The ROQ domain of Roquin recognizes mRNA constitutive-decay element and double-stranded RNA

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A conserved stem-loop motif of the constitutive decay element (CDE) in the 3′ UTR of mRNAs is recognized by the ROQ domain of Roquin, which mediates mRNA degradation. Here we report two crystal structures of the *Homo sapiens* ROQ domain in complex with CDE RNA. The ROQ domain has an elongated shape with three subdomains. The 19-nt *Hmgxb3* CDE is bound as a stem-loop to domain III. The 23-nt *TNF* RNA is bound as a duplex to a separate site at the interface between domains I and II. Mutagenesis studies confirm that the ROQ domain has two separate RNA-binding sites, one for stem-loop RNA (A site) and the other for double-stranded RNA (B site). Mutation in either site perturbs the Roquin-mediated degradation of *HMGXB3* and *IL6* mRNAs in human cells, demonstrating the importance of both sites for mRNA decay.

The cellular levels of mRNAs are regulated through many different mechanisms, which control mRNA biosynthesis, stability and degradation. For example, microRNAs can recognize appropriate sequence motifs in the mRNAs and can lead to mRNA destruction and/or repression of translation by the ribosome. Other sequence elements in the mRNAs can recruit protein factors to regulate mRNA levels. A well-known example is the AU-rich element (ARE) found in the 3′ untranslated region (3′ UTR) of many mRNAs for cytokines, proto-oncogenes and transcription factors 1–3. The ARE mediates the rapid decay of these mRNAs by recruiting deadenylation and decapping enzymes as well as other proteins, in response to defined cellular signals. Therefore, the ARE functions primarily as a regulated-decay element for these mRNAs.

Recent studies have identified a constitutive-decay element (CDE) in the 3′ UTR of tumor necrosis factor α, inhibitor of κB, inducible T-cell co-stimulator (ICOS) and other mRNAs 4,5. The CDE mediates the post-transcriptional, microRNA-independent repression of these mRNAs, irrespective of the cellular environment. A conserved stem-loop motif in the CDE is recognized by the ROQ domain of paralogous proteins Roquin and Roquin-2 (ref. 5), which recruit the deadenylation and decapping machineries to initiate mRNA degradation 4,5. Roquin has a central role in repressing autoimmunity, and mice deficient in both Roquin and Roquin-2 in T cells or those carrying a single-point mutation in the ROQ domain of Roquin (M199R, sanroque mutation) have increased ICOS expression and exhibit a lupus-like autoimmune phenotype 6–11. However, overexpression of Roquin in T cells promotes the development of arthritis in a mouse model 12. Roquin-knockout mice show periarticular lethality but not autoimmunity, thus suggesting that Roquin may have important developmental functions as well 9,13.

Human Roquin contains 1,133 amino acid residues (125 kDa, Fig. 1a). The N-terminal RING-finger, ROQ and CCCH-type zinc-finger (ZF) domains are well conserved among metazoans (Supplementary Fig. 1). The ROQ domain does not share recognizable sequence conservation with other proteins. It is required for binding the CDE, whereas the RING and ZF domains are dispensable for this interaction 5. The ROQ domain of Roquin-2 is 88% identical to that of Roquin (Supplementary Fig. 1), and Roquin-2 can complement Roquin’s function in repressing autoimmunity 9,10. The C-terminal segment of Roquin following the ZF is poorly conserved in sequence (Supplementary Fig. 1), but it recruits the deadenylation machinery and is required for CDE-mediated decay 5,6. 

To understand the molecular basis for CDE recognition by the ROQ domain, we have determined the crystal structures of this domain from human Roquin in complex with two different CDEs from *Hmgxb3* and *TNF* mRNAs. The ROQ domain is actually composed of three subdomains, I, II and III, and the structural analysis reveals previously unrecognized similarity of domains II and III to the helix-turn-helix (HTH) and winged-helix (WH) motifs. The *Hmgxb3*CDE stem-loop interacts with the WH motif of domain III, which primarily recognizes the 5′ arm and the loop of the RNA (A site). Two bases of the 3′-nt loop are flipped out to make close contacts with the protein. Unexpectedly, the *TNF* RNA is bound as a duplex, owing to its higher AU content in the stem and hence lower stability of the stem-loop conformation. This RNA is located in a separate binding site (B site) at the interface between domains I and II. Our mutagenesis, biochemical and cellular experiments confirm the importance of both RNA-binding sites for the functions of Roquin in mediating mRNA decay.

