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RNA recognition by Roquin in post-transcriptional gene regulation

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Abstract

Post-transcriptional regulation of gene expression plays a central role in the initiation of innate and adaptive immune responses. This is exemplified by the protein Roquin, which has attracted great interest during the past decade owing to its ability to prevent autoimmunity. Roquin controls T cell activation and T helper cell differentiation by limiting the induced expression of costimulatory receptors on the surface of T cells. It does so by recognizing *cis* regulatory RNA-hairpin elements in the 3' UTR of target transcripts via its ROQ domain – a novel RNA-binding fold – and triggering their degradation through recruitment of factors that mediate deadenylation and decapping. Recent structural studies have revealed molecular details of the recognition of RNA hairpin structures by the ROQ domain. Surprisingly, it was found that Roquin mainly relies on shape-specific recognition of the RNA. This observation implies that a much broader range of RNA motifs could interact with the protein, but it also complicates systematic searches for novel mRNA targets of Roquin. Thus, large-scale approaches, such as crosslinking and immunoprecipitation (CLIP) or systematic evolution of ligands by exponential enrichment (SELEX) experiments coupled with next-generation sequencing, will be required to identify the complete spectrum of its target RNAs. Together with structural analyses of their binding modes, this will enable us to unravel the intricate complexity of 3' UTR regulation by Roquin and other *trans*-acting factors. Here, we review our current understanding of Roquin-RNA interactions and their role for Roquin function.

Post-transcriptional gene regulation is emerging as an important element in the control of innate immunity (1) and the development and regulation of the adaptive immune system (2, 3). Over the past two decades large numbers of RNA-binding proteins (RBPs) have been identified that are involved in the regulation of RNA processing and turnover, including pre-mRNA maturation (4), alternative splicing (5), quality control, degradation (6), transport/localization (7) and translational regulation (8). Recent genome-wide studies have revealed the presence of previously unknown RNA-binding proteins (RBP) that interact with mRNAs (9, 10) through one or more RNA-binding domains (RBDs). While such studies greatly facilitate the prediction of novel potential RNA-binding proteins based on sequence homology, as yet unrecognized types of RBDs may be present in many known RBPs. Experimental approaches such as CLIP usually identify binding of RBPs to linear RNA motifs. In contrast, structured *cis*-regulatory RNA elements are very difficult to determine computationally, because their primary sequences are often degenerate (11-14). However, the recently introduced hiCLIP method can also detect RBP binding to RNA secondary structures (15). As single-stranded RNA motifs are short and typically have relatively low affinity for a given RBD, multiple RBDs often cooperate in the recognition of extended single-stranded *cis* elements (16-19). This enables the specific recruitment of RBPs, but also provides additional opportunities for regulation of RNA binding by combinatorial domain interactions and dynamics coupled to RNA binding. Similar mechanisms might extend to the recognition and function of structured *cis* RNA elements, but are still poorly understood. These considerations underline the complexity of gene regulation via untranslated regions (UTRs) of mRNAs and complicate the task of identifying RNA motifs that are recognized by RBPs.

Roquin is a relatively recently recognized member of the family of RBPs that target *cis*-regulatory elements, and was first described in the context of lupus-like autoimmune disease (20). It has since been found to play an essential role in controlling the levels of mRNAs coding for various key

proteins involved in immune regulation (21-25) (**Fig. 1**). Roquin has been shown to bind to 3' UTRs of target RNAs via its amino-terminal half (26), while its effector functions, e.g. mRNA decay, reside in the carboxy-terminal region (21). The structural mechanisms employed by Roquin to recognize its target RNAs long remained elusive. Recently, however, several groups have reported high-resolution structures for the N-terminal region of Roquin, and identified the ROQ domain as a novel RBD (27-30). These studies reveal molecular details of the recognition of the *cis*-regulatory RNA hairpin elements, the so-called constitutive decay elements (CDEs), by Roquin. Here, we review our current understanding of the recognition of RNA *cis* elements by Roquin in its target mRNAs, and their role in downstream regulatory processes. We focus particularly on the definition and characterization of the ROQ domain and summarize emerging principles that define its functional target RNA elements. Potential roles for the other regions of Roquin are also discussed, and we provide perspectives for future developments in Roquin research.

Roquin's function in immune responses

Roquin is remarkable for its central role in regulating immune responses and autoimmunity. Roquin-1 (Rc3h1) and its paralogue Roquin-2 (Rc3h2) share a common domain organization and the ability to bind nucleic acids (31). Two recent publications have demonstrated functional redundancy between the two proteins (23, 32) by showing that Roquin-2 can compensate for the loss of Roquin-1 action. Roquin mRNA is expressed in many tissues (20), but protein levels (especially of the Roquin-1 paralogue) are significantly increased in thymus and lymph nodes. One of the main functions of Roquin is to prevent T cell activation and Tfh cell differentiation by mRNA suppression in CD4+ T cells (20, 22, 23, 33, 34). Roquin was found to destabilize the mRNAs for the inducible co-stimulator ICOS (22, 35), Ox40 (23), IFN-γ (36) and TNF-α (21) (**Fig. 1A**). Loss of regulation of co-stimulatory receptors and cytokines correlates with autoimmunity in Rc3h1$^{san/san}$ mice, which are homozygous for the M199R point mutation (20) that maps to the novel ROQ domain of Roquin.

Functional roles of Roquin domains

Roquin (i.e. paralogues 1 and 2) is located in the cytoplasm and is enriched in stress granules or P bodies (20, 26, 37). The 125-kDa protein is highly conserved, e.g. the murine Roquin-1 is more than 90% identical to its human orthologue. Indeed, sequence identity exceeds 99% over the first 450 amino acids, which comprise identifiable domains, while the remainder of the protein is predicted to form an intrinsically disordered region of almost 600 residues. An extended proline-rich stretch between amino acids 600 and 800 could serve as a docking site for proline-rich sequence recognition domains (**Fig. 1B)**, a feature that is often found in signaling adaptor proteins (38), including spliceosomal proteins (39). Indeed, the Heissmeyer group found that the C-terminus of Roquin-1 is crucial for repression of ICOS mRNA (26), and Stoecklin and coworkers subsequently reported that the Ccr4-Caf1-Not deadenylase complex is recruited by the C-terminal region of Roquin-1 to initiate target degradation (21). Q/N-rich stretches in the C-terminal segment of Roquin are suggestive of its co-localization with P-body components, as such sequences enable protein-protein interactions important for the formation of ribonucleoprotein complexes during post-transcriptional regulation (40). Bioinformatic analysis of the Roquin C-terminal portion also predicts the existence of a coiled-coil region (amino acids 946 and 981 of murine Roquin-1), which could mediate protein-protein interactions (**Fig. 1B**).

Based on the presence of a RING (*Really Interesting New Gene*) domain (41) at the N-terminus (**Fig. 1B**), Roquin could be a potential E3-ubiquitin ligase. The Roquin-1 RING domain adopts a canonical

cross-brace zinc-binding topology, although a recent crystal structure revealed atypical Cys_2-His-Asp coordination of the zinc ion in the second coordination site (30). E3 ligase activity has indeed been reported for the Roquin homolog in *C. elegans*, RLE-1 (42). More recently, Roquin-2 (43) has been found to ubiquitinate apoptosis signal-regulating kinase 1 (ASK1 or MAP3K5)1, while formal evidence for Roquin-1-mediated ubiquitination of specific substrates is so far lacking. ASK1 mediates cellular responses to environmental changes, and ASK1 signaling is abrogated by Roquin-2-mediated K48 polyubiquitination and subsequent proteasomal degradation. Interestingly, deletion of the RING domain or mutation of cysteine 14 to alanine was shown to impair localization of Roquin to stress granules (32) and a recent report suggests that the Roquin-1 RING domain enables autoubiquitination of Roquin-1 and antagonizes the catalytic α1 subunit of Adenosine Monophosphate-activated Protein Kinase (44).

