high-glucose conditions, Puf3 destabilizes the transcripts that encode mitochondrial proteins. However, glucose deprivation or growth on nonfermentable carbon sources induces mitochondrial biogenesis, which results in a switch from degradation to preferential translation of Puf3 target genes. The switch in Puf3 activity is mediated by phosphorylation induced by a lack of glucose (78). This example illustrates the RBP-mediated control of protein abundance upon environmental changes. It also illustrates how a single RBP can determine opposite outcomes for bound mRNAs. Such dual functions of RBPs have also been described for DDX6, HuR, and many others (137, 150).

THE 3′-UTR-SPECIFIC RNA-BINDING PROTEOME AT A GIVEN MOMENT DETERMINES THE FUNCTIONS OF A 3′-UTR

The function of a given 3′-UTR regulatory element is influenced by both the RBP that directly binds to it and the RBPs that bind in its vicinity. This includes neighboring elements as well as distal elements that come into proximity through mRNA folding events. Thus, the functions of a 3′-UTR are determined by the ensemble of bound RBPs at a given moment. Notably, some RBPs can be loaded onto the mRNA in the nucleus, whereas others are added locally. Some RBPs are bound to 3′-UTRs with higher affinity, whereas others associate more transiently. To identify all RBPs bound to a single 3′-UTR, methods can be applied that were initially developed to determine the proteome associated with the long noncoding RNA Xist (23, 89). ChIRP-MS and RAP use biotinylated oligonucleotides to pull out endogenous RNAs followed by the identification of bound proteins by mass spectrometry. A similar method, called TRIP, was developed to examine the RBPs bound to mRNAs (84).

Fifteen years ago, the posttranscriptional operon theory was proposed by Keene & Tenenbaum (66). It stated that RBPs regulate subpopulations of mRNAs in a coherent manner and predicted that the mRNAs bound by an RBP would be functionally related. The best examples for RNA regulons are found in yeast, where, for example, Puf3 binds to a group of mRNAs that encode mitochondrial proteins (38, 128). To identify mRNAs that are bound by a single RBP, RNA-IP, and later CLIP (including HITS-CLIP, PAR-CLIP, iCLIP and eCLIP) were invented and widely applied (44, 71, 129, 132). CLIP uses an affinity tag or antibody against an RBP and precipitates the in vivo bound mRNAs after cross-linking. CLIP uncovered that the core motifs bound by RBPs are usually 3–7 nt long, are often degenerate, and are found in the majority of mRNAs (39). It also revealed that although many RBPs have hundreds or thousands of target mRNAs (76), a specific RBP usually binds only to 6–14% of its potential motifs in vivo (122, 132, 145).

Increased Specificity of RNA-Binding Proteins Through Cooperativity

Considering that each RBP binds to thousands of mRNAs, it seems a daunting task to identify specific RBP–mRNA relationships that are functional in a given context. The fact that RBPs use

interactions with other proteins to increase the binding specificity to 3′-UTRs makes this process less overwhelming.

Posttranslational modifications or protein interaction partners of RNA-binding proteins can change the binding specificity of RNA-binding proteins. Campbell et al. (14) used an in vitro selection method called SEQRS, to identify the binding motifs of RBPs. They determined the motif of an RBP alone or when bound to a domain of a PIP. In both cases the core motif of the RBP was detected, but the sequence motif in the neighboring bases differed. This study demonstrated that the specificity of an RBP can be altered through association with a PIP, even if the PIP does not bind to the RNA. Similarly, the binding motif of an RBP can be altered through PTMs of the RBP. This was shown for HuR, which prefers binding to AU-rich sequences but shifts toward U-rich sequences upon phosphorylation (110).