**RESULTS**

**Overall structure of the ROQ domain**

We determined the structure of the ROQ domain of human Roquin in complex with a 23-nt CDE of human *TNF* mRNA (TNF23) at 1.9-Å resolution (Table 1). TNF23 contains the 17-nt conserved stem-loop
as well as 3-nt flanking sequences at its 5′ and 3′ ends (Fig. 1b and ref. 5). We next determined the structure of the ROQ domain in complex with a 19-nt CDE of mouse Hmgxb3 mRNA (Hmg19) at 2.9-Å resolution, in a different crystal form. This CDE is more GC-rich in the stem but has the same 3-nt loop as TNF23 (Fig. 1b). Efforts at crystallizing the ROQ domain alone have not been successful.

The structure of the ROQ domain is mostly helical, with 13 helices (αA–αM) and a small three-stranded β-sheet (β1–β3) (Fig. 1c). The structure has an elongated shape, with dimensions of approximately 25 Å × 40 Å × 75 Å, and it can be divided into three subdomains. Subdomain I has six tightly packed antiparallel helices, three from the N-terminal end of the ROQ domain (αA–αC) and three from the C-terminal end (αK–αM). The ROQ domain has generally been defined in the literature as containing residues 131–360. Our structure shows, however, that the domain actually covers residues 90–400 (Fig. 1a), and the extra ~40 residues at the N and C termini contribute to helices αA and αM, respectively.

Subdomain III is formed by residues in the middle of the ROQ domain (195–271), and it contains three helices (αE–αG) and the three-stranded β-sheet (Fig. 1c). Unexpectedly, the backbone fold of this domain is similar to that of the WH motif, especially the WH-B domain of the RING ubiquitin-protein ligase cullin14–16 and related proteins17. For example, the r.m.s. distance among equivalent Cα atoms of this domain and the WH-B domain of cullin 1 is 2.3 Å (Supplementary Note 1 and Supplementary Fig. 2), although the amino acid sequence identity of the aligned residues is only 11%, on the basis of an analysis with Dalili (Z score of 9.2)18. Another structural homolog of this domain is the WH motif of elongation factor SelB (Supplementary Fig. 2), which is responsible for selenocysteine incorporation during translation19. The r.m.s. distance is 2.7 Å, and the sequence identity is 13% (Z score of 5.6).

Subdomain II, with four helices (αD and αH–αI), connects domains I and III, and there are no direct contacts between them (Fig. 1c). It has tight contacts with domain III, with ~900 Å² of surface-area burial, whereas the interface with domain I is more limited (~200 Å² of surface-area burial) and mostly hydrophilic in nature. To our surprise, helices αH–αI form another HTH motif in the ROQ domain, and its structural homologs include other HTH and WH proteins, such as the Zα domain of human adenosine deaminase 1 (Supplementary Fig. 2 and ref. 20). The r.m.s. distance is 1.8 Å for 48 equivalent Cα atoms, and the sequence identity is 14% (Z score of 4.9).

In the arrangement of helices αH–αI in domain II is also similar to that of helices αE–αG in domain III, even though domain II lacks the three-stranded β-sheet (the ‘wing’) compared to domain III (Fig. 1d and Supplementary Note 1). The r.m.s. distance is 2.1 Å for 44 equivalent Cα atoms, and the sequence conservation is 16% (Z score of 3.8, indicating a more remote similarity between the two domains). Helix αD of domain II is not a part of the HTH motif but has close interactions with helices αH and αI.