A Cys_3-His zinc finger (ZnF) between residues 411 and 450 (**Fig. 1B**) is predicted to resemble known structures of other C_3H zinc fingers. In the light of RNA binding by the ROQ domain, the ZnF may provide additional RNA contacts, e.g. via recognition of AU-rich elements (ARE) found adjacent to ROQ domain-bound *cis* elements. In fact, the ZnF has been shown in two recent studies to contribute to RNA binding (37, 45).

Roquin - a novel member of the RNA-binding protein family

Although it was proposed early on that Roquin might bind directly to RNA, biochemical proof has remained elusive. The ROQ domain and the flanking zinc finger (**Fig. 1B**) have both been implicated in RNA binding. Recent publications have provided evidence that Roquin's function as a key regulator of immune homeostasis involves recognition of various structural elements of RNAs, such as stem-loops, as well as double-stranded RNA (dsRNA) and miRNAs (21, 27-30).

The modular domain composition of Roquin resembles that of other proteins in which RNA binding is mediated by multiple RBDs (19, 46). Most RBPs are composed of highly abundant RBDs, such as the RRM, KH, zinc finger and dsRBD domains (18). While – apart from dsRBDs – most RBDs recognize single-stranded RNA (ssRNA), relatively few examples are known of RBDs that specifically recognize stem-loop RNA structures. These include SAM domains (47, 48) and the Stem-Loop Binding Protein (SLBP)/3'hExo (49), which mediates recognition of a stem-loop in the 3' UTR of replication-dependent histone mRNAs. In the latter case both SLBP and the exonuclease comprise short helix-turn-helix motifs to specifically interact with the stem-loop.

The identification and definition of Roquin's RNA-binding region has been a particularly challenging task. Until 2014 the existence of a Roquin RNA-binding domain, called ROQ, was predicted solely based on sequence conservation and the observation that a stretch of residues between 130 and 360 can mediate RNA binding (21, 26) (**Fig. 1B, 2A-C**). Secondary-structure prediction indicated that the region between amino acids 60 and 410, i.e. the segment that connects the RING domain to the ZnF, is predominantly helical. Limited proteolysis of this region of Roquin-1 (residues 64-411) identified a stable domain of approximately 20 kDa, and the crystal structure revealed a globular fold between residues 176 and 326 (27). Interestingly, we identified the same core domain in crystals of the N-terminal portion of Roquin-1 (1-411) (unpublished), indicating that the regions flanking the stable ROQ core are susceptible to proteolytic cleavage. Similar findings were reported by Schuetz *et al.* (29). The ROQ domain fold shows an extended winged helix-turn-helix (WH) motif (**Fig. 2D-F**) comprising additional helices packed against the core motif (residues 191-274). A recently reported

crystal structure of the ROQ domain of Roquin-2 reveals similar domain boundaries after tryptic digestion of a longer fragment (50). The structural similarity between the ROQ domains of the two paralogues is consistent with their largely redundant function (23, 32).

Two groups have succeeded in crystallizing a considerably longer fragment of Roquin-1 (28, 30). Tan *et al.* identified three structural modules in a Roquin-1 construct comprising residues 89 to 400, which they named domains I, II and III. Domain III (residues 195-271) corresponds to the winged-helix portion of the ROQ domain (28) and is inserted into the primary sequence of domain II (**Fig. 2B**). Therefore, domains II and III together (comprising residues 176-326) constitute the ROQ domain (**Fig. 2D**) as defined by limited proteolysis and by crystallography (27). The protein crystallized by Tan *et al.* harbors two additional helical regions (residues 89-175 and 327-400, respectively), which together form a helical bundle referred to as domain I (**Fig. 2B, E**). Presumably, the flexible nature of the unstructured linkers flanking the ROQ domain (between domains I and II) is responsible for their sensitivity to proteolytic degradation.

Very recently, the Vinuesa group reported another structure for the N-terminal region of Roquin-1 including the RING domain (residues 6-75) (30) (**Fig. 2C**). Here, the ROQ domain (residues 176-326) is flanked by two helical regions that the authors called $HEPN_N$ (128-176) and $HEPN_C$ (327-399), as they resemble the HEPN fold of nucleotide-binding domains (51). This fragment is very similar to the one reported by Tan *et al.* (**Fig. 2B, C,E, F**) and thus the additional N-terminal helix (residues 86-112) seen by Srivastava *et al.* should be considered as part of $HEPN_N$. It is important to note that the overall folds and domain arrangements in all reported structures of Roquin-1 are essentially identical. In particular the ROQ domain (residues 177-326) is unambiguously defined by the structural data. This is noteworthy, as none of the individual domains within the N-terminal region of Roquin was predicted from the primary sequence. The structural studies demonstrate that the ROQ domain adopts a novel fold, even though its central region resembles WH domains that are found in DNA-binding proteins of the Forkhead-box family (52) and in the DNA/RNA-binding protein ADAR1 (27, 53).

The ROQ domain structures reported by Schlundt *et al.* (4QI0), Srivastava *et al.* (3XI0), Zhang *et al.* (4YWQ, unpublished) and Schuetz *et al.* (4ULW) show almost identical dimeric arrangements in the crystal lattice. However, all studies conclude that the ROQ domain is monomeric in solution, while non-specific, concentration-dependent oligomerization is observed. This is supported by the observation of line-broadening of NMR signals at the crystallographic dimer interface and by SAXS data obtained at different protein concentrations (27). The structures reported for larger portions of Roquin-1 by Tan *et al.* (4QIK and 4QIL) and Srivastava *et al.* (4TXA) also showed additional protein-protein contacts in the crystal lattice, albeit involving different interfaces of the proteins. Even the two structures of the same protein fragment bound to different stem-loop RNAs (4QIL and 4QUIK (28) exhibit distinct dimer interfaces. Taken together, these observations suggest that dimerization of the N-terminal half of Roquin-1 does not occur in solution.

Heinemann and colleagues engineered an artificially dimerized Roquin-1 (2-452) fragment and found that it displayed enhanced affinity for both ICOS stem-loop and duplex RNA. However, it is not yet known whether full-length Roquin forms dimers *in vivo*, for instance through homodimer formation via the C-terminal region, heterodimerization with Roquin-2, complex formation with other factors such as Regnase (24), binding to multiple concatenated RNA motifs, or non-specific oligomerization

at significantly increased concentration when localized in P bodies and stress granules. As Heinemann and colleagues suggest, oligomerization of Roquin could have a substantial impact on its functional activity.

Roquin-RNA interactions

The Roquin ROQ domain recognizes stem-loop RNA structures

Since the initial finding that Roquin is an RNA-binding protein the identification of *cis* elements in its target mRNAs has been a focus of research. In 2013, the Stoecklin group reported that Roquin binds to a short stem-loop motif and that this interaction mediates mRNA decay (21). This CDE (constitutive decay element) motif is present in a set of approximately 50 target transcripts coded for by all vertebrate genomes. Interestingly, that study also revealed a large number of Roquin-bound RNAs that do not harbor a recognizable CDE. The structural basis for the recognition of CDE-containing RNA by Roquin-1 has been reported by the Tong group, as well as the Sattler and Niessing labs (27, 28) (**Fig. 3 and 4**) and recently by Sakurai *et al.* for Roquin-2 (50). Schlundt *et al.* present the Roquin-1 core ROQ in complex with the canonical *Tnf* CDE (**Fig. 3A, B and 4A-D**), while Sakurai *et al.* describe the Roquin-2 ROQ domain (171-325) bound to a CDE in the Roquin-1 (*Rc3h1*) mRNA. In contrast, Tan *et al.* found that their extended ROQ domain recognizes a fully complementary dsRNA helix formed by dimerization of two *Tnf* CDE elements during crystallization (**Fig. 3A, C and 4E, F**). However, these authors also report the recognition of a stem-loop RNA found in a CDE derived from the *Hmgxb3* mRNA. The unexpectedly different interactions of the ROQ domain with CDE stem-loop RNAs and double-helical RNA immediately raises the question whether Roquin is able to bind to several distinct RNA species. Strikingly, recognition of stem-loop RNAs and dsRNA involves two distinct regions in Roquin-1 (90-400). Binding of the stem-loops occurs at the so-called A-site, while dsRNA binds at a "B-site" located between domains I and II, and thus spatially remote from the A-site (**Fig. 3, 4**). Even though the dsRNA recognition observed by Tan *et al.* is likely to be an artifact of the high RNA concentrations used during crystallization (28, 29), their findings nevertheless indicate that Roquin-1 can bind to dsRNA at a second RNA-binding site. Mutations introduced at each site were used to dissect their individual contributions, and the data suggested that, while the A site is critical for mRNA decay (27, 28), residues in the B-site may also have an impact on the process (28).