Ternary complex formation increases the specificity of mRNA–protein interactions. RBPs often physically interact with other RBPs and were shown by CLIP studies to coassemble on mRNAs (93). The binding of two RBPs to neighboring sequence motifs in an mRNA has been shown in numerous examples to increase the binding specificity (1, 48). Such cooperativity was shown for the RBPs She2p and She3p when bound to *ASH1* mRNA. All binary interactions between She2p and *ASH1* mRNA, She3p and *ASH1* mRNA, and She2p and She3p resulted in low-affinity and low-specificity complexes (96). In contrast, ternary complex formation increased their affinity and enabled binding of the RBPs to *ASH1* mRNA with higher selectivity than to other hairpin mRNAs (96).

Another example of increased specificity through formation of a cooperative ternary complex was demonstrated by the crystal structure of the *Drosophila* RBPs Sxl and Unr and the *msl2* mRNA (49). Translational repression of *msl2* mRNA is required for sex chromosome dosage compensation in female flies. Sxl binds to U-rich sequences, and the RNA-binding motifs of Sxl and Unr are very abundant in the transcriptome. The extended RNA-binding motif in *msl2* mRNA contains 16 nt and is recognized by Sxl together with Unr. Cooperative protein complex formation resulted in a 1,000-fold increase in RNA binding affinity. Thus, this and other structures demonstrated how general and widespread mRNA motifs can be specifically recognized by cooperative ternary complex formation (49, 80).

Binding of RNA and Proteins in the Nucleus Can Influence Cytoplasmic 3′-UTR Functions

Although most 3′-UTR functions are carried out in the cytoplasm, some RBPs bind to 3′-UTRs in the nucleus (118). For example, it is important to recruit the RBP She2p to *ASH1* mRNA in the nucleus, as mutant She2p that is unable to enter the nucleus results in impaired cytoplasmic localization of *ASH1* mRNA (98). Nuclear She2p is also necessary for the nuclear recruitment of the translational repressor Puf6p to *ASH1* mRNA, although it acts in the cytoplasm (114). Furthermore, nuclear events are important for *oskar* mRNA localization, as deposition of the exon junction complex during splicing is required for cytoplasmic localization of *oskar* mRNA (43).

Additional nuclear events are mediated through cotranscriptional loading of RBPs at promoters and seem to play a general role in the regulation of cytoplasmic processes of mRNAs. In yeast, promoters control mRNA stability, translation, and mRNA localization (12, 126, 151). Although glucose starvation leads to the translational repression of most yeast mRNAs, a subset remains actively translated. Binding of HSF1 to promoters determines the group of mRNAs that is being translated during glucose starvation. HSF1 binding may result in the recruitment of an RBP that

is exported with the mRNAs and specifies translation in the cytoplasm (151). A similar mechanism was proposed for the influence of promoters on mRNA stability, where swapping the upstream activating sequence (UAS) of a promoter changed the mRNA decay rate of the transcript. As changing the UAS did not alter the mRNA sequence, promoter-mediated recruitment of specific factors that influence cytoplasmic processes seems likely (12). Such promoter-mediated recruitment of an RBP was recently demonstrated in *Drosophila*. Loading of HuR at specific promoters influences the recognition of alternative pA sites (101). Thus, RBPs recruited to promoters can change whether regulatory elements are functional in 3′-UTRs.

Local mRNA Functions Are Enabled Through Local Binding of RNA-Binding Proteins to 3′-UTRs