**Binding mode of the Hmgxb3 CDE**

We chose the Hmgxb3 CDE for our studies because it has four G-C base pairs in the stem and is therefore expected to be more stable as a stem-loop than the TNF CDE (Fig. 1b). The stem of Hmg19 contains six Watson–Crick base pairs and assumes a slightly distorted A-form RNA structure (Fig. 2a). A U1-G17 wobble at the base of the stem has been proposed in an earlier report5, but this base pair was not formed, and in fact G17 was disordered (Fig. 2a). These two nucleotides are present in TNF and Hmgxb3 but are not conserved in other CDEs (Supplementary Fig. 3).

The majority of the interactions between Hmg19 and the ROQ domain are mediated through the 3′-nt loop and the 5′ arm of the stem, which contact primarily domain III (helices αF and αG of the HTH motif and the β2–β3 loop of the wing) of the ROQ domain (Fig. 2b–d). In contrast, the 3′ arm of the stem has few contacts with the protein and weaker electron density (Fig. 2a).

We will refer to this binding site for the Hmg19 RNA as the A site (Fig. 1c). It is located in an electropositive surface patch in the ROQ domain (Supplementary Fig. 4). The protein–RNA interface involves hydrogen-bonding and ionic interactions with the backbone phosphate groups of the RNA and π-stacking interactions for a few of the bases (Supplementary Note 2). Both pyrimidine bases of the U8-G9-U10 triloop are flipped out and have close contacts with the protein (Fig. 2b,c). The base of G9 is stacked with the last base pair of the stem as well as the guanidine group of Arg219 (αF). The base of U1 is flipped ~180° from the helical pattern of the stem (Fig. 2a) and is π-stacked with the side chain of Trp184, in helix αD of domain II (Fig. 2d).

Amino acids in this interface are highly conserved among the ROQ domains (Supplementary Fig. 1). However, none of the...
nucleotides of Hmg19 appear to be recognized specifically by the A site. Nonetheless, this site recognizes the shape of the 3-nt loop (Fig. 2c) and is unlikely to accommodate other types of loops. This provides a degree of selectivity in the interactions between the ROQ domain and Hmg19 and may explain why all known CDEs have a triloop with pyrimidines at the first and third positions and purine at the second position.

### Binding mode of the TNF23 RNA

Unexpectedly, our crystallographic analysis revealed that the TNF23 RNA is not in a stem-loop conformation in the crystal. Instead, we found the two TNF23 molecules in an antiparallel, double-helical structure formed by the stem regions of the two RNAs (Supplementary Fig. 5). The 3-nt loop gives rise to an unpaired region in the middle of this duplex, which has weaker electron density, thus suggesting that this region is somewhat flexible. In fact, one nucleotide in this region (U10) is disordered in the crystal (Supplementary Fig. 5). Our further experiments showed that the formation of this duplex was due to the lower stability of the stem-loop conformation (low GC content) and the high concentration that was needed for crystallization (Supplementary Note 3).

Under physiological conditions, the TNF CDE exists as a stem-loop and is expected to be recognized by Roquin through the A site in domain III.

The TNF23 duplex contains six Watson-Crick base pairs from each of the stem regions of the TNF23 RNA (Supplementary Fig. 5). The two ROQ domains in the asymmetric unit have contacts with the base-paired regions of the TNF23 RNA but have no interactions with the nucleotides in the loop region in the middle of the duplex (Supplementary Fig. 5). The RNA is positioned in an electropositive surface depression at the interface of domains I and II (Supplementary Fig. 4), and we will refer to this binding site as the B site (Fig. 3a).

Both arms of the base-paired region interact with the ROQ domain (Fig. 3b, Supplementary Note 4 and Supplementary Fig. 6). For the 5′ arm, nucleotides U4, U5 and U6, near the middle of the base-paired region, have hydrogen-bonding interactions with residues in helix αB of domain II (Fig. 3c). In addition, the interactions with the base of U4 are consistent with pyrimidines because they involve the carbonyl oxygen on the C2 atom that is shared between U and C. For the 3′ arm, nucleotides A14, A15 and C16 near the end of the duplex contact residues in helices αB and αC of domain I (Fig. 3d). These residues that interact with the RNA are highly conserved among the ROQ domains (Supplementary Fig. 1). However, neither arm of the RNA appears to make strong contacts with the ROQ domain. Therefore, simultaneous binding to both arms may be required for association with the B site and this site is likely for binding double-stranded RNA (dsRNA). Moreover, the exact sequence of the RNA is not specifically recognized in this binding site either.