Recognition of stem-loops and double-stranded RNA by Roquin

Recently it has been reported, based on RNA-IPs, PAR-CLIP and Selex approaches, that Roquin-1 can bind to non-CDE-like motifs and that these interactions contribute to mRNA destabilization (21, 27, Janowski et al in revision, 45). Nevertheless, the *Tnf* CDE stem-loop motif originally identified is currently the only RNA *cis* element for which binding to Roquin and its functional consequences have been comprehensively characterized. The initially proposed CDE consensus sequence (21) is characterized by a pyrimidine-purine-pyrimidine tri-loop and a closing C-G base-pair, followed by two U-A pairs (**Fig. 4A-D and 5A,B**). The remainder of the stem region typically comprises another 2-5 base-pairs. Notably, the function of the stem-loop motif is unaffected by most individual mutations that preserve the stem-loop fold (27) (**Fig. 5C**). This suggests that RNA elements recognized by the ROQ domain are governed by shape recognition, while sequence-specific contacts are less important (see also **Fig. 4A-D**). Neither an extension of the stem nor substitution of individual purines/pyrimidines in the loop that conserve the base type have any significant effect on binding to the ROQ domain A-site. The same was observed for replacement of the closing base-pair or the two U-A pairs by C-G. However, exchange of the base type in the Py-Pu-Py tri-loop from U-G-U to A-C-A

(Pu-Py-Pu) completely prevented Roquin binding (**Fig. 5C**). Nevertheless, it remains to be seen whether or not mutations with mild effects on RNA binding *in vitro* have a more severe impact on regulation by Roquin *in vivo*. This reservation is partially motivated by the fact that *in vitro* binding and functional studies have yielded different consensus motifs for these interactions (21, 27, 45, 54) (**Fig. 5D-E**). In particular, multiple nucleotide substitutions might influence the secondary structure of the 3' UTR, either locally or more widely. While two reports confirm the expected base pairing of all variant stem-loops tested by NMR analysis (27, 54), the particular stem-loop structure might be different or underrepresented *in vivo*, and thus alter the overall functional response. It has also been proposed that the presence of an A-tract in the 3' half of the CDE stem is important for recognition by the ROQ domain due to its adoption of a unique RNA helix conformation (27). This has been supported by recent NMR structures of unbound wild-type and variant CDE RNA stem loops (54).

The available structures of Roquin-RNA complexes clearly suggest that the ROQ domain may also accommodate RNA elements that harbor more extended loops and/or have altered/extended 5' strands, as only few protein-RNA contacts have been observed for these parts of the RNA ligand and no steric clashes are expected. In fact, this is supported by recent observations that longer U-rich loops in stem loops of target mRNA 3'UTRs are efficiently bound by Roquin and are functionally active as *cis* elements in post-transcriptional gene regulation (45). It is conceivable that – as long as shape requirements and key interactions are fulfilled – a much broader range of stem-loop RNA structures can be recognized by the ROQ domain and thus act as functional *cis* elements to trigger Roquin-dependent mRNA repression. The definition of a relaxed consensus for the original CDE (27) may well lead to the recognition of alternative decay elements that have extended and diverse loop motifs (45, and Janowski et al in revision) (**Fig. 5F**).

Binding of the Roquin-1 B-site to dsRNA is sequence independent (**Fig. 3A, C and 4E, F**). It is thus conceivable that non-specific interactions with dsRNA motifs are used to enhance the overall affinity of Roquin proteins for RNA, and complement the specific recognition of stem-loop elements by the ROQ domain in target mRNAs. The simultaneous and efficient binding of an RNA stem loop by the A-site and of dsRNA by the B-site of Roquin could in principle be enhanced by a particular spacing of both RNA elements. Such an increase in specific binding by the combined recognition of a bipartite RNA element with a defined linker length has previously been shown for other RBPs, such as ZBP1 in complex with β-actin mRNA (55, 56). In addition, dsRNA could be bound so as to allow sliding of Roquin along stretches of target RNAs and thus facilitate encounters with *bona fide* stem-loop elements. This could potentially augment the overall stability of Roquin-RNA binding, considering that dsRNA regions are typically very abundant, especially in long 3' UTRs (15).

No binding of single-stranded RNA (ssRNA) was observed at either of the RNA-binding sites in Roquin (28, 29). This is confirmed by NMR titrations (Schlundt *et al.*, unpublished, **Fig. 5C**), and strongly argues that Roquin primarily binds to structured RNA elements and does not recognize a specific single-stranded RNA sequence *per se*. However, the zinc finger (ZnF) adjacent to the ROQ domain (**Fig. 1B**) could provide an additional contribution to RNA binding by recognizing single-stranded RNA elements. The CCCH-type Roquin ZnF resembles zinc fingers in other proteins involved in ARE recognition in 3' UTRs, and shares high homology with the tandem zinc fingers of TIS11d (57) and tristetraprolin (58). So far, the few studies on single-site ZnF mutants, or on the impact of knockdown of full-length Roquin-1 have revealed only minor effects on RNA binding (26, and

unpublished data). On the other hand, Landthaler and coworkers have very recently presented evidence for the involvement of the Roquin-1 ZnF in RNA binding (45).

Another recent publication suggests that Roquin-1 may directly bind to miR-146a (30), a micro RNA that regulates ICOS abundance (59). However, in that study, Pratama *et al.* permitted RNA secondary structures to form before analyzing the protein-RNA complex using surface plasmon resonance. Under such conditions, the miRNA could adopt a duplex or stem-loop structure that is recognized by Roquin proteins. However, although Roquin-mediated regulation of ICOS apparently depends on binding to CDE-like elements in the ICOS mRNA, it may also involve additional interactions with miRISC complexes.

Interestingly, the M199R mutation within the ROQ domain, which is associated with dysregulation of several RNA targets by full-length protein, does not impair RNA binding (21). While in the crystal structure the mutation causes minor alterations in the ROQ domain (30), the relevant residue is not involved in interactions with RNA. This suggests that the M199R mutation alters interactions with a protein partner.

Cooperativity and dynamics in RNA interactions by Roquin

It is becoming ever clearer that the conformational dynamics and flexibility of RNA are coupled to its functional activity (60-62). In this respect, the structure and conformational flexibility of mRNA may affect recognition by regulatory proteins by modulating the accessibility of target *cis* elements. In fact, it has been shown that RNA secondary structure is under permanent selection pressure for resistance to inappropriate recognition and denaturation by helicases and other RBPs (63). In this context, the targeting of Roquin to mRNAs may be enhanced by the presence of two RNA-binding domains that recognize two different types of structural elements. For example, the combination of ROQ and ZnF domains could ensure efficient identification of target RNAs by sequence-, shape- and context-specific recognition. We speculate that Roquin might require a threshold RNA-binding affinity for complete functional activity in cells. Such a threshold could, in principle, be reached in various ways: 1) by an optimal fit of the CDE motif, 2) by cooperative interactions of multiple RNA motifs in a given mRNA with Roquin, i.e. involving simultaneous A- and B-site binding, or 3) by cooperative mRNA binding involving the ROQ and flanking zinc-finger domains. This more complex picture of Roquin-mRNA interactions could potentially explain the differences in consensus motifs proposed for Roquin-1 interactions with CDEs, which vary depending on whether they were derived from *in vitro* binding studies or functional read-outs (27) (**Fig. 5D-F**). In this respect, cellular activity might be inhibited even though RNA binding affinity is reduced by only 2- to 3-fold *in vitro*. A similar explanation may account for the observation that single-site mutations are sufficient to inhibit binding to the CDE *in vitro*, while only triple mutants of important residues in the A- or B-site were able to completely abolish Roquin-1 function *in vivo* (27, 28).