Local recruitment of RBPs to 3′-UTRs can also change 3′-UTR functions. One of the classical examples for mRNA transport in neurons is the localization of *β-actin* mRNA from the soma to the synapse. The zipcode binding protein IGF2BP1 (ZBP1, IMP-1) binds to a localization element in the 3′-UTR of *β-actin* mRNA, which is necessary for mRNA localization, but *β-actin* mRNA is released from IGF2BP1 binding after reaching the synapse. This event is triggered by phosphorylation of IGF2BP1, as only unphosphorylated Y396 can bind *β-actin* mRNA. Phosphorylation is mediated by Src kinase, whose expression is locally restricted to the synapse. During transport, *β-actin* mRNA is translationally repressed, but phosphorylation of IGF2BP1 also leads to translational activation of *β-actin* mRNA (53). Thus, the new local environment, characterized by expression of Src kinase, defines the end point of β-actin mRNA localization and enables its local functions. PTMs that occur in a spatially or temporally restricted manner are widely used to achieve dynamic changes in RBP composition at 3′-UTRs and, thus, in 3′-UTR function. For example, in certain stress conditions, HuR is ubiquitinated through addition of a K29 chain, a modification that does not induce degradation of HuR, but rather releases bound HuR from several of its target mRNAs. The release of HuR changes the fate of the mRNAs from stabilization to destabilization (150).

In addition to local expression of enzymes that add PTMs, local expression of RBPs can also change 3′-UTR functions. For example, *nanos* mRNA localizes to the posterior pole of the *Drosophila* embryo and establishes a morphogen gradient (82). However, it does not appear to be actively transported to the posterior pole because *nanos* mRNA is found throughout the embryo. Also throughout the embryo, the RBP Smaug binds to the *nanos* 3′-UTR and recruits a deadenylase, resulting in *nanos* mRNA decay. However, at the posterior pole, it was suggested that the RBP Oskar replaces Smaug, preventing Smaug from binding to the *nanos* 3′-UTR, which results in active translation and accumulation of Nanos protein at the posterior pole (147). This shows how the binding of locally restricted *trans*-acting factors to 3′-UTRs can determine local functions of mRNAs and their encoded proteins.

Each Alternative 3′-UTR Isoform Has a Specific RNA-Binding Protein Composition and, Thus, Function

Differentiation and alterations in the cellular environment are associated with changes in signaling pathways that are able to change RBP composition at 3′-UTRs both in *cis* and in *trans*. Changes in the PTMs of RBPs can alter the association of an RBP with a 3′-UTR and, thus, alter the function of a 3′-UTR (53, 110, 150) (see section titled Posttranslational modifications or protein interaction partners of RNA-binding proteins can change the binding specificity of RNA-binding proteins). Furthermore, signaling can also change 3′-UTR ratios (32, 63, 81, 87, 106,

125). This can result in a switch in protein localization, protein abundance, and protein function, which has been shown for BDNF, the oncogene IMP-1, and for CD47, respectively (3, 9, 75, 87).

Functional discrepancy of 3′-UTR regulatory elements when analyzed in isolation or within a larger sequence context. APA results in the expression of mRNAs with either short or long 3′-UTRs. The sequence of shorter 3′-UTRs is fully contained in longer 3′-UTRs (**Figure 1*c***), therefore, it is commonly expected that an RBP that binds to the shorter 3′-UTR should also bind to the longer 3′-UTR. However, this is not necessarily the case, as the longer 3′-UTRs form their own distinct secondary and tertiary RNA structures that may occlude binding sites that are accessible in the context of shorter 3′-UTRs. Although this relationship has not been conclusively established for alternative 3′-UTRs, it was demonstrated that the functional effects of expressing smaller 3′-UTR pieces led to substantially different protein abundances when compared to full-length 3′-UTRs (72). Whereas most smaller 3′-UTR pieces (50-mers to 400-mers) had activating effects on protein abundance, the two full-length 3′-UTRs (*PIM1*, approximately 1,300 nt; *Hmga2*, approximately 2,900 nt) that were examined were both highly repressive (72, 88).