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**Figure 2** Interactions between the Hmg19 CDE and the ROQ domain. (a) Composite omit $F_o - F_c$ electron density map at 2.9-Å resolution for the Hmg19 RNA, contoured at 3σ. (b) Interactions of the 3-nt UGU loop and the top base pair of the stem with domain III of the ROQ domain. Hydrogen bonds and ionic interactions are shown as red dashed lines. (c) Molecular surface of the ROQ domain near the binding site for the 3-nt UGU loop, showing the close contacts between domain III of the ROQ domain and the triloop. (d) Interactions between the 5′ arm and U1 of Hmg19 with domains III and II of the ROQ domain. Amino acids are labeled in black and nucleotides in orange.

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**Table 1** Data collection and refinement statistics

|                  | ROQ-Hmg19 complex | ROQ-TNF23 complex |
|------------------|-------------------|-------------------|
| **Data collection** |                   |                   |
| Space group      | $P2_1$            | $P2_12_12_1$      |
| Cell dimensions  | $a$, $b$, $c$ (Å) | 49.6, 170.0, 51.1 | 90.8, 93.1, 100.2 |
| $\alpha$, $\beta$, $\gamma$ (°) | 90, 97.0, 90 | 90, 90, 90 |
| Resolution (Å)   | 40.2-2.9 (3.0-2.9)$^a$ | 40.0-1.9 (2.0-1.9) |
| $R_{merge}$ (%)  | 9.4 (41.5)        | 6.9 (43.9)        |
| $I / \sigma I$   | 9.1 (1.7)         | 19.4 (3.4)        |
| Completeness (%) | 97 (88)           | 100 (100)         |
| Redundancy       | 2.1 (1.9)         | 3.8 (3.8)         |

One crystal was used for each data collection.
$^a$Values in parentheses are for highest-resolution shell.
$^b$The Friedel pairs were kept as separate reflections during the refinement.

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Helix αJ in domain II that mediates the interactions with TNF23 is the second helix of the HTH motif. However, the binding mode of TNF23 to this domain is rather different from that of nucleic acids to other HTH motifs (Supplementary Fig. 2). Moreover, the position of TNF23 relative to domain II is also strikingly different from that of Hmg19 relative to domain III (Fig. 1d). The helical axes of the two RNAs are at a nearly 90° angle, probably because of the involvement of domain I in the binding of TNF23.

Mutations in the interfaces abolish RNA binding

We next introduced mutations in the ROQ-domain residues that are in the interfaces with RNA and assessed their effects on binding by electrophoretic mobility shift assay (EMSA). Mutations in the interface with Hmg19 (A site) included K239E T240A (Fig. 2d) and Q247A Y250A R251E (Fig. 2b), and those in the interface with TNF23 (B site) included Q318A S319A of domain II (Fig. 3c), R135E K136E D322A K323A of both domains. The mutants produced similar elution profiles from cation-exchange chromatography as that for the wild-type ROQ domain, thus suggesting that the mutations did not disrupt the overall structure of the domain.

To confirm that the B site is truly for binding dsRNA, we created another RNA molecule by replacing the 3′-nt loop of TNF23 with two GC base pairs (TNFds, Fig. 1b). We mixed and annealed the two separate chains to form the dsRNA and found that mutations in the B site completely blocked binding to this RNA (Fig. 4a). Wild-type ROQ domain as well as the mutants in the A site bound to this RNA, but the complex migrated as a smear on the gel, probably because of its poor stability. We then decreased the NaCl concentration to 50 mM and observed a clear band for the complex in the EMSA experiment, whereas mutations in the B site still abolished the interactions with the TNFds RNA (Fig. 4b). The lower ionic strength also stabilized the complex with the stem-loop RNA, such that the effects of mutations in the A site were reduced. This is consistent with the structural observations in which the interface is mostly ionic and hydrophilic in nature (Fig. 2b). Finally, the single-stranded RNA species of TNFds did not cause a gel shift (data not shown), thus proving that the B site is a dsRNA-binding site.