Functional consequences of Roquin-RNA interactions

Protein-protein interactions involved in Roquin function

In contrast to RNA binding by the N-terminal region of Roquin, little is known about its interactions with other proteins. Roquin is believed to play a role in RNA storage and downstream processing in stress granules and P-bodies (20, 26, 37). The latter serve as a compartment for mRNA degradation, and several studies have confirmed a co-localization of Roquin with P-body markers such as the Enhancer of decapping Edc4 and the helicase Rck, even though any direct involvement of Roquin in

decapping remains to be proven. Interestingly, the protein immediately relocalizes to stress granules upon treatment with arsenite (23, 26). mRNA decay is now the best described downstream effect related to Roquin function (21, 28). Recruitment of the deadenylation complex was demonstrated by Leppek *et al.* and Murakawa *et al.* (21, 45); in fact all complex components (Ccr4, Caf1, Not) were found to associate with both paralogs of Roquin independently of RNA. For both studies, however, the significance of the physical interactions and the functional importance of deadenylation and decapping pathways for the different Roquin target mRNAs remain to be confirmed. Altogether, these findings underline a role for Roquin in mRNA degradation pathways. Whether different mRNA degradation pathways cooperate in Roquin-induced mRNA decay and whether all mRNA targets are destabilized by the same molecular mechanisms is currently unknown.

The complexity of gene regulation at the level of mRNA can be explained by the fact that certain of its target RNAs carry a combination of ARE and CDE and are therefore also subject to translational regulation through ARE elements (64). There is no evidence for a role for Roquin in this process; however, the protein has been implicated in the regulation of mRNA stability by links with the miRNA pathway (30, 59). While an earlier study had excluded a requirement for miRNAs and RISC in Roquin functions (26), Srivastava *et al.* provided evidence for microRNA-146a binding by Roquin-1 and its interaction with Ago2, a RISC component. Thus, Roquin could act in the regulation of both miRNA and mRNA. This potentially adds yet another functional role to the overall picture of post-transcriptional regulation by Roquin (**Fig. 6A**). From a structural point of view it is quite conceivable that Roquin could bind to stem-loop motifs in pre-miRNA, but binding to dsRNA or to miRNA/mRNA duplexes in mature miRNAs is equally possible.

Roquin in the regulation of target mRNA expression

Recently, the endonuclease Regnase-1 was identified as a functional partner of Roquin with which it shares a common subset of target mRNAs (24). While analyzing regulation via a minimal response element from the *Tnf* 3' UTR, the Heissmeyer group observed that Roquin-mediated regulation depends on the expression of Regnase-1 in fibroblasts. Conversely, Regnase-1 was fully active only in the presence of Roquin and, most importantly, fusing of the ROQ domain of Roquin-1 to Regnase-1 relieved its dependence on Roquin. This suggested a mandatory cooperation in which Roquin interacts with the RNA and directly or indirectly recruits Regnase-1 to destabilize the target, at least for the response element studied (24). However, the inference that the two proteins have to act in concert has recently been challenged (25). Mino *et al.* confirmed that they share some common targets, but they found that regulation of mRNA decay by Regnase-1 required active translation and was dependent on Upf-1 helicase activity, which was not the case for Roquin-mediated repression. Most surprisingly, transcriptome-wide mapping of Regnase-1 binding sites in HITS-CLIP experiments identified specific tri-loop structures, in which pyrimidine-purine-pyrimidine loops atop 3- to 7-nt stems as well as a novel hexa-loop structure in the Ptgs2 RNA were required for Regnase-1-mediated repression (25). Structural information on how Regnase-1 can physically interact with these stem-loop structures is currently lacking. Also, no comparison of individual and combined loss-of-function mutations in the mouse designed to probe the nature and physiological significance or redundancy of Roquin-Regnase-1 interaction has yet been reported.

It is likely that composite *cis* elements bearing multiple stem-loops, as in *OX40* or *ICOS* (23, 27) (**Fig. 6B**), could recruit several *trans*-acting factors, i.e. Roquin, Regnase and others as yet unidentified. They may co-regulate or act cooperatively to control mRNA expression through the same or

different post-transcriptional pathways. We and others (45) have recently confirmed that regulatory stem-hexa-loop motifs are recognized by Roquin and are functional in gene regulation (Janowski *et al*., under revision). These observations indicate that alternative decay elements and CDEs may together regulate target 3' UTRs. A recent example of this is *OX40*, the stability of which is regulated by two distinct decay elements with similar affinities. The existence of stem-loop RNA elements distinct from the CDE tri-loop identified by Leppek *et al.* could explain why a remarkable number of target mRNAs that lack consensus CDEs were enriched in anti-Roquin RNA immunoprecipitations (21). No doubt the identification of yet more novel 3' UTR loop motifs that can be recognized and targeted by Roquin will expand this multilayer mode of co-regulation.

How is Roquin itself regulated?

An obvious parameter for post-transcriptional gene regulation is the presence, activity and availability of RNA-regulatory proteins. For Roquin, this question has been addressed only very recently (24, 65). Schaefer *et al.* have described a modest upregulation of the Roquin-1 mRNA by the interleukin IL-10. In contrast, Jeltsch *et al.* (66) reported that cleavage of Roquin C-terminal to the ZnF by the paracaspase MALT1 promotes the differentiation of Th17 cells (24) (**Fig. 6B**). MALT1-mediated cleavage inactivates Roquin-1 and Roquin-2 downstream of TCR signaling, and a positive correlation between the strength of cognate antigen recognition and the graded inactivation of Roquin has been demonstrated. An "inactive" protein comprising the N-terminal half of Roquin could hypothetically block cognate *cis* elements and prevent binding of active, full-length Roquin molecules. Interestingly, MALT1 also acts in the proteolytic inactivation of Regnase-1 (67), which, together with the overlap between their target sets, strengthens the case for functional cooperation between the two. The recently published crystal structure of the Roquin-2 ROQ domain in complex with a *RC3H1* and *RC3H2* mRNA CDE (50) suggests that Roquin could auto-regulate its own mRNA levels. However, for Roquin it is likely that additional factors act at different stages to modulate its activity and thus its effectiveness in mRNA repression, cell-fate decisions, and immune responses.

Future perspectives

The recent structural data highlight the important function of Roquin-1 and -2 as novel RNA-binding proteins, and have substantially enhanced our understanding of the known biological functions of these proteins. The structural insights suggest that a much wider range of functional *cis* elements than hitherto assumed are targeted by Roquin. To enhance our understanding of the functional roles of other Roquin domains and their possible co-regulation with other *trans*-acting factors in mRNA suppression, future research should address a number of open questions. Little is known about Roquin's unstructured C-terminal half, and its roles in protein-protein interactions and the regulation of Roquin itself. Furthermore, it will be important to investigate the role of RNA structure and accessibility for its function. Finally, further exploration of the activity of Roquin as an E3 ligase might be a key to answering some unresolved issues in Roquin-mediated regulation.

Roquin as an E3 ligase in a broader context?