Such a discrepancy was also seen when even smaller 3′-UTR regulatory elements (8-mers) were tested in isolation or within the context of a larger endogenous sequence (142). When newly identified functional 3′-UTR elements were tested as 8-mers with respect to the regulation of protein abundance, all of them were either activating or repressive. However, when they were placed within a 500-nt endogenous sequence context, a significant fraction had opposite effects and the great majority had no regulatory effects on protein abundance (142). A likely explanation for this phenomenon is that a new local secondary structure is formed within the larger sequence context that prevents the accessibility or changes the folding of the isolated 8-mers. It is also possible that most RBPs do not act alone but rather compete or cooperate with RBPs that bind adjacently (14, 62). Thus, within the new sequence context, cooperativity with another RBP may alter the functional outcome of the RBP. It is also possible that binding of the RBP alone may regulate protein abundance, but interaction with a binding partner may result in the recruitment of a 3′-UTR-dependent PIP. Thus, the element would still be functional, but it would mediate a function that was not interrogated by the assay.

The observations that functional RBP-binding sites can be silent or have opposing effects when put into a new sequence context have important implications for alternative 3′-UTRs. They suggest that each alternative 3′-UTR isoform will have its own repertoire of functional RBP-binding sites, even if the sequence of the shorter 3′-UTRs is fully contained within longer 3′-UTRs. Thus, in a specific cellular condition, shorter 3′-UTRs may be associated with different RBPs than their corresponding longer 3′-UTRs (**Figure 2*d***) (79, 85). However, each 3′-UTR isoform likely can adopt several different configurations that depend on the activity of signaling pathways, expression of RBPs, their cofactors, their PTMs, and their subcellular localization (53, 110, 150). Taken together, it seems that longer 3′-UTRs in particular have a lot of latent regulatory capacity that is regulated through accessibility (122). Yet, our understanding of how accessibility of 3′-UTR regulatory elements is controlled is still rudimentary. 3′-UTR structure analyses will help to elucidate this, but, ideally, they need to be performed in vivo on full-length 3′-UTRs in different contexts.

Different biophysical properties of short and long 3′-UTRs. For instance, short 3′-UTRs are predominantly found in the cytoplasm, whereas long 3′-UTRs can be part of liquid droplets and hydrogels (46, 65). The reasons for the difference in distribution are currently not known. It

is possible that long 3′-UTRs bind more RBPs than short 3′-UTRs. Because RBPs are enriched in protein domains with an overabundance of a few amino acids, called low-complexity (LC) domains (15), they are often intrinsically disordered. This causes most of their residues to be accessible and, thus, these residues represent major interacting domains for other proteins (40, 138). This feature enables RBPs to function as network hubs, as they were shown to have, on average, more PIPs than other protein classes (5). The multivalency of RBPs together with their LC domains allows liquid–liquid phase transitions of messenger ribonucleoproteins (65, 138). Phase transitions seem to play a very important role in the regulation of RNA–protein interactions, as they enable an increase in the local concentration and compartmentalization of factors without the need for membranes, providing a unique local environment that is distinct from the cytoplasm (46, 65, 134). Thus, in addition to a specialized local environment found on the surface of organelles (131), localization to liquid droplets can also change the range of RBPs that are available for binding to 3′-UTRs. There also seems to be interdependency between the mRNA and protein components within the droplet. On the one hand, as mRNAs are differentially bound by RBPs, mRNAs are able to determine the protein composition and the biophysical properties of the droplet (148). On the other hand, the biophysical properties of the droplet determine which RNAs are retained in the droplet (99). As liquid droplet formation may serve as a dynamic compartmentalization tool to enable local mRNA processes, 3′-UTR length itself may promote localized processes and may contribute to compartmentalization of cellular functions (133).