Two separate RNA-binding sites in the ROQ domain

The structures of the Hmg19 and TNF23 complexes demonstrate that the ROQ domain has two separate RNA-binding sites with no overlaps between them (Fig. 5a). The binding mode of Hmg19 RNA in the A site clearly indicates that a dsRNA cannot be accommodated there because the loop makes intimate interactions in the B site.
Functional study of Roquin in human HEK293T cells. (a) mRNA levels as measured by quantitative real-time PCR. Roquin and Roquin-2 mRNA levels in HEK293T cells are shown after control knockdown (ConKD), double knockdown of Roquin and Roquin-2 (Roquin1/2KD) and complementation by add back with shRNA-resistant Roquin (WT), Roquin (A site) or Roquin (B site) mutants. Complementation with the vector plasmid lacking the Roquin cDNA (vector) is shown as a control. Error bars, s.d. (n = 3 cell cultures). (b) Plot showing the stability of the HMGXB3, IL6 and PPP1R10 mRNAs. Degradation of the mRNAs was measured by quantitative real-time PCR in the same HEK293T cells as in (a). Level of mRNA remaining following actinomycin D-directed transcriptional silencing at the indicated times is shown. Error bars, s.d. (n = 3 cell cultures). Half-lives (t1/2) are shown relative to GAPDH mRNA and are derived from three independent experiments. Comparison of the various complementation data relative to the control are indicated by the bars, and P values from comparison of the decay rates are presented with asterisks (**P < 0.05; ***P < 0.001, from two-tailed extra sum-of-squares F test).
knocked down human Roquin and Roquin-2 in HEK293T cells and complemented with exogenous wild-type and mutant Roquin (Fig. 6a), which we confirmed to be stable and expressed at comparable protein levels (M.Z. and M.K., unpublished data). We tested the stability of HMGXB3 and PPP1R10 mRNAs, which were shown to be responsive to Roquin in mouse NIH3T3 cells, and the pleiotropic cytokine IL6 mRNA, which may possess a CDE (Supplementary Fig. 3). Reduction of endogenous Roquin and Roquin-2, by 70% and 90%, respectively, led to stabilization of the human HMGXB3 and IL6 mRNAs that was reversed upon complementation with short hairpin RNA (shRNA)-resistant wild-type Roquin (Fig. 6b). In contrast, despite levels of expression comparable to that of wild type (Fig. 6a), neither the A-site nor the B-site Roquin mutants could restore the decay of these mRNAs, thus demonstrating that both sites are necessary for efficient Roquin-mediated decay of target mRNAs in cells. The effect of Roquin knockdown on IL6 stability is smaller, in agreement with the presence of other mechanisms that regulate this mRNA. As expected, an mRNA lacking a predicted CDE, PAQR8, was not responsive to changes in Roquin levels. Surprisingly, the PPP1R10 mRNA was also nonresponsive to a reduction in Roquin levels or complementation with Roquin in human cells, thus indicating some functional differences between the human and mouse cells for this mRNA.

**DISCUSSION**

Our structural studies reveal that the ROQ domain actually consists of three subdomains. Moreover, although the ROQ domain does not share recognizable sequence homology with other proteins, the structures show that it contains two HTH motifs in tandem: a WH motif in domain III and an HTH motif in domain II. These motifs are known to bind DNA and RNA as well as to mediate protein-protein interactions. Our mutagenesis and biochemical studies show that the WH motif in domain III is crucial for binding stem-loop RNA (the A-site) and that the HTH motif in domain II contributes to binding dsRNA (the B-site). The tight interface between domains II and III suggests that they may function together as a unit. Domain I of the ROQ domain, with a unique arrangement of helices, is also involved in dsRNA binding. Therefore, all three domains of the ROQ domain mediate or contribute to RNA binding.