At least 15 E3 ubiquitin ligases contain putative RNA-binding domains (68). Of these, Roquin and MEX-3C have been studied in greatest detail. For the latter the RING domain was shown to be essential for the degradation of the HLA-A2 mRNA, which implies a critical role for ubiquitin ligase activity in mRNA degradation (68). To date, the function of the RING domains of Roquin-1 and Roquin-2 has not been studied in comparable detail. However, mice expressing only RINGless forms of Roquin-1 and Roquin-2 in T cells exhibit weak loss-of-function phenotypes (32). Furthermore,

Ramiscal *et al.* have recently shown that Roquin-1 uses its RING domain for automonoubiquitination and can negatively regulate Adenosine Monophosphate-activated Protein Kinase (AMPK) (44). Mechanistically, it is completely unclear how E3 ligases could directly promote mRNA degradation, while effects on post- or co-translational regulation are more readily understandable. Interestingly, 12-15% of newly translated proteins were found to be ubiquitinated in mammalian cells. This co-translational ubiquitination (CTU) further increased to approximately 50% in situations where proteins contained errors or were misfolded. Quality-control mechanisms, such as nonsense-mediated decay, no-go decay or non-stop decay (69) can cause stalling of the translation complex and under such circumstances CTU may contribute to the removal of the nascent polypeptide chains (69). CTU of nascent polypeptides has also been described in association with active translation, but the E3 ubiquitin ligases responsible are currently unknown. It is therefore conceivable that E3 ligases with RNA-binding domains, like Roquin-1 and -2, can affect gene-specific mRNA translation via CTU or even co-opt components of quality-control mechanisms for differential gene expression. However, Roquin may also act as an E3 ligase that regulates ubiquitin-dependent control of mRNAs by regulating protein degradation of other *trans*-acting factors.

Multi-motif regulation and the role of RNA secondary structure

The presence of distinct motifs in 3' UTRs offers a broader range of opportunities for regulation by RNA *cis* elements. The accessibility of individual regulatory motifs can be modulated by *trans*-acting factors that may bind regulatory motifs, unfold higher-order structures in the RNA (63) or maintain a preference for duplex structures, as was shown recently for mRNAs that are recognized by Staufen-1 (15). We further hypothesize that the combination of multiple binding sites may be more widely used to enhance the functional activity of Roquin (**Fig. 6B**). At the same time, the combination of *cis* elements with similarly low or high binding affinities may be important for differential regulation, as composite *cis* elements with lower affinity may be less sensitive to Roquin. This will lead to less effective repression of targets with low affinity compared to those with high affinity *cis* elements when antigen recognition is of moderate signal strength and cleavage of Roquin by MALT1 reduces the cellular amount of Roquin in T cells (24). To understand the intricate complexity of 3' UTR regulation, future work will have to combine large-scale approaches, such as CLIP experiments to identify RNA-binding sites and context-dependent occupancy of these, with structural biology to dissect the underlying molecular mechanisms (70). With regard to identifying potential target motifs within complex RNA stretches such as 3' UTRs, it remains difficult to unequivocally predict RNA secondary structure, either *in vivo* or on the basis of the effects of temperature, buffer and concentration observed in *in vitro* studies, although a number of promising approaches to the prediction of complex RNA folding are under development (11, 71-73).

Conclusion

The recent advances in understanding of Roquin's function and mode of action have greatly benefitted from high-resolution structural insight into its interactions with RNA. The mode of recognition of the CDE RNA by Roquin suggests a broader range of target mRNAs that may be regulated by Roquin. The successful identification of novel target motifs is important for a full understanding of the regulation of mRNA repression by Roquin. While the role of the ROQ domain as a central module for RNA recognition is now clear, the functions of the zinc-finger and RING domains need to be studied further.

We are only beginning to understand the roles of post-transcriptional regulation by Roquin, especially when one considers the opportunities for regulation offered by processes that act up- and downstream of RNA target recognition. These include regulation of Roquin expression and turnover, target mRNA decay or storage and Roquin's potential role in translational inhibition. Our understanding of the involvement of additional protein factors that act together with or counteract Roquin is still fragmentary. The role of RNA herein is closely related to factors such as size, secondary structure and the protein factors bound to it. The presence of multiple motifs that can be targeted by Roquin raises the question how regulation of mRNA surveillance is fine-tuned. In this picture a complex matrix of individual affinities for loop motifs, their availability, and competition by other regulators like the recently identified nuclease Regnase-1 is envisioned. Future studies will show how post-transcriptional regulation is governed by the existence and longevity of targetable states in a dynamic set-up.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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Figure captions

Figure 1. Roquin function and domain organization. (**A**) Roquin acts as an mRNA repressor. The scheme shows that the Roquin protein binds to a *cis* regulatory element in the 3' UTR of a target mRNA. Typical target mRNAs encode costimulatory T cell receptors, cytokines and transcription factors. Subsequent to mRNA binding Roquin recruits the deadenylation machinery (CCR4-Not-Caf) and stimulates decapping of the mRNA. (**B**) Roquin domain organization. The murine Roquin-1 protein consists of 1130 amino acids and comprises an N-terminal RING domain, a ROQ domain (blue: ROQ domain, ocher: extended helical domain (HEPN), see Figure 2) and a zinc-finger (ZnF) domain. The C-terminal half of Roquin harbors a proline-rich sequence (PRS) and a short coiled-coil stretch.

Figure 2. The Roquin ROQ domain. Overview of proteolytic fragments harboring the N-terminal RNA-binding region of Roquin-1. (**A**) The ROQ domain identified by Schlundt *et al.*, with additional flanking helical motifs reported by Tan *et al.* (**B**) and by Srivastava *et al.* (**C**). The core WH motif fold and the helical extension/domain II are shown in dark and light blue, respectively. The additional helical bundle representing domain I/$HEPN_{N/C}$ and the separate helical extension (black) reported by Srivastava *et al.* (30) are depicted in gold and black, respectively. The white sections in **C** indicate regions for which no structural information could be obtained due to lack of electron density. The double-headed arrow at the top delineates the extent of the core ROQ domain as defined by Schlundt *et al.* (27). Cartoon representations of the three-dimensional structures of these fragments are also shown. (**D**) The ROQ domain (PDB 4QI0) as defined in **A** (27, 29). (**E**) The region shown in **B** (PDB 4QIK, (28)). (**F**) The region shown in **C** (PDB 4TXA, (30)). This last is also shown in a rotated view that reveals the RING domain. Colors are as in **A-C**. Note that the structure shown in **E** depicts the conformation of the ROQ domain when bound to RNA, but for clarity only the protein part is illustrated.

Figure 3. The ROQ domain contains two RNA-binding sites. (**A**) Schematic representation of the extended Roquin ROQ domain (with sub-domains color coded as in Fig. 2) in complex with either a CDE-type stem-loop RNA (pink) at the A-site or dsRNA (orange) at the B-site. While canonical A-site binding involves the core WH motif, dsRNA is bound at a distinct site remote from the A site. (**B**) Structure of the CDE stem-loop bound to the A site in the ROQ domain (PDB 4QI2, (27)). (**C**) Structure of duplex RNA bound at the B site (PDB 4QIK (28)). In this structure (PDB 4QIK) the dimerization of ROQ observed in the crystal lattice may be a consequence of, or be facilitated by binding to the dsRNA.

Figure 4. Details of Roquin-RNA recognition at the A- and B-sites. (**A**) Recognition of the *Tnf* CDE motif by the ROQ domain of Roquin (PDB 4QI2). The ROQ domain interacts with the 5'-half of the CDE stem of the *Tnf* mRNA by non-sequence-specific contacts to the RNA backbone. Key interactions are annotated. (**B-D**) Close-up views of the recognition of the Py-Pu-Py tri-loop of the *Tnf* CDE (U11, G12 and U13). (**E**) Recognition of duplex *Tnf* dsRNA at the B site interface by the extended ROQ domain involves mainly non-sequence-specific contacts with the RNA backbone (PDB 4QIK). (**F**) A different view of the Roquin-dsRNA complex in the B-site from that shown in **(D)**. Colors in all panels are the same as in Figure 3.