NEW GLOBAL APPROACHES IDENTIFY NOVEL RNA-BINDING PROTEIN MOTIFS AND FUNCTIONAL 3′-UTR ELEMENTS

In yeast, Puf3 preferentially binds to mRNA transcripts that encode mitochondrial proteins (38, 128). However, binding of Puf3 can result in translational repression or activation (78). This indicates that it is not the sequence element per se, but rather the status (PTMs, PIPs) of the *trans*-acting factor, that determines the functional outcome. This interpretation is supported by recent studies that used fluorescence-based screens to identify new functional elements in 3′-UTRs (100, 142, 149). All of the screens used small 3′-UTR fragments to search for 3′-UTR elements that regulate protein abundance and found similar numbers of activating and repressive elements. However, interestingly, when the functional 3′-UTR fragments were placed within a larger endogenous sequence context, a large fraction showed either no effect or the opposite effect on protein abundance (142). This suggests that functional screens that use small 3′-UTR fragments can identify elements with a potential for regulation, but they are not able to identify the biological context or endogenous 3′-UTR that will produce a specific phenotype when harboring the element. Therefore, such screens are able to catalog RBP-binding sites with potential function and are comparable to bioinformatic predictions of miRNA targets (2). Further experiments are necessary to examine if a specific binding site is functional within a particular 3′-UTR as well as within a certain biological context.

The Giraldez laboratory (144) recently developed a screen that may go a step further in identifying the functional 3′-UTR elements that regulate mRNA stability in a specific biological context. The RNA-element selection assay (RESA) uses random fragmentation of 3′-UTR sequences to detect regions that contain stabilizing and destabilizing elements. For a proof-of-principle analysis, it was used to identify functional 3′-UTR elements at the onset of zygotic gene expression during zebrafish development. At this stage, all maternal mRNAs need to be degraded, and RESA confirmed that this is predominantly accomplished by miR-430 (144). This novel approach may also identify functional RBP regulons in different biological contexts. The combination of such

functional screens with newly developed in vitro methods that identify high-affinity motifs of RBPs (14, 74, 104) will help to shed light on the multilayered regulation accomplished by 3′-UTRs.

CURRENT ISSUES AND HOW THEY CAN BE OVERCOME

It is currently not known how many RBPs bind to a specific 3′-UTR or to a pair of alternative 3′-UTRs. If such data were to exist for many 3′-UTRs, it would tremendously increase our understanding of 3′-UTR biology. It is also important to learn about the dynamics of RBP binding to specific 3′-UTRs in steady-state or altered cellular conditions. This could be accomplished by performing ChIRP-MS, RAP, or TRIP (23, 84, 89) on many 3′-UTRs in different cellular conditions.

Many RBPs bind to U-rich regions. Assuming that this observation is not a CLIP-generated artifact (121), there is a need to develop assays to examine the in vivo binding sites and functions of these RBPs while they are bound to their PIPs (14). This would enable the integration of cooperative binding events with functional assays.

RBPs mediate 3′-UTR functions, but they are only the adaptors that connect the 3′-UTR *cis*-regulatory elements to effector proteins. The effector proteins are the true mediators of a specific phenotype. As the effector proteins are generally PIPs of RBPs, there is a need to comprehensively identify the protein interactome of RBPs (11).

Researchers who set out to globally examine 3′-UTR elements with these methods used small 3′-UTR fragments to probe for functional elements (100, 142, 144, 149). When the effects of 3′-UTR fragments on protein output were compared with the effects of full-length 3′-UTRs, substantially different outcomes were observed (72, 142) (see section titled Functional discrepancy of 3′-UTR regulatory elements when analyzed in isolation or within a larger sequence context). This indicates that 3′-UTR elements function in the context of the whole 3′-UTR or mRNA. Thus, methods need to be developed that identify functional regulatory elements within each alternative 3′-UTR isoform, as the presence of the motif often does not correlate with its functionality. Additionally, there is a need to develop functional assays that investigate global 3′-UTR functions that use full-length and alternative 3′-UTR isoforms as input.

Lastly, the majority of assays that assessed global 3′-UTR functions have used protein abundance as a functional readout. Although these readouts are both well established and highly reliable, regulation of protein abundance is only one of the many functions of 3′-UTRs. Thus, new readout systems are needed that are able to globally determine additional 3′-UTR functions such as mRNA localization (123) or 3′-UTR-mediated transfer of PIPs, which can be investigated by performing the experiment shown in **Figure 2*c*** (9, 79).

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