The presence of two RNA-binding sites in the ROQ domain accords with and explains the current data on Roquin. Immunoprecipitation experiments with Roquin have identified a large number of mRNAs, many of which do not contain the conserved CDE stem-loop. Within the ICOS 3′UTR, Roquin also recognizes sequences in addition to the CDE. Therefore, Roquin may bind sequences other than the CDE stem-loop, and the B site may have an important role in recognizing such RNAs.

The distance from the 3′ end of the dsRNA in the B site to the 5′ end of the stem-loop in the A site is ~30 Å, a span that can be bridged by a linker of at least 7 nt (Fig. 5d). However, the distance from the 3′ end of the stem-loop in the A site to the 5′ end of the dsRNA in the B site is ~60 Å, and a longer linker would be needed to connect these two ends (Supplementary Fig. 3). Alternatively, the two binding sites in the ROQ domain may function independently and may bind separate RNA molecules.

Our studies show that domain III has a crucial role in recognizing the CDE stem-loop. The functional importance of this domain is further supported by the fact that it also contains the M199R mutation that produces the sanroque phenotype in mice. The Met199 residue is located at the N-terminal end of helix αE, but it is not in the interface with RNA (Fig. 1c). The side chain is partially exposed to the surface (Supplementary Fig. 6), and the mutation to arginine can be accommodated without substantially perturbing the structure. The structural observations are consistent with reports showing that the M199R mutant has similar binding affinity for the CDE as that of the wild-type protein and that the mutation does not affect the stability of Roquin but partially reduces its ability to repress ICOS expression. The role of the M199R mutation in the sanroque phenotype is probably due to a different mechanism, for example disrupting interactions with a protein partner, because the arginine side chain introduced by the mutation is expected to change the surface shape and electrostatic properties of that region of domain III.

Overall, our structural and biochemical studies have defined the molecular basis for the recognition of the CDE stem-loop by the ROQ domain and have unexpectedly revealed a separate site for dsRNA recognition by this domain. Our functional studies showed that both binding sites are important for Roquin-mediated decay of target mRNAs. The structures also provide a foundation for understanding the functions of Roquin and the CDE in mediating mRNA decay as well as other cellular processes.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 4QIK (TNF23 complex) and 4QIL (Hmg19 complex).

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

D.T. carried out protein expression, purification and crystallization, X-ray diffraction data collection and processing, structure determination and refinement, mutagenesis and EMSA experiments. M.Z. carried out the mRNA stability experiments. L.T. and M.K. supervised the experiments, analyzed the data and wrote the manuscript. All authors commented on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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**Online Methods**

**Protein expression and purification.** Residues 89–407 of human Roquin (containing the ROQ domain) were subcloned into the pET26b vector (Novagen), and the resulting recombinant protein carried a C-terminal hexahistidine tag. Escherichia coli BL21 Star (DE3) cells transformed with this plasmid were induced with 0.5 mM IPTG and grown at 37 °C for 18 h. The soluble protein was purified through nickel-affinity agarose affinity (Qiagen), cation-exchange (SP Sepharose Fast Flow, GE Healthcare) and gel-filtration (Sephacryl S-300, GE Healthcare) chromatography. The purified protein was concentrated to 20 mg/ml and stored at −80 °C in a solution containing 20 mM Tris, pH 8.5, 250 mM NaCl, 5 mM DT and 5% (v/v) glycerol. The histidine tag was not cleaved for crystallization.

The selenomethylion protein sample was produced in E. coli B834 (DE3) cells, and the bacteria were grown in defined LeMaster media supplemented with selenomethionine24. The purification procedure was the same as that for the native protein.

The RNA samples were purchased from Integrated DNA Technologies (IDT). The TNF23 and Hmg19 RNAs were diluted to 300 and 50 µM concentration, respectively, in a buffer containing 20 mM Tris, pH 8.5, 250 mM NaCl and 4 mM MgCl2, and the solution was heated at 98 °C for 5 min and then slowly cooled to room temperature. To form the ROQ–TNF23 complex, 60 nmol of the protein and 72 nmol of RNA were mixed in a solution (total volume 3 ml) containing 20 mM Tris, pH 7.9, 150 mM NaCl and 5 mM MgCl2. The Hmg19 complex and 72 nmol of the RNA were mixed in a solution (total volume 3 ml) containing 20 mM Tris, pH 8.5, 250 mM NaCl, 5 mM DT and 5% (v/v) glycerol. The histidine tag was not cleaved for crystallization.