Figure 5. The ROQ domain of Roquin recognizes a relaxed stem-loop RNA consensus. (**A**) Wild-type *Tnf* CDE stem-loop (23 nt). (**B**) Structure of the *Tnf* CDE stem-loop RNA when bound to the ROQ

domain (PDB 4QI2; note that the protein is not shown) (27). (**C**) The table shows variants of the 23-mer CDE in **A** that were tested in *in-vitro* affinity assays by Schlundt *et al.* (27) and Leppek *et al.* (21). The relative change in affinity compared to the WT is indicated by the color code as follows: no change (green), less than five-fold reduction (orange), complete loss of binding (red). The nucleotide variations are highlighted in yellow or deleted from the scheme. Binding of single-stranded RNA was tested by NMR titrations. (**D**) Relaxed CDE consensus based on panel **C**. (**E**) Adapted CDE consensus based on mRNA decay assays in references (21, 54) and suggested by Codutti *et al.* (54). (**F**) A general suggestion for a CDE consensus based on all available data (i.e. those reflected in **D** and **E**, recent findings with U-rich hexa-loops (45), and our own data (Janowski *et al.*, under revision).

Figure 6. Summary of RNA binding by Roquin and its regulation. (**A**) Roquin domains and their involvement in RNA- and protein binding. The extended ROQ domain can bind to stem-loop RNA at the A site and double-stranded RNA at the B site, colors as in Figures 2 and 3 (CDS: coding sequence, Ub: ubiquitin). Note that only the Roquin N-terminal region is depicted schematically. The C-terminal region (dashed line) is involved in subsequent downstream events that are not indicated here. A role for the Roquin RING domain for E3-ligase activity is still under investigation; possible targets are unknown with the exception of the data published by Ichijo and colleagues (43). The zinc-finger probably supports target recognition by binding to AU-rich elements in the target mRNA. (**B**) The efficacy of mRNA repression by the Roquin protein depends on its concentration in the cytoplasm (regulated by the Malt1 protease), the affinity and number of *cis* RNA elements in the mRNAs available for Roquin binding, and possibly on simultaneous binding of dsRNA regions. The degree of suppression of mRNA translation ranges from “not measurable” (light blue transcript, arrow indicates translation) via “medium” (blue, partial suppression) to “strong” (lilac, complete blockage), where multiple elements are bound and a high concentration of Roquin facilitates non-specific interaction with dsRNA to guide Roquin to *cis* regulatory response elements or possible engagement of non-consensus response elements.

Related Articles

Article ID	Article title
x	RNA structure and the regulation of gene expression
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Figures

Figure 1

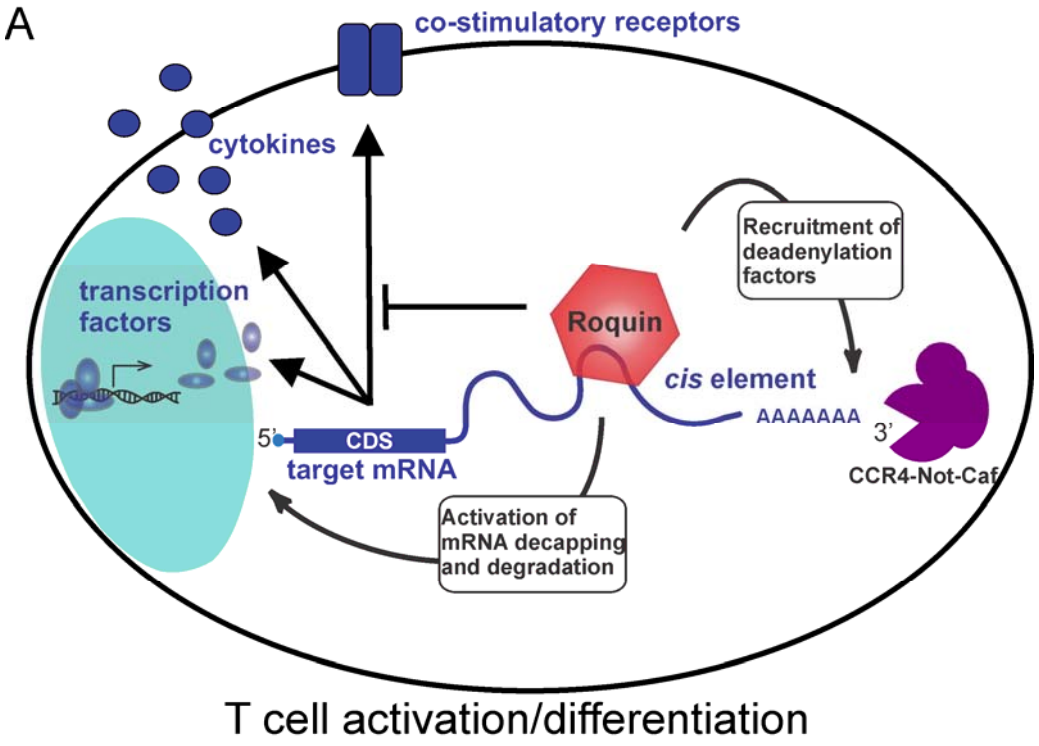


Figure 2

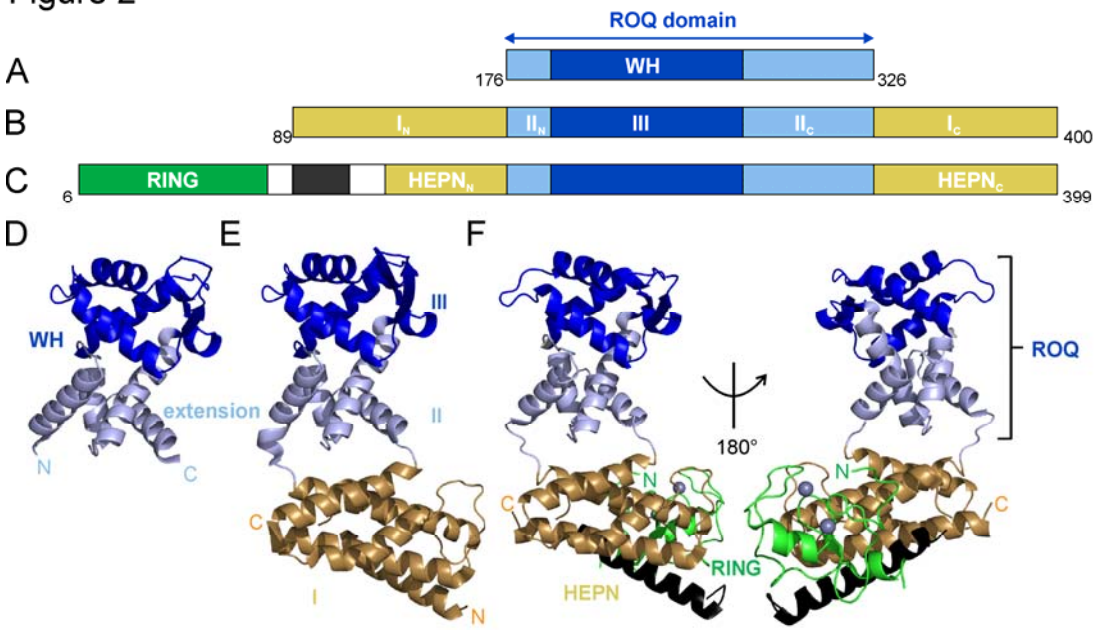

ROQ domain
A
176
WH
326
B
89
I_N
II_N
III
II_C
I_C
400
C
6
RING
$HEPN_N$
$HEPN_C$
399
D
E
F
WH
extension
N
C
III
II
C
I
N
180°
N
C
RING
HEPN
N
C
ROQ

Figure 3

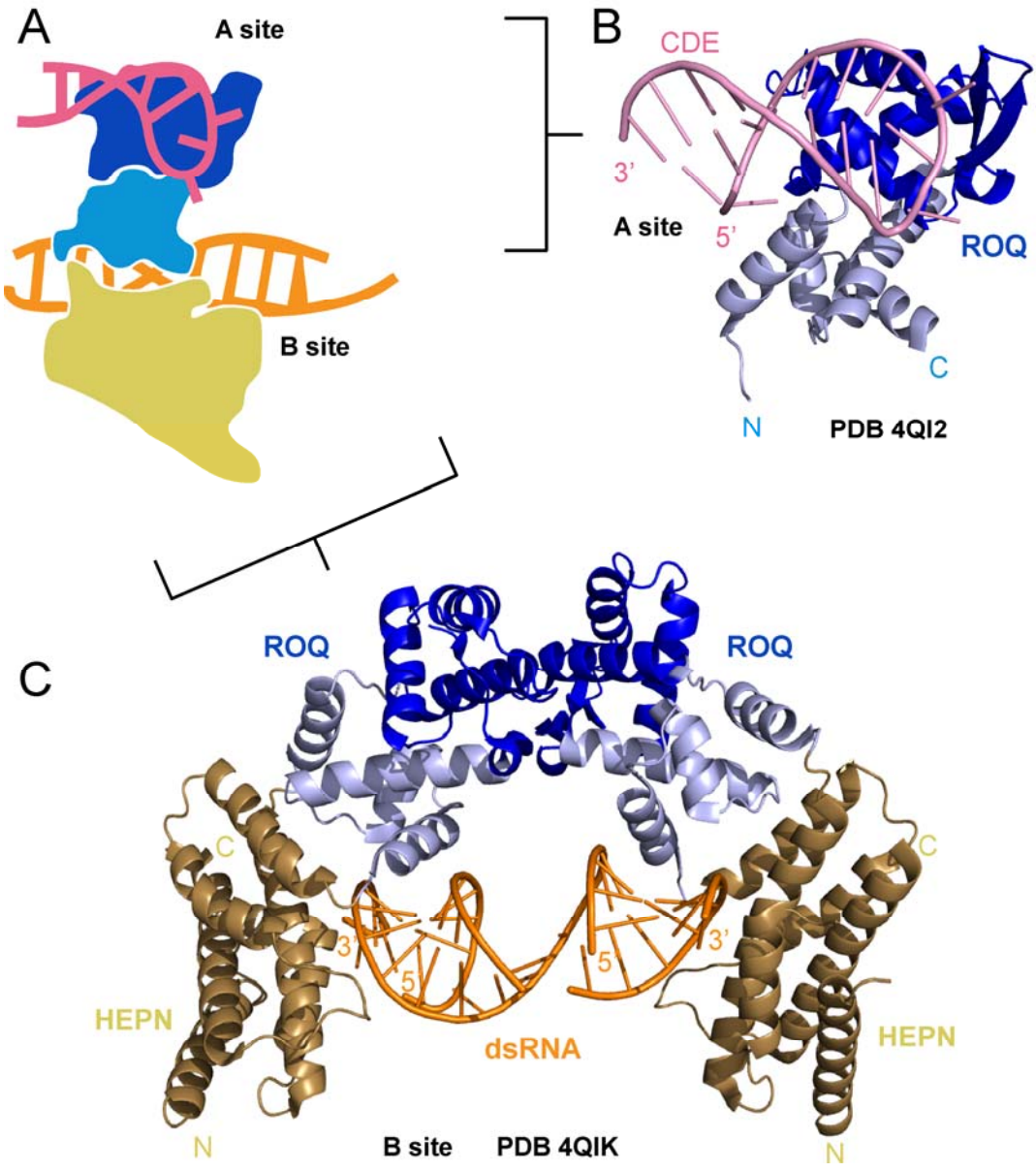

A
A site
B site
B
CDE
3'
A site
5'
ROQ
C
N
PDB 4QI2
C
ROQ
ROQ
C
3'
5'
5'
3'
HEPN
dsRNA
HEPN
N
B site
PDB 4QIK
N