**Protein crystallization.** Crystals of the ROQ–TNF23 complex were obtained with the sitting-drop vapor-diffusion method at 20 °C. The reservoir solution contained 100 mM sodium citrate, pH 5.7, 1.5 M LiCl, and 18–21% (w/v) PEG 6000. 1 µl of the protein–RNA complex solution at 1.5 mg/ml concentration (diluted with the storage buffer without glycerol) was mixed with 1 µl of the reservoir solution. Crystals grew to full size in one week. They belonged to space group P212121, with unit-cell parameters of a = 90.8 Å, b = 93.1 Å, and c = 100.2 Å. There are two molecules of the ROQ domain and a TNF23 duplex in the asymmetric unit.

Crystals of the ROQ–Hmg19 complex were obtained with the hanging-drop vapor-diffusion method at 20 °C. The reservoir solution contained 100 mM sodium citrate, pH 8.2, 0.3 M calcium acetate and 12–14% (w/v) PEG 8000. 1 µl of the protein–RNA complex solution at 3 mg/ml concentration was mixed with 1 µl of the reservoir solution. Crystals grew to full size in two weeks. They belonged to space group P212121, with unit-cell parameters of a = 49.6 Å, b = 170.0 Å, c = 51.1 Å, and β = 97°. There are two copies of the ROQ–Hmg19 complex in the asymmetric unit.

The crystals were cryoprotected by the reservoir solution supplemented to 35% (w/v) PEG 6000 (TNF23 complex) or 6.4 mg/ml (Hmg19 complex) in a buffer containing 20 mM Tris, pH 8.5, 250 mM NaCl, 5 mM DT and 5% (v/v) glycerol.

**Electrophoretic mobility shift assay (EMSA).** The Hmg19, TNF23 and TNFds 5’ arm RNAs with 6-FAM fluorescent label at the 5’ end and the unlabeled TNF23 3’ arm were purchased from IDT. The RNAs were diluted to 5 µM and heated at 98 °C for 5 min and then slowly cooled to room temperature. The protein–RNA mixtures were prepared in a buffer containing 20 mM Tris, pH 8.2, 5 mM MgCl2, and 50 or 200 mM NaCl. The samples were loaded to 0.8% native agarose gel. The electrophoresis was performed in 1× TAE buffer, pH 8.0, at 4 °C, and then the RNA was visualized on a UV illuminator.

**Plasmid construction.** The Flag-tagged pTK-IREShyg-Flag plasmid was constructed in three steps. First, the polylinker of pIREShypo3 (Clontech) was replaced with the Flag tag–containing polylinker in pCDNA3 (ref. 33) with SacI and NotI restriction sites to generate pIRESh-flag-puro3 vector. Second, the puromycin-resistance gene in pIRESh-flag-puro3 was removed by SmaI and XbaI digestion and replaced with the hygromycin B–resistance gene derived from pTRE2hyg (Addgene) with StuI and XbaI to generate pIRESh-Flag vector. In the last step, pIRESh-Flag cassette from the pIRESh-Flag vector was PCR amplified with the addition of adaptors containing Nhel and NotI restriction sites at 5’ and 3’ ends, respectively. Then, the Renilla luciferase cdNA within pRL-TK (Promega) was replaced by the Nhel–NotI–flanked IREShyg-Flag cassette to generate the pTK-IRESh-Flag plasmid. The full-length human Roquin cdNA was PCR amplified with primers 5’ GTAATCGTAGATCCGATGCGGCTGGTGAAGCTGCCTTTT 3’ and 5’ GAATTCTCGTGCTGGCTGCCGCTTAAGGAGCAGAACTGG 3’ containing the EcoRV and NotI restriction endonuclease sites, respectively, and inserted into the same restriction sites of the pTK-IRESh-Flag plasmid in frame and downstream of the Flag tag to generate pTK-IRESh-Flag Roquin. shRNA-resistant Roquin was generated by PCR mutagenesis with primers 5’ AAGGAGTAGTTAATTCA CTTCCTGCTTGAAGCTGCCTTTTA 3’ and 5’ GTAATCGTAGATCCGATGCGGCTGGTGAAGCTGCCTTTT 3’ to alter the shRNA target sequence to generate pRoquin (WT). shRNA-resistant mutants, pRoquin (A site) and pRoquin (B site) were constructed by replacing the wild-type ROQ domain region within pRoquin (WT) with the corresponding A-site mutant (Q247A Y250A R251E) or B-site mutant (R153E K136E D322A K323A) with Bpl/BstEII or Bpl/MfeI restriction sites, respectively. All constructs were confirmed by sequencing.