Figure 4

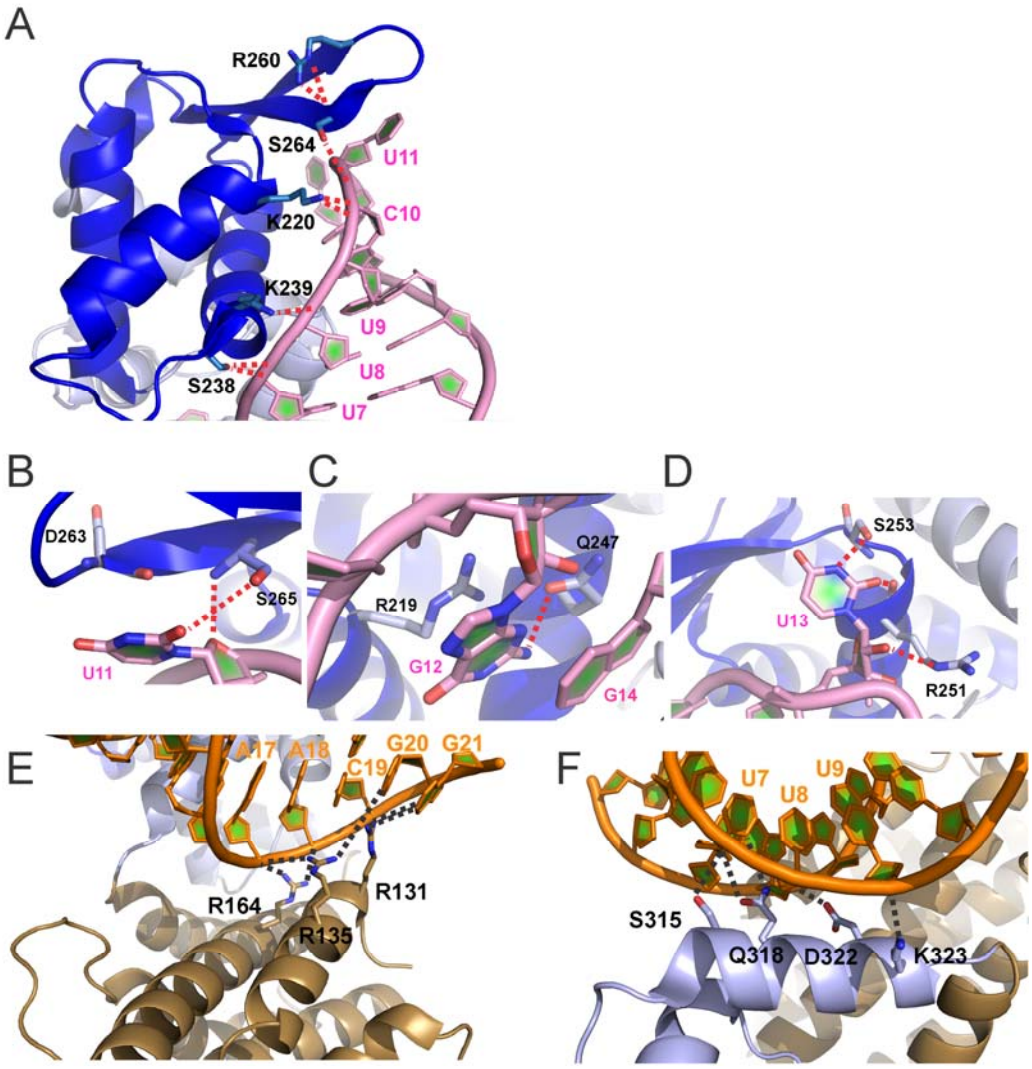
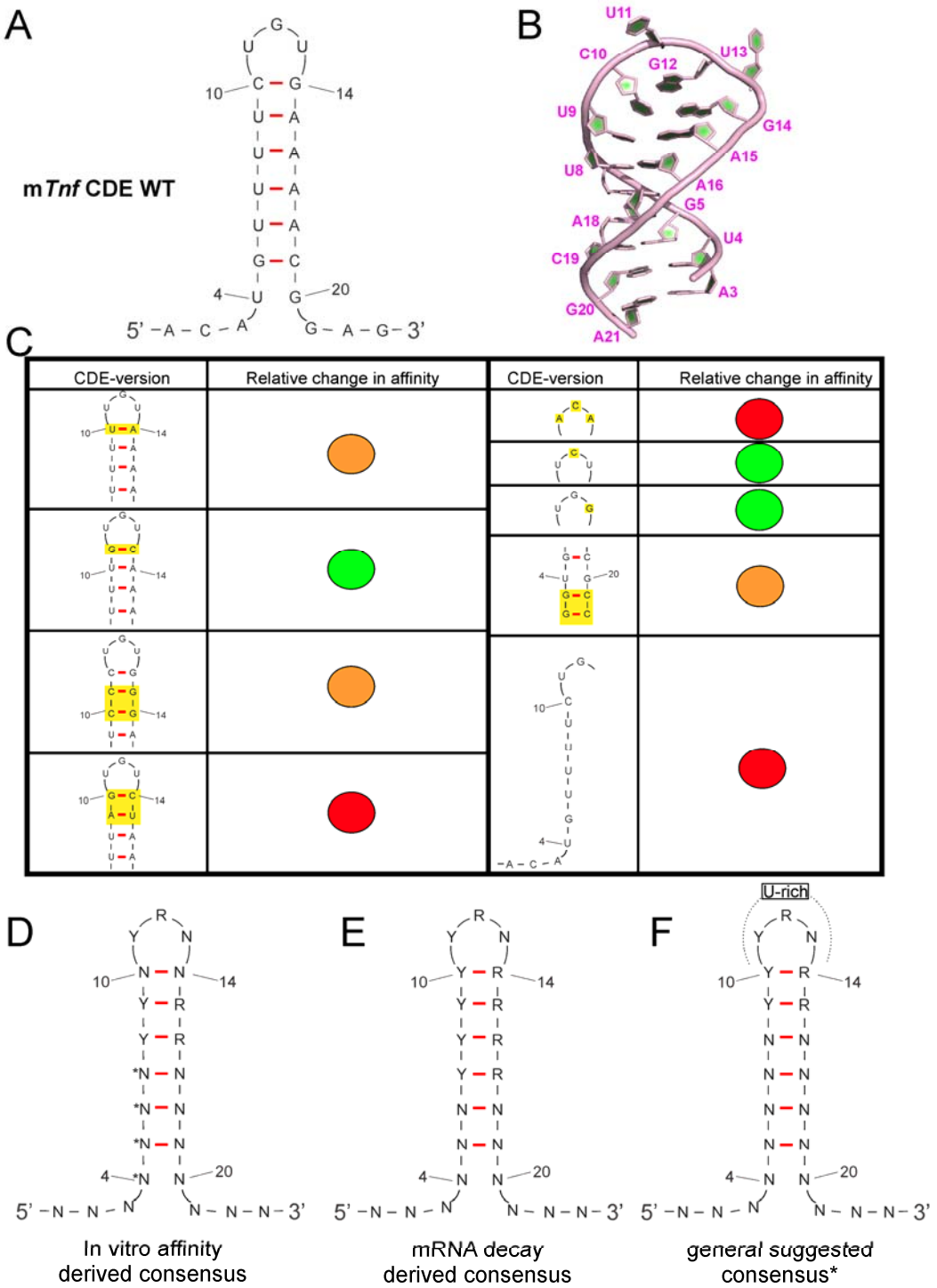
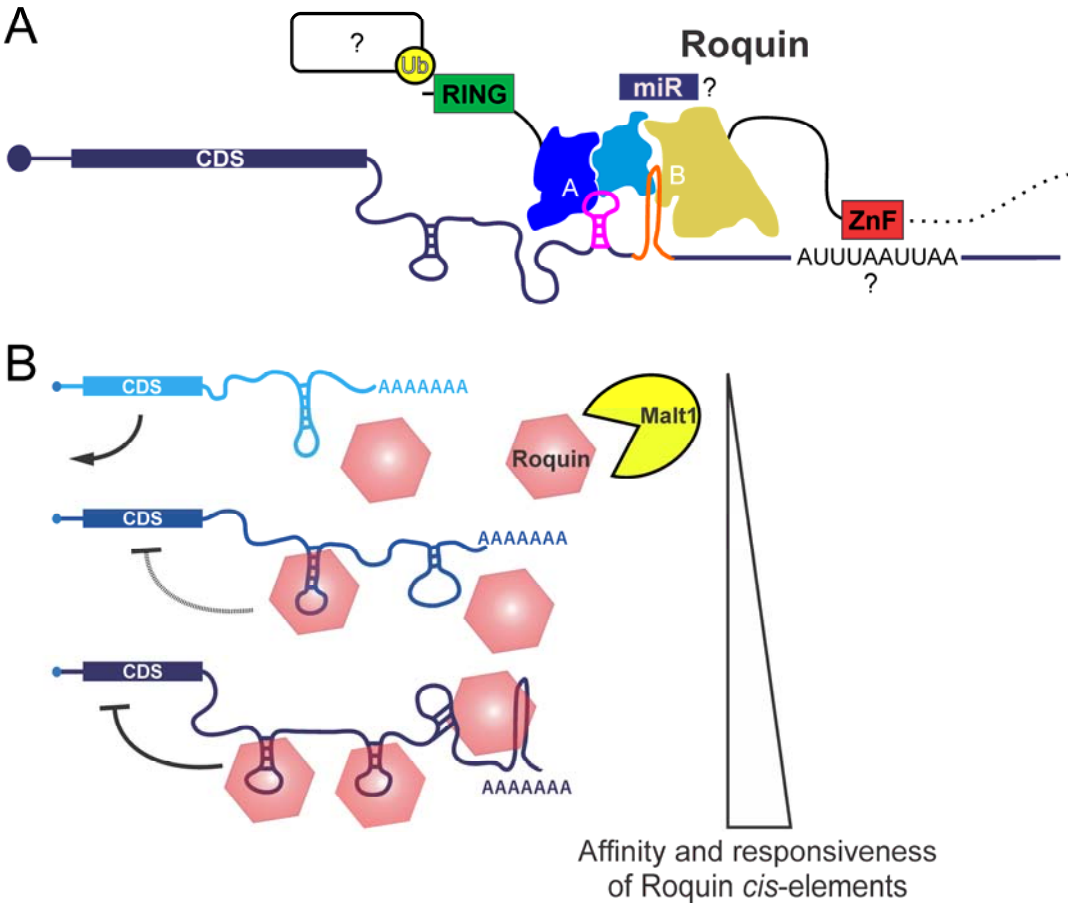

A
R260
S264
U11
K220
C10
K239
U9
U8
S238
U7
B
D263
S265
U11
C
Q247
R219
G12
G14
D
S253
U13
R251
E
A17
A18
C19
G20
G21
R164
R131
R135
F
U7
U8
U9
S315
Q318
D322
K323

Figure 5



*No systematic mutational analysis has been performed for these base pairs or stem-loop yet.

Figure 6


A
?
Ub
RING
Roquin
miR
?
CDS
A
B
ZnF
AUUUAAUUAA
?
B
CDS
AAAAAAA
Malt1
Roquin
CDS
AAAAAAA
CDS
AAAAAAA
Affinity and responsiveness
of Roquin cis-elements