**Generation of lentiviral particles.** All shRNA plasmids and packaging plasmids were purchased from Sigma. HEK293T cells were transfected by pLKO.1-shRoquin (Sigma TRCN0000144045)/pLKO1-shRoquin-2 (Sigma TRCN0000294353), and pCMV-VSVG and psPAX2 with Lipofectamine 2000 (Life Technologies) to generate viral particles. Culture medium was harvested 2 days after transfection and frozen at −80 °C for storage.

**Determination of mRNA half-lives.** HEK293T cells infected by control shRNA–expressing lentivirus (ConP/2) or both Roquin- and Roquin-2–specific shRNA lentivirus (Roquin1/2KD) were transiently transfected with pTK-IRESh-Flag vector, pRoquin (WT), pRoquin (A site) or pRoquin (B site). Cells were treated with actinomycin D (5 mg/L, Sigma) 48 h after transfection/infection, and were then harvested and RNA was isolated (TRIzol Reagent; Life Technologies) at the indicated time intervals. RNAs were then reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Promega) and oligo(dT) primer according to the manufacturer's instructions. Values were quantified by real-time PCR with SYBR green PCR core reagent (Applied Biosystems), and the abundance of specific mRNAs was quantified with the standard-curve method according to the recommendations of the manufacturer. mRNA levels were normalized to the GAPDH mRNA and plotted against time. The primers used for real-time PCR are listed in Supplementary Table 1. Real-time PCR was carried out with an ABI Prism 7900HT sequence-detection system. An equation assuming first-order decay rate was fitted to the data points by linear regression: \( y = 100 \times e^{b \times t} \), where \( y \) stands for the percentage of mRNA remaining, and the time. mRNA half-lives were determined by \( b = (\ln 2)/t \). P values for the decay rate in Figure 6b were derived from the extra sum-of-squares \( F \) test calculated by PRISM software.

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**Table 1**

| Primer Name | Sequence |
|-------------|----------|
| GAPDH F/P | GAATTCGATATCCATGCCTGTACAAGCTCC/CTCTGTCTCTGGAGTGGTGG |
| Renilla F/P | GAAGTTGAGCAGAACTGG/GAATTCTCGTGCTGGCTGCCGCTTAAGGAGCAGAACTGG |
| ACTIN F/P | GAAGTTGAGCAGAACTGG/GAATTCTCGTGCTGGCTGCCGCTTAAGGAGCAGAACTGG |
| ROQ/HPK F/P | GAAGTTGAGCAGAACTGG/GAATTCTCGTGCTGGCTGCCGCTTAAGGAGCAGAACTGG |

**Supplementary Table 1**

| Primer Name | Sequence |
|-------------|----------|
| GAPDH F/P | GAATTCGATATCCATGCCTGTACAAGCTCC/CTCTGTCTCTGGAGTGGTGG |
| Renilla F/P | GAAGTTGAGCAGAACTGG/GAATTCTCGTGCTGGCTGCCGCTTAAGGAGCAGAACTGG |
| ACTIN F/P | GAAGTTGAGCAGAACTGG/GAATTCTCGTGCTGGCTGCCGCTTAAGGAGCAGAACTGG |
| ROQ/HPK F/P | GAAGTTGAGCAGAACTGG/GAATTCTCGTGCTGGCTGCCGCTTAAGGAGCAGAACTGG |